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Research paper

Spray freezing into liquid nitrogen for highly stable protein nanostructured microparticles

Zhongshui Yu[a], Ana S. Garcia[b], Keith P. Johnston[b,*], Robert O. Williams III[a,*]

[a]College of Pharmacy, The University of Texas, Austin, TX, USA
[b]Department of Chemical Engineering, The University of Texas, Austin, TX, USA

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Abstract

The objectives of this study were to produce nanostructured protein microparticles with the spray freezing into liquid (SFL) cryogenic process and to demonstrate a smaller degree of protein denaturation and aggregation than observed in spray freeze drying (SFD). Nanostructured microparticles were formed by atomization of an aqueous buffer solution containing bovine serum albumin (BSA) with and without excipients beneath the surface of a cryogenic liquid. Lyophilization was used to sublime the water in the frozen particles. The resulting BSA dry powder was characterized by size exclusion chromatography, Fourier-transform infrared spectroscopy, scanning electron microscopy (SEM), light scattering, and specific surface area analysis. SEM revealed highly porous microparticle with features smaller than 500 nm. The specific surface area of the BSA microparticles ranged from 19.2 to 97.7 m^2/g as a function of the total protein and excipient content in the aqueous feed solution. SFL produced less denaturation and aggregation of protein monomer than SFD, despite the extremely high surface areas in both processes. The intense atomization and ultra-rapid freezing in the SFL process lead to nanostructured BSA microparticles with high surface areas. Protein denaturation and aggregation are reduced in SFL relative to SFD. The more rapid freezing in SFL lowers the time for proteins to aggregate or diffuse to water–air and water–ice interfaces where they may be denatured.

Keywords: Bovine serum albumin (BSA); Spray freezing into liquid (SFL); Microparticle; Cryogenic liquid

1. Introduction

In biopharmaceutical applications, chemical and physical instability of proteins and peptides is problematic in formulation and storage [1]. Proteins and peptides are more often stable in the solid state than in solution as reviewed elsewhere [2,3]. Although protein particles may be formed either by lyophilization or by spray freeze drying (SFD), loss of stability during particle formation remains a major challenge [4].

In the spray-freeze drying process [5–7], a solution containing dissolved protein is sprayed into a cold vapor phase over a cryogenic liquid to form droplets, which do not freeze immediately. The droplets may begin to freeze while falling through the cold vapor phase; however, most of the freezing takes place upon contact with the cryogenic liquid phase [5]. Atomization generates fine droplets with a large air–liquid interfacial area. It was reported by Webb et al. [8] that the time of flight for the fine liquid droplets to pass through the vapor phase was approximately 1 s. Proteins and peptides are often surface active at the air–water interface and unfold upon interfacial adsorption thereby disrupting the native structure which leads to loss of biological activity. This hydrophobic air–water interface is considered to destabilize proteins more than the hydrophilic ice–water surface [8,9]. It was reported that recombinant human growth hormone (rhGH) aggregates and precipitates upon adsorption at the air–liquid interface. This type of aggregation was reduced by addition of a surfactant [10,11]. Webb et al. [8] showed that adsorption of rhIFN-γ at the air–liquid interface resulted in significant aggregation of the protein during passage of the droplets through the vapor

* Corresponding authors. Robert O. Williams. Address: College of Pharmacy, The University of Texas, Mailstop A 1920, Austin, TX 78712 1074, USA. Tel.: +1-512-471-4681; fax: +1-512-474-7474; Keith P. Johnston. Address: Department of Chemical Engineering, The University of Texas, Austin, TX 78712-1074, USA. Tel.: +1-512-471-4617; fax: +1-512-475-7824.

E-mail addresses: kpj@che.utexas.edu (K.P. Johnston), williro@mail.utexas.edu (R.O. Williams).

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phase in SFD. On the other hand, freezing the protein solution slowly without forming droplets did not induce protein aggregation because there was minimal protein adsorption at the ice–water interface [8]. In their study, the SFD process was isolated into separate processing steps: spray freezing and lyophilization, and the influence of each step on protein stability was determined. The protein solution was also frozen slowly, then allowed to thaw back to the unfrozen state, and the protein stability was determined. They concluded that the majority of the aggregation in the SFD process was due to protein adsorption at the air–liquid interface in the nebulization step.

During spray freezing, the concentrations of protein and other solutes including electrolytes increase markedly in the remaining unfrozen solution as ice crystals are formed [12]. The large increase in protein concentration in the remaining unfrozen liquid, as well as increases in the ionic strength and changes in pH, further contributes to protein denaturation and aggregation. The time for freezing of the aqueous protein solution has been minimized in our study to avoid these undesirable increases in concentration in the unfrozen liquid phase before the droplets are completely frozen.

