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Research paper

Stable high surface area lactate dehydrogenase particles produced by spray freezing into liquid nitrogen

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Abstract

Enzyme activities were determined for lactate dehydrogenase (LDH) powder produced by lyophilization, and two fast freezing processes, spray freeze-drying (SFD) and spray freezing into liquid (SFL) nitrogen. The 0.25 mg/mL LDH aqueous feed solutions included either 30 or 100 mg/mL trehalose. The SFL process produced powders with very high enzyme activities upon reconstitution, similar to lyophilization. However, the specific surface area of 13 m^2/g for SFL was an order of magnitude larger than for lyophilization. In SFD activities were reduced in the spraying step by the long exposure to the gas–liquid interface for 0.1–1 s, versus only 2 ms in SFL. The ability to produce stable high surface area submicron particles of fragile proteins such as LDH by SFL is of practical interest in protein storage and in various applications in controlled release including encapsulation into bioerodible polymers. The SFL process has been scaled down for solution volumes <1 mL to facilitate studies of therapeutic proteins.

Keywords: Lactate dehydrogenase (LDH); Spray freezing into liquid (SFL); Spray freeze-drying (SFD); Gas–liquid interface; Atomization

1. Introduction

Due to the limited physical and chemical stability of proteins in solution, it is often necessary to produce a solid protein formulation to achieve an acceptable shelf life [1–6]. Protein particles are also of interest for encapsulation into bioerodible microspheres or other matrix geometries for subcutaneous, intramuscular, transdermal and pulmonary delivery [7–14]. To achieve high protein loadings and to minimize burst release, high surface area, submicron protein particles have been encapsulated uniformly into 10–50 μm diameter microspheres [8,9,15–17]. However, it is challenging to produce stable submicron protein particles when the surface area exceeds 10 m^2/g [9,18,19], relative to less than 1 m^2/g for powders produced by lyophilization.

Protein particles may be precipitated from aqueous solution by a variety of processes including spray-drying [20–23], supercritical CO_2-assisted aerosolization and bubble drying ($scCO_2$A-BD) [24], spray freeze-drying (SFD) [5,18–20], and spray freezing into liquids (SFL) [25–27]. The spraying, freezing and drying steps in these processes must be designed to minimize stresses that cause destabilization [1,3,4,6]. Loss in stability may be characterized in terms of protein denaturation, aggregation, and loss of enzyme activity upon rehydration [4,20,28]. During spraying and freezing, stresses arise from changes in solute concentration and pH in the remaining unfrozen solution, as well as exposure of protein to gas–liquid and/or ice–liquid interfaces [3,6]. During drying, dehydration stress from removal of water molecules can subsequently lead to protein unfolding [1,6,29]. Stabilizers, such as disaccharide

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sugars, often increase the thermodynamic stability of the native folded protein during freezing and prevent the protein from unfolding during drying through replacement of hydrogen bonds to the protein upon loss of water [1,3,29–31].

In SFD, an aqueous solution containing dissolved protein and stabilizers is atomized into the cold gas above a cryogenic liquid (Fig. 1) [18,19,32–34]. The micron-sized droplets travel through the cryogenic gas, where they may begin to freeze [35], and then freeze completely after contact with the liquid cryogen. Decreasing the aqueous droplet diameter leads to more rapid freezing resulting in a greater specific surface area (SSA) of the final powder [3,5,18]. As the SSA increases, the particles become more friable and may be micronized into smaller particles upon sonication [9,18]. By systematically studying the effects of the separate spraying, freezing and drying steps in SFD, it was shown that the large gas–liquid interface in the spraying step was the primary cause of protein aggregation for recombinant human interferon-γ (rhIFN-γ) [19] and lysozyme [26]. The protein adsorbs at this interface and subsequently unfolds and forms aggregates [19,36]. The freezing and drying steps had a minimal effect on protein stability. For rhIFN-γ, Webb et al. [19] reported that a protein in the freeze concentrate during rapid freezing would not have sufficient time to diffuse to the ice–liquid interface and denature before being trapped in an amorphous glass [19]. To minimize gas–liquid interface in the SFD process, the size of the atomized droplets may be increased, but at the expense of the SSA of the powder [5,18]. Therefore, the formation of high SSA protein powder by SFD has often resulted in an undesirable degree of denaturation and aggregation for a variety of proteins [5,18–21].