Recently, we presented a process to produce porous microparticles containing insulin [13] and to produce water insoluble drugs [14–16] by spray freezing into liquid (SFL). The liquid is typically nitrogen. The liquid solution was sprayed directly into liquid nitrogen through an insulated nozzle, rather than into cold vapor as in SFD. The intense atomization of the droplets in the liquid phase of the cryogenic liquid results from the high pressure drop through a fine orifice nozzle and the friction generated by passing the droplets through a liquid rather than a vapor phase. Insulin particles produced by SFL had a low tap density, high surface area, a narrow particle size distribution and no loss in stability as characterized by the concentration of covalent dimer [13]. The secondary structure of the insulin and physical aggregation were not studied. As insulin solutions are lyophilized, significant denaturation has been observed with an increase in structure as the alpha helix and beta sheet/turn fractions grow at the expense of the random coil [17].

Low density microparticles are of interest in pulmonary delivery of proteins and peptides to enhance lung deposition [18–20]. In addition, high surface area small diameter protein nanoparticles may be encapsulated more uniformly in biodegradable polymer microspheres for depot drug delivery [6]. The more uniform distribution may be expected to reduce the initial burst release. However, the degree of aggregation was found to increase significantly with surface area, consistent with the observation that proteins unfold and aggregate at the air–water interfaces [8]. The formation of high surface protein particles stable against unfolding and aggregation remains a formidable challenge.

The objectives of this study were to produce nanostructured BSA microparticles by the SFL process and to demonstrate less protein aggregation (monomer loss) relative to the SFD process [6]. The denaturation process for our model protein, BSA (68 kDa), is complicated since it has three homologous domains that assemble to form a heart-shaped structure [21]. For BSA, we examine the physical aggregation due to unfolding, which was not addressed in our previous study of insulin (5.5 kDa). For insulin, the primary loss in stability resulted from chemical degradation. Our hypothesis is that the process induced instability of protein produced by the SFL process may be significantly less than that produced by the SFD process, even for comparably similar surface areas of the protein powders, because the air–liquid interface may promote protein denaturation in the SFD process. High surface area stable protein powders may be utilized in a variety of drug delivery applications including microspheres for depot delivery and pulmonary delivery.

2. Materials and methods

2.1. Materials

BSA ($M_w \sim 68$ kDa, fraction V powder, approx. 99% purity, protease-free, essentially γ-globulin free) was purchased from Sigma Chemical Co. (St Louis, MO) and was approximately 83% monomeric. Tyloxapol, trehalose, sodium hydroxide, potassium phosphate monobasic, potassium phosphate dibasic, potassium bromide, sodium chloride, and acetonitrile were purchased from Spectrum Laboratory Products, Inc. (Gardena, CA). All chemicals were A.C.S. grade or higher. Poly(lactic-*co*-glycolic acid) (PLGA) (Resomer RG502H, 50:50 lactide:glycolide nominal, $M_w \sim 10$ kDa) was supplied by Boehringer Ingelheim Chemicals, Inc. (Petersburg, VA).

2.2. Methods

2.2.1. Spray freezing into liquid process

The SFL process, described in detail in a previous study was modified slightly [13]. The aqueous feed solution was prepared by dissolving 100 mg of BSA powder in 20 ml of 5 mM phosphate buffer pH 7.4. In the final powder, the ratio of the phosphate salt to BSA was about 5:1. The aqueous feed solution was loaded into the solution cell and then sprayed beneath the surface of 1 l of cryogenic liquid (e.g. liquid nitrogen) through a 63.5 μm polyetheretherketone (PEEK) capillary nozzle at a pressure of 5000 PSI which was provided by an ISCO syringe pump (Isco, Inc., Lincoln, NE) with a flow rate of 12 ml/min. The frozen particles were collected and lyophilized using a Virtis Advantage Lyophilizer (The Virtis Company, Inc., Gardiner, NY). All other process conditions were reported previously [13].

2.2.2. Lyophilization cycle

The shelf temperature of the lyophilizer was precooled to −40 °C before the frozen samples were loaded. After the samples were loaded, the shelf temperature was increased to −25 °C over 2 h and held for 36 h at 100 mTorr. The shelf temperature was then increased to 25 °C over 4 h and held for 12 h to complete the cycle. For the slow-freeze lyophilization control, samples were frozen in a lyophilizer from room temperature to −40 °C over a 2 h period, held for 2 h at −40 °C, and then lyophilized using the above lyophilization cycle.

2.2.3. Scanning electron microscopy

A Hitachi Model S-4500 scanning electron microscope (Hitachi Ltd, Tokyo, Japan) was used to obtain the SEM photographs. The powder samples were mounted on an aluminum stage using double adhesive carbon conductive tape and coated with gold for 30 s using a Pelco Model 3 sputter-coater in an argon atmosphere.

2.2.4. Fourier-transform infrared spectroscopy (FT-IR)

Lyophilized samples containing 2 mg of BSA were mixed with 300 mg of potassium bromide and pressed at 9000 kg/cm^2 into pellets using a Carver Laboratory Press (Fred. S. Carver, Inc., Menomonee Falls, WI) for secondary structure analysis. Spectra were collected by a Perkin-Elmer Spectrum 2000 Spectrometer. The optical bench and sample chamber were continuously purged with dry N_2. For each spectrum, a 256 scan interferogram was collected in single beam mode, with a 2 cm^{-1} resolution.

2.2.5. Specific surface area

Specific BET surface area was measured using a NOVA-2000 Verion 6.11 instrument with NOVA Enhanced Data Reduction Software Version 2.13 (Quantachrome Corporation, Boynton Beach, FL). A known amount of powder (30 mg) was loaded into a sample cell and degassed for at least 3 h prior to analysis. The samples were measured three times.

2.2.6. Particle size analysis

Twenty milligrams of SFL BSA powder were suspended in 20 ml methylene chloride containing 10% PLGA and sonicated 4 min (Model 450, Branson Ultrasonics Co., Danbury, CT) with an output of 20 W. The particle suspensions in methylene chloride were diluted in acetonitrile and the particle size distribution was determined with a Malvern Mastersizer-S (Malvern Instrument Co., UK). The measurement was repeated three times. Particle diameter is presented as 10% ($D_{v,10}$), 50% ($D_{v,50}$), and 90% ($D_{v,90}$) of the volume distribution.

2.2.7. Size exclusion chromatography

The HPLC system used for size exclusion chromatography (SEC) consisted of a LC-10AT vp pump, SIL-10A autoinjector, and SPD-MI10A vp Diode array detector (Shimadzu USA Manufacturing, Inc., Columbia, MD). A 30 cm × 7.8 mm TSK-Gel G2000SWXL column (Tosoh Biosep, Japan) provided good selectivity. The mobile phase was comprised of 50 mM phosphate buffer containing 0.3 M sodium chloride, pH 7.0. The flow rate was 0.4 ml/min and the injection volume was 50 μl of a 0.4 mg/ml solution. The eluent was detected by measuring UV absorbance at 214 nm.

3. Results and discussion

3.1. Particle morphology and particle size

Protein powders were prepared by SFL or by slow freezing and drying in a lyophilizer (slow freezing control), and each was further compared to the results reported for similar protein powders prepared by SFD [6]. The protein solutions were sprayed beneath the surface of liquid nitrogen without passing through a vapor gap in order to minimize the time for protein denaturation at an air–liquid interface present in the SFD process. The differences in protein stability between SFL and SFD result in part from the deleterious effects of air–liquid interfaces inherent in the SFD process, which are not present in the SFL process. Differences in particle morphology between SFL powders and slow-freeze lyophilization powders are shown in the SEM photographs (Fig. 1). The porous particle surfaces of the SFL powders were composed of interconnected features with sizes smaller than 500 nm (Fig. 1B and E). For the SFD powders, the morphology depended on the atomization conditions [6]. At the best atomization condition reported, the morphology of SFD powders was similar to the morphology of SFL powders seen in Fig. 1B and E. Large smooth sheets that appeared to have little porosity were observed for the powders produced by slow-freeze lyophilization (Fig. 1C and F). The specific surface areas of the SFL and slow-freeze lyophilization samples are given in Table 1. Compared to the particles produced by slow-freeze lyophilization (Fig. 1C and F), the particles generated by the SFL process were highly porous. The specific surface area of the SFL powders varied between 19.2 and 97.7 m^2/g, and the magnitude depended on the particle composition. The specific surface area of the slow-freeze lyophilization sample was about 1 m^2/g (Table 1). The significant increase in specific surface area for the SFL powders is consistent with its highly porous structure and the submicron primary particle domains. For SFD powders, the surface areas were between a few square meters per gram to 160 m^2/g [6]. The magnitudes of the surface areas were similar to SFL powders [6].