In spray-drying, the protein is exposed to the gas–liquid interface of the atomized droplets which are typically of the order of 10 μm in diameter [20,23]. In spite of the relatively large gas–liquid interface formed upon atomization in spray-drying, Nguyen et al. showed that the protein darbepoetin alfa remained stable, whereas it aggregated in the SFD process [21]. Adler and Lee produced ~9 μm diameter particles by spray-drying of a formulation of lactate dehydrogenase (LDH) with 100 mg/mL trehalose as an excipient and showed that approximately 10% of the activity was lost [23]. The addition of Tween 80 to the formulation improved the initial activity, but it was harmful to the storage stability [23]. Although spray-drying can produce particles that are between 1 and 10 μm in diameter and relatively stable [21], the SSAs of protein powders are much lower than in SFD and SFL [21,22].

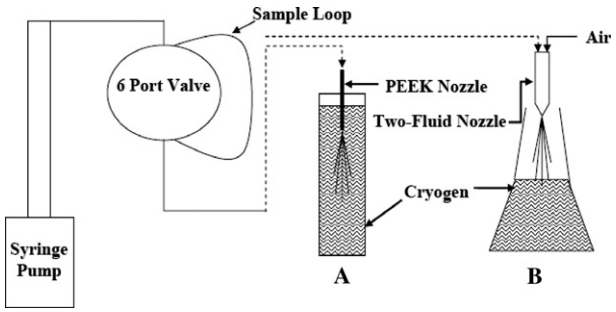

Fig. 1. Apparatus for SFL (A) and SFD (B) sprays of solution volumes of 1 mL.

A novel spray freezing into liquid (SFL) process has been developed to minimize exposure of protein to the gas–liquid interface and yet sustain rapid freezing rates to produce high SSA powder [25–27]. As shown in Fig. 1, the feed nozzle is immersed under the cryogen surface to minimize the time of exposure to nitrogen gas–liquid interface [25–27]. The SFL process has also been used for freezing dilute organic solutions of poorly water soluble drugs [37–40] to produce powders with high SSA and enhanced dissolution rates. For dilute (5–10 mg/mL) aqueous peptide and protein solutions the resulting powder SSAs have been greater than 100 m^2/g [9,25–27,39]. For lysozyme, a reduction in the time of exposure to the gas–liquid interface in the spraying step for SFL led to a smaller degree of aggregation and loss in enzyme activity relative to SFD. In addition, the subsequent freezing and drying steps for SFL and SFD were shown to produce an insignificant amount of additional protein destabilization [19,26]. Since lysozyme is a relatively stable protein, it would be interesting to compare protein stabilities for SFL and SFD for much more fragile proteins.

Lyophilization [41–43], spray-drying [23] and SFD [44] of the fragile protein LDH have been studied extensively. LDH consists of 4 subunits that form a tetramer in solution with an approximate molecular weight of 144 kDa and a hydrodynamic radius of approximately 4 nm, as measured by dynamic light scattering [45,46]. It has already been shown to be destabilized by exposure to the gas–liquid interface in both spray-drying and SFD studies [23,44]. In order to achieve high LDH activities, it was necessary to use a high trehalose concentration (>100 mg/mL) and a surfactant such as Tween 80 to minimize dissociation of tetrameric LDH at the gas–liquid interface [23,44].