The nanostructured microparticles are produced by atomizing a feed solution beneath the surface of a cryogenic liquid. Because the nozzle is inserted beneath the surface and directly into the cryogenic liquid, the droplets begin to freeze instantaneously after they are

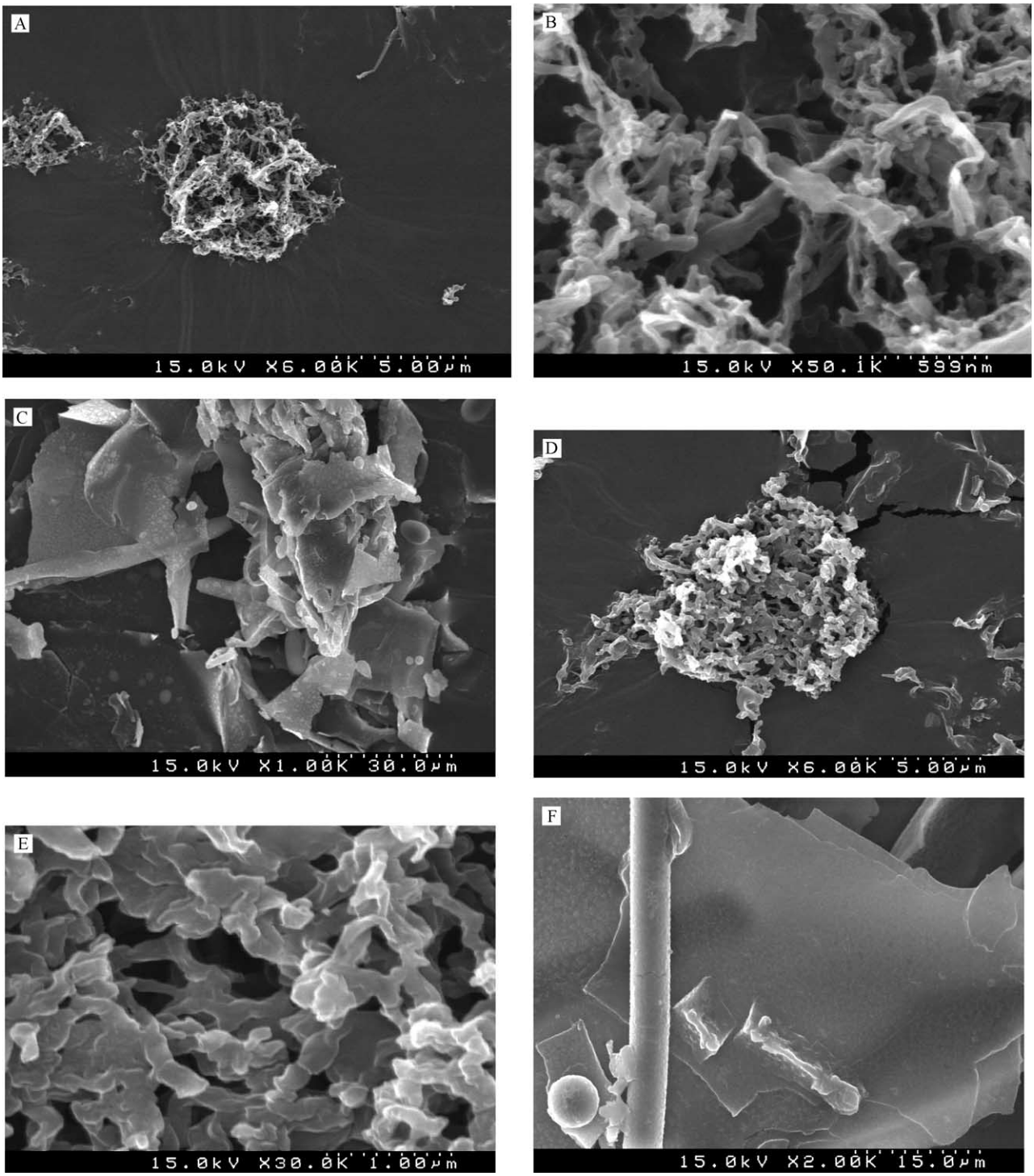

Fig. 1. Scanning electron micrographs of SFL BSA powders. A solution of BSA at a concentration of 5 mg/ml with or without excipients was sprayed into liquid nitrogen to form frozen particles, which were then lyophilized to produce a powder. The same BSA solution was also frozen slowly on the shelf of the lyophilizer and then lyophilized. SFL BSA (A); Close-up view of SFL BSA (B); Slow freezing and lyophilization BSA (C); SFL BSA/tyloxapol (D); Close-up view of SFL BSA/tyloxapol (E); Slow freezing and lyophilization BSA/tyloxapol (F).

formed. The intense atomization of the droplets resulting from the high pressure drop through the small orifice nozzle produces extremely small droplets. The low temperature of liquid nitrogen (−196 °C) and small droplet sizes allowed for very rapid freezing rates to be achieved. The rapid freezing rates obtained in the SFL process generate significant supersaturation and thus rapid nucleation rates of dissolved substances. The rapid nucleation and restricted growth that occurs after the fine droplets are formed and frozen leads to

Table 1
Effect of BSA concentration and composition on the physicochemical properties of powders produced by SFL versus freezing slowly and drying in a lyophilizer

	BSA feed (mg/ml)	Specific surface area (m^2/g)		Monomer loss (%)		Secondary structure content of SFL powders (%)	
		SFL	Control[a]	SFL	Control[a]	α-Helix content	β-Sheet content
BSA control as received	–	–	–	–	–	51	19
BSA[b]	5	32.3 ± 5.6	1.03 ± 0.1	0.4 ± 0.2	1.5 ± 0.4	45 ± 4	23 ± 3
BSA and 1 mg/ml tyloxapol[b]	5	27.7 ± 3.1	1.12 ± 0.22	ND	0.2 ± 0.1	43 ± 4	27 ± 2
BSA and 25 mg/ml trehalose[b]	5	19.2 ± 3.4	0.94 ± 0.15	ND	0.6 ± 0.4	46 ± 5	25 ± 3
BSA[b]	10	71.0 ± 5.4	–	0.5 ± 0.1	–	45 ± 3	26 ± 4
BSA[b]	1	23.5 ± 3.4	–	0.4 ± 0.2	–	43 ± 2	21 ± 6
BSA[c]	5	97.7 ± 3.5	–	0.8 ± 0.2	–	43 ± 6	27 ± 2

ND: none detected.
[a] Control was prepared by freezing slowly and drying in a lyophilizer of the corresponding BSA solution.
[b] BSA and excipients were dissolved in 5 mM phosphate.
[c] BSA was dissolved in deionized water.

extremely small submicron domains following lyophilization.

In a recent SFL study [13], the median particle size of SFL insulin microparticles was in the range of 5–7 μm. It was recently reported that the particle sizes of SFD powders were between 3 and 19 μm depending on atomization conditions [5]. In the present study, SEM photographs (Fig. 1A and D) showed that the particle size of the particle aggregates was about 5 μm. The particle size was about 1/10 the nozzle diameter. One possibility is that the droplets broke up by Rayleigh instabilities and were comparable to the nozzle diameter. In this case, multiple particles would be produced in each droplet. In SFD where droplets of known size are formed before contacting the liquid nitrogen, the particle size should reflect the size of the frozen droplet [5,6]. A second explanation is that the intense atomization in SFL produced aqueous droplets much smaller than the nozzle diameter. In this case, the protein particle size may reflect the size of the droplets produced by the turbulent jet break up in the viscous liquid nitrogen. Also it can be seen in Fig. 1B and E that the 5 μm diameter particles were composed of nanoparticles measuring less than about 200 nm, depending on the composition of the aqueous feed solution containing the BSA. As the water vitrifies to form glassy water domains, the protein is concentrated in the unfrozen solution that quickly freezes. Upon lyophilization of the powder, the water is removed to produce highly porous protein particles. The extremely small submicron protein domains indicate that small domains of water vitrified.

In the slow-freeze lyophilization process, the ice crystals grew slowly in large domains, thus the protein-rich domains were also large. The long time for growth of protein crystals in the aqueous phase led to large protein particles with smooth surfaces and a small specific surface area [12]. The time for nucleation and growth was too long to form submicron protein primary particles.

3.2. Particle size distribution after sonication

The particle size and size distribution are important parameters for incorporation of drug particles into depot delivery systems. SFL powders are of interest in numerous applications in drug delivery, e.g. microspheres for depot delivery and pulmonary delivery because of the desirable physical properties and protein stability. It would be desirable to encapsulate small particles into polymer microspheres to minimize initial release. For 50 μm particles, solid protein particles in the order of a few hundred nanometers may be distributed more uniformly than commonly used particles larger than 1 μm. In addition, small particle size has been linked to improved encapsulation efficiency [6].