Proteins such as LDH are much more stable in aqueous buffer solutions in the presence of high sugar concentrations. A recent companion study has shown differences in the morphologies of lysozyme powders produced by SFL and SFD with liquid nitrogen (LN2) for concentrated (>50 mg/mL) feed solutions [47]. It was shown with flow visualization, calculated heat transfer rates, and SSAs of lysozyme powder that the cooling rate in SFL (7200 K/s) was approximately 2–3 orders of magnitude slower than in SFD [47]. The slower cooling rate in SFL was a result of LN2 boiling around the liquid jet forming an insulating gas layer, known as the Leidenfrost effect. For concentrated feed solutions, the slower cooling rate of the SFL process led to surface areas of the order of 30 m^2/g, compared to 30–120 m^2/g for SFD [47]. However, the powder SSA was still more than an order of magnitude higher than in conventional lyophilization. Therefore, it can be expected that LDH

formulations processed by SFL will also have high powder SSAs.

The primary objectives of this study were: (1) to produce LDH powders with surface areas greater than 10 m^2/g and high enzyme activities approaching those for low surface area particles (<1 m^2/g) produced in lyophilization, (2) to compare enzyme activities in SFL relative to SFD, particularly in view of differences in the time of exposure to the gas–liquid interface. LDH is a highly fragile tetramer, and thus sugars such as trehalose have been used to stabilize LDH in solution. Two concentrations of trehalose, 30 and 100 mg/mL were utilized to influence the particle surface area and protein stability. Three types of freezing processes were considered in this study. The first type, as exemplified by tray lyophilization and freezing of large slowly falling droplets into liquid nitrogen, is characterized by a slow cooling rate and low exposure to gas–liquid interface during freezing. The second and third types were the rapid freezing processes, SFD and SFL. They will be shown to differ in two primary ways: the longer exposure to the gas–liquid interface in SFD, and the less intense atomization in SFL, as a consequence of the low viscosity of evaporating N_2. The droplet size in the SFD spray was varied to investigate the effect of the gas–liquid interfacial area and the final SSA of the powder. In SFL, the time of exposure of the liquid jet to nitrogen gas will be shown to be orders of magnitude shorter than for exposure of atomized droplets to gas in SFD. This pronounced reduction in exposure to the gas–liquid interface will be shown to lead to high enzyme activities, without the need to add a surfactant such as Tween 80. The ability to minimize the addition of surfactant excipients can often be beneficial, especially as they can be detrimental to long term stability [1,23]. Furthermore, the high SSAs of particles produced by SFL relative to lyophilization can be advantageous for the dispersion of these particles within drug delivery matrices including bioerodible polymers [9]. A secondary objective of this study was to reduce the volume of feed solution to 1 mL relative to volumes greater than 20 mL in previous studies [9,26,27] in order to facilitate studies of expensive proteins.

2. Materials and methods

2.1. Materials

L-LDH from porcine heart suspended in a 3.2 M ammonium sulfate solution was purchased from Roche Applied Science (Indianapolis, IN). Trehalose, NADH and pyruvate were purchased from Sigma Chemical Company (St. Louis, MO). The water was deionized by flowing distilled water through a series of 2×7 L mixed bed DI vessels (Water and Power Technologies, Salt Lake City, UT) containing 60:40 anionic/cationic resin blends.

2.2. Enzyme preparation and catalytic activity assay

The LDH in ammonium sulfate was dialyzed against 10 mM KPO_4 buffer (pH 7.5) at 4 °C for 3 h before use [41,42] with Spectra/Por® Dialysis Membrane tubing with a molecular weight cutoff of 12,000–14,000 (Fisher Scientific, Houston, TX). Protein concentration was determined with a μQuant Model MQX200 spectrophotometer (Biotek Instruments Inc., Winooski, VT) with an absorbance at 280 nm. A value of 1.37 mL/mg cm was used for the LDH extinction coefficient [41]. LDH samples were then diluted to 0.25 mg/mL in both 30 and 100 mg/mL trehalose solutions in 10 mM KPO_4 buffer (pH 7.5).

The LDH assay was prepared by adding 0.1 mL of 20 mM pyruvate and 0.05 mL of 11 mM NADH prepared in 0.1 M KPO_4 buffer (pH 7) to 2.5 mL of 0.1 M KPO_4 buffer (pH 7) at 25 °C into 3 plastic cuvettes which were then mixed by inversion [23]. Prior to adding LDH to the reagents, each LDH formulation was diluted with 10 mM KPO_4 buffer to form LDH concentrations of 2, 3, and 4 μg/mL. Aliquots of 0.05 mL of each LDH dilution were added simultaneously with an 8 channel Transferpette® pipet (Brand, Wertheim, Germany) to the 3 plastic cuvettes filled with the reagents to make final protein concentrations of 37, 56, and 74 ng/mL, respectively. The 3 samples in the plastic cuvettes with protein were mixed again by inversion and then 3 aliquots of 230 μL of each solution were immediately placed in a 96-well Falcon plate with the Transferpette® pipet and set in the spectrophotometer. The total time from adding the LDH to the reagents to measuring absorbance data was 30 s. Units of LDH activity (U) were calculated by measuring the decrease in absorbance of NADH at $\lambda = 340$ nm every 15 s for 1 min due to the conversion of NADH to NAD over time ($U = \Delta\mu$mol NADH/min) and then dividing by the mass (mg) of the LDH protein in solution to determine specific activity (U/mg). For the protein concentration range tested during the 1 min the measurements were made, the rate of NADH decrease remained linear and began to show significant deviation from linearity between 2 and 4 min after making the measurement. For final protein concentrations greater than 74 ng/mL, the activity measurement could not be obtained quickly enough since deviation from the linear rate of absorbance occurred in less than 1 min.

For the LDH formulations with 30 and 100 mg/mL trehalose the stability of the LDH in buffer solution over time was measured. For the formulation with 30 mg/mL trehalose the LDH specific activity remained stable for an hour and then began to decrease. For the formulation with 100 mg/mL trehalose the LDH specific activity remained stable for up to 6 h before LDH activity began to decrease. All experiments were performed in the time period where the LDH specific activity had not decayed. During this time period, the specific activity was defined as 100%. Here, the measured specific activities of LDH formulated with 30 or 100 mg/mL trehalose were not significantly different.

2.3. Freeze procedures

2.3.1. Spray freezing into liquid (SFL)

The SFL process was modified slightly from a previous study [25,47]. To accommodate the smaller volumes of LDH sample, the protein solution was loaded into a 1 mL sample loop in a six port rotary valve (Valco Instruments Company, Inc., Houston, TX). DI water was used to displace the LDH sample through the sample loop and then through the SFL nozzle. The liquid flow rate was 10 mL/min resulting in a pressure drop of 17.2 MPa through a 63 μm ID poly-ether-ether-ketone (PEEK) nozzle 5 cm in length (Upchurch Scientific, Oak Harbor, WA) (Fig. 1). A 1 L plastic bottle 8.5 cm in diameter and 17.6 cm in height containing a 2.54 cm length, 0.952 cm diameter octagonal magnetic stir bar (Fisher Scientific, Somerville, NJ) was filled with LN2 and placed in a 4 L insulated bucket (Fisher Scientific, Somerville, NJ) also filled with LN2. The ice bucket was placed on a stir plate. The depth of the LN2 vortex was approximately 2.5 cm and the nozzle was placed 2.5 cm beneath the minimum LN2 vortex level. Once the spray was completed, stirring was stopped allowing the frozen slurry to settle. Excess LN2 was decanted, and the slurry was then transferred to 50 mL polypropylene tubes (Part No. UP2255, United Laboratory Plastics, St. Louis, MO) 2 cm in diameter and 16 cm in height using a spatula pre-cooled in LN2. The plastic tubes were held in a −80 °C freezer to remove residual LN2 and then were transferred to the pre-cooled lyophilizer shelf.