The porous particles were micronized into smaller particles by sonication. Sonication has been used in the initial processing step in solid-in-oil-in-oil (s/o/o) microencapsulation processes in order to disperse solid protein powder in an organic solvent [6]. Results for the particle size distribution for all SFL BSA samples are presented in Table 2. The $D_{v,10}$, $D_{v,50}$ and $D_{v,90}$ indicate the measured particle size at 10, 50 and 90% of the volume distribution, respectively, for the powders sonicated in methylene chloride containing 10% PLGA. PLGA was included in methylene chloride in order to simulate conditions that would be used in an encapsulation process, as has been done previously [6]. The $D_{v,10}$ of the sonicated powders was about 100 nm. The $D_{v,50}$ of sonicated powders was between 300 and 400 nm indicating the extremely rapid nucleation and prevention of growth in the SFL process. Particles in this size range are consistent with the primary particles in the SEM photographs (Fig. 1). Two peaks in the curve in Fig. 2 indicate the presence of nanoparticles and nanoparticle aggregates. The particle size of SFL powder before sonication is also displayed. The loosely flocculated SFL aggregates are readily broken up into nanoparticles upon sonication. A similar size distribution was reported

Table 2
Particle size distribution of powders produced by SFL

	Particle size distribution[a]			
	$D_{(v,10)}$ (μm)	$D_{(v,50)}$ (μm)	$D_{(v,90)}$ (μm)	Span index[b]
BSA	0.11 ± 0.05	0.39 ± 0.08	3.94 ± 0.41	9.82
BSA and Tyloxapol	0.12 ± 0.03	0.31 ± 0.04	1.24 ± 0.25	3.61
BSA and trehalose	0.25 ± 0.1	2.10 ± 0.32	4.88 ± 1.12	2.20

SFL powders were sonicated for 4 min in methylene chloride containing 10% PLGA and particle size was measured by a Malvern Mastersizer.
[a] Values represent mean and standard deviation of sonicated SFL particles.
[b] Span index was calculated from $(D_{90} - D_{10})/D_{50}$.

previously for particles produced by SFD followed by sonication [6].

3.3. Influence of composition on moisture content, particle size and surface area

The composition of the SFL feed solution also influenced the particle size distribution and particle morphology. In Fig. 1B and E, the SFL particles were highly porous and were composed of submicron domains. The SFL/tyloxapol sample prepared by SFL in Fig. 1E had a smoother surface morphology than the other samples. The particles after sonication were similar to the excipient-free sample. For the sample with trehalose, the $D_{v,50}$ of particles after sonication was significantly higher than that of other two samples ($P < 0.05$). This was caused by the use of a high concentration of trehalose. The ratio of BSA to trehalose was 1:5 (Table 1). The high concentration of trehalose led to larger nanoparticles, with much lower surface area, compared to the excipient free sample. The much higher concentration of solutes for this system may be expected to produce larger particles for various reasons. For a given aqueous droplet size, the larger solute concentration will lead to a larger number of collisions between solute molecules as the water freezes, enhancing particle growth. The greater viscosity of the aqueous solution may also lead to a smaller degree of atomization as has been seen for polyethylene oxide solutions [22]. The trehalose in the samples also changed the moisture content (Table 3). Samples without trehalose contained more moisture compared to the samples with trehalose. The moisture content of 2–7% was in the same range as reported by Constantino et al. [6].

The concentration of BSA in the liquid feed solutions influenced the specific surface area of the resulting SFL powder. As shown in Table 1, the specific surface areas of SFL BSA powders were 23.5, 32.3 and 71.0 m^2/g for BSA concentrations of 1, 5, and 10 mg/ml, respectively. As the concentration of BSA increased, the surface area increased. The ratio of BSA to phosphate buffer salt was low for low BSA feed concentrations, and the resulting SFL powder was much denser than for the case of higher BSA to salt ratios. When the concentration of BSA was1 mg/ml, the weight ratio of protein to buffer was about 1:1. To understand the effect of the phosphate buffer, BSA was dissolved in deionized water at the same concentration. The specific surface area was 97.7 m^2/g as compared to 32.3 m^2/g for samples in which the BSA was dissolved in 5 mM phosphate buffer. This result demonstrated that presence of phosphate buffer salt influenced the specific surface area of SFL powder.

3.4. Stability of SFL protein

Proteins undergo physical and chemical degradation, and these instabilities present unique and challenging difficulties in their formulation, production and storage. The preservation of secondary, tertiary and quaternary, as well as primary structure is necessary in order to retain biological activity of proteins [3]. Denaturation aggregation and chemical degradation are frequent manifestations of protein instability in the formulation and storage of protein pharmaceuticals.

Aggregation of BSA was measured by SEC. Data are reported as a decrease in percent monomer compared to the protein prior to SFL processing (Table 1). In cases where the monomer content was higher in the dried protein than in the starting material, the decrease in percent monomer is reported as zero. SFL samples without excipient had 0.4–0.8% monomer loss for various BSA concentrations (Table 1). The monomer loss was extremely small even though the surface area of powder ranged from 19.2 to 97.7 m^2/g. In a recent SFD study, the monomer loss ranged

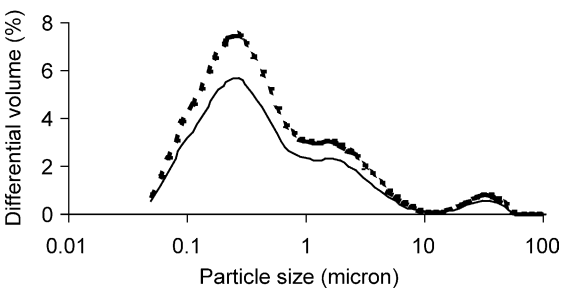

Fig. 2. Particle size distribution of sonicated BSA dry powder. SFL powders were sonicated for 4 min in methylene chloride and the particle size distribution was measured by a Malvern Mastersizer.------ SFL BSA.— SFL BSA/tyloxapol.

Table 3
Moisture content of SFL samples

	BSA feed (mg/ml)	Moisture Content (%)
BSA[a]	5	6.29 ± 0.63
BSA and 1 mg/ml tyloxapol[a]	5	7.12 ± 0.32
BSA and 25 mg/ml trehalose[a]	5	2.87 ± 0.82
BSA[a]	10	7.57 ± 2.36
BSA[a]	1	8.47 ± 2.04
BSA[b]	5	4.64 ± 0.98

[a] BSA and excipients were dissolved in 5 mM phosphate.
[b] BSA was dissolved in deionized water.

from a few percent to 16% [6]. Furthermore, the monomer loss increased with decreasing particle size, and was correlated with specific surface area [6]. The monomer loss of BSA powder was 7.2% when the specific surface area was 90 m^2/g (Table 4). This represents approximately a nine-times greater monomer loss for SFD as compared to the monomer loss produced by SFL for a similar surface area.

Part of the difference in monomer loss may be due to differences in the as-received BSA. The starting material in SFD [6] had a monomer of 71%, whereas it was 83% in our study, despite the fact that the source was the same.

Protein adsorption at the air–liquid interface plays a key role in the SFD process [8]. In the SFD process, the protein solution was atomized into the vapor phase of liquid nitrogen using a two fluid nozzle. This initial atomization step creates small droplets measuring less than 50 μm diameter which corresponds to 0.12 m^2/ml air–liquid interface [8]. During the time the droplets pass through the vapor until they contact the surface of the liquid nitrogen, surface active protein migrates to the air–liquid interface leading to denaturation and aggregation. Webb et al. [8] reported that this interface was the primary reason for monomer loss. However, during the SFL process presented in the present study, protein solution was directly sprayed into liquid nitrogen and atomized by intense impingement. The protein did not pass through the vapor phase as in previous studies of SFD. Costantino et al. [6] reported the atomization conditions of their SFD process and showed that specific surface area increased with more intense atomization. At the same time air–liquid interfacial area increased because of smaller droplet size. The higher monomer loss may have been caused by the larger air–liquid interfacial area, on the basis of Webb et al. [8].

Table 4
Monomer loss versus surface area for particles produced by spray freeze drying and spray freezing into liquid (SFL) nitrogen

SFD[a]		SFL	
Surface area (m^2/g)	Monomer loss (%)	Surface area (m^2/g)	Monomer loss (%)
28	2.0	23.5	0.4
37	3.8	32.3	0.4
77	6.0	71	0.5
90	7.2	97.7	0.8

[a] Values estimated from Figure 4 of Costantino et al. [6].