2.3.2. Spray freeze-drying (SFD)

The SFL apparatus described above was also modified for SFD to handle 1 mL sample volumes (Fig. 1). The aqueous feed solution was sprayed with a 0.7 mm diameter two-fluid nozzle (Büchi, Laboratory-Techniques, Switzerland) at a constant flow rate of 10 mL/min resulting in a pressure drop of 0.827 MPa (Fig. 1). Droplet sizes were tuned by varying the air flow rates. Droplet size distributions were measured by laser light scattering (Malvern Mastersizer-S, Malvern Instruments, Ltd., Worcestershire, UK) by mounting the nozzle 10 cm above the laser beam and spraying the droplets normal to the laser beam [47]. Obscuration values were between 8% and 12%.

The SFD setup was comparable to that of Maa et al. [22], who used a flow rate of 15 mL/min, and droplet sizes were shown to be similar [47]. The SFD nozzle was mounted 10 cm above the surface of the cryogen. Cryogen preparation and post-spray slurry collection were performed as presented in the SFL spray section, with the exception that 1 L of LN2 was utilized in a 2 L Erlenmeyer flask. For SFD air flow rates of 250 mL/s the LN2 splashed 5 cm above the liquid surface inside the Erlenmeyer flask which quickly dropped the gas temperature surrounding the air plume to −196 °C 10 cm above the cryogen surface. Reducing the air flow rates to 50 mL/s decreased LN2 splashing and increased the gas temperature to −140 °C. For no air flow, the gas temperature above the LN2 was −30 °C.

2.3.3. Falling droplets into liquid nitrogen

The aqueous feed solution was passed through a 127 μm ID, 1.59 mm OD PEEK nozzle at a flow rate of 2 mL/min producing 3.6 mm diameter droplets. The nozzle was held 10 cm above a beaker of the stirred cryogen.

2.4. Lyophilization

Lyophilization samples were prepared by placing 1 mL of formulation in a 50 mL polypropylene tube (Part No. UP2255, United Laboratory Plastics, St. Louis, MO) and placing it on a pre-cooled −80 °C shelf. The samples remained on the shelf until they had completely frozen which took approximately 20 min. The samples were then transferred to the pre-cooled lyophilizer shelf.

2.5. Spray into air (SA)

To test the effect of gas–liquid interface on LDH activity independently of the freezing and drying steps in the SFD process, 1 mL of the protein solution was sprayed into an empty beaker with the two-fluid nozzle. In this spray into air (SA) process, 10 μm diameter droplets were produced, and LDH activities were measured immediately after spraying.

2.6. Drying and shelf loading

A Virtis Advantage Lyophilizer (The Virtis Company, Inc., Gardiner, NY) was used to dry the frozen slurries. The 50 mL plastic tubes containing the frozen slurries were covered with a single layer Kim-wipe held in place by a rubber-band in order to prevent powder from leaving the vial. Primary drying was carried out at −40 °C for 36 h at 300 mTorr and secondary drying at 25 °C for 24 h at 100 mTorr. A 12 h linear ramp of the shelf temperature from −40 to +25 °C was used at 100 mTorr.

2.7. Reconstitution and protein concentration assay

Dried LDH powders were reconstituted with 1 mL of DI water and then the enzyme assay was immediately performed. After all protein samples had been assayed, the protein concentration was measured with the BCA analysis kit (Sigma Chemical Company, St. Louis, MO). Once protein concentrations were determined, the specific activity from each measurement could be calculated. The activity of each LDH sample, formulated either in 30 or 100 mg/mL trehalose, was normalized by the specific activity of the control measured immediately before the freezing process.

2.8. Transfer and storage of dried powders

After the lyophilization cycle was complete, the lyophilizer was purged with nitrogen. The 50 mL plastic tubes were then rapidly transferred to a dry box held at 14% RH, and the powders were transferred to 20 mL scintillation vials. The vials were then covered