Protein adsorption at air–liquid interfaces requires sufficient time for diffusion of the protein to the interface. Based on the calculation by MacRitchie [23], the diffusion time for 0.03 mg/ml BSA was about 6 s to form a monolayer at the air–liquid interface. However, concentration of BSA is much higher in SFD and the diffusion distance is much smaller thus, the time for diffusion to the interface is much shorter. Webb et al. [8] estimated that the induction time for 5 mg/ml human serum albumin to diffuse to the interface was in the order of milliseconds for 10 μm droplets. The time for droplets falling from an open nozzle to the surface of liquid nitrogen is about 1 s for spray-freeze drying [5]. This time (e.g. 1 s) was sufficient of allow for protein diffusion to the air–liquid interface. The adsorbed protein may unfold at the interface and is then subject to aggregation [8]. However, the SFL process avoids the time in which droplets pass through the vapor phase, thus lowering time for protein to diffuse to the interface. The intense atomization of the droplets produced extremely small droplets in the order of about 10 μm in diameter based on the size of the larger particles found in the protein size distribution. The low temperature of liquid nitrogen (−196 °C) and small droplet size allow for very rapid freezing rates to be achieved. Based on previous reports, the maximum cooling rates achievable with liquid nitrogen are in the order of 10^3 K s^{-1} when the droplet size is about 10 μm [24,25]. With the median droplet diameter of about 5 μm, the time for the droplet temperature to reach the freezing point of the solution is less than 1 ms [4] after it contacts the liquid nitrogen. In the SFL process in which the nozzle is inside the liquid nitrogen, the ultra-rapid freezing of the small droplets provides minimal time for diffusion of protein to an interface. The low degree of monomer loss observed in this study is consistent with the ultra-rapid freezing.

An additional factor that must be considered is the influence of the low temperature stresses of the freezing on protein stability. An early quantitative study on low temperature denaturation of a model protein was conducted by Shikama and Yamazaki [26]. They demonstrated a specific temperature range in which ox liver catalase was denatured during freezing followed by thawing. Cold denaturation of catalase at 8.4 μg/ml in 10 mM phosphate buffer (pH 7.0) started at −6 °C. Loss of catalase activity reached 20% at −12 °C, remained at this level between −12 °C and near −75 °C, then decreased gradually from −75 to −120 °C. There was almost no activity loss between −129 and −192 °C. Similar results were also obtained for ovalbumin by Koseki et al. [27]. Maa and Prestrelski [4] pointed out in their recent review that rapid freezing rates (e.g. 1 ml solution in a glass vial submerged in liquid nitrogen) at very low temperature may denature protein.

Rapid freezing can promote supercooling which produces small ice crystals which increase the ice–water interface and cause the denaturation of protein [28]. However, it was also reported that the ice–water interface was much milder than other interfaces (e.g. air–liquid) for protein denaturation [8]. In SFL, the time that the protein in the unfrozen solution is exposed to ice crystals or a water–glass interface is much shorter than in the case of submersion of a vial into liquid nitrogen. The results in this study show very little aggregation due to any of the steps in the SFL process.

3.5. Examination of SFL protein secondary structure

FT-IR spectroscopy offers non-invasive determination of secondary structure for proteins both in solution and in the dried state [29]. Table 1 lists the α-helix content of BSA in the solid state. The overall content of α-helix of all the samples was more than 40% and β-sheet content was about 25%. The α-helix content dropped modestly from the value of 51% for the crystallized native protein. No correlation was observed between secondary structure in the powders and either specific surface area or protein monomer loss. The α-helix contents were not statistically different ($P > 0.05$). This observation is similar to that reported for SFD for BSA [6]. However, the α-helix content of SFL protein samples was higher than those for slow freezing–lyophilization and most SFD protein samples recently reported [6]. The α-helix content reached 46% when the SFD samples contained trehalose [7]. However, in our study, the excipient-free formulation contained up to 40% α-helix structure relative to 30% in the previous study. Addition of trehalose only increased the α-helix content slightly. It may be assumed that some of the loss in helix structure may recover upon rehydration, given that the aggregation of the SFL powders after dehydration was very small.

4. Conclusions

Protein microparticles produced by SFL were highly porous with high surface areas and extremely low levels of monomer loss, less than 1%. For these high surface area particles, monomer loss is reduced relative to SFD. Some of the difference may be due to modest differences in the properties of the as-received BSA in the two studies, thus further study is warranted. The more rapid freezing in SFL lowers the time for proteins to aggregate or diffuse to water–ice and water–air interfaces where they may be denatured. The time of exposure of protein to the large air–liquid interface, which is known to cause aggregation [8] is much longer in SFD than in the SFL process. The intense atomization and ultra-rapid freezing enabled formation of nanostructured BSA microparticles with surface areas of tens of m^2/g. These high surface areas are not produced by slow freezing and lyophilization in vials. The secondary structure of BSA was better preserved by SFL compared to slow-freezing/lyophilization and SFD. The ability to produce high surface area protein nanoparticles with minimal aggregation may be expected to be advantageous in many drug delivery applications including depot delivery from biodegradable microspheres (more uniform encapsulation) and pulmonary delivery.

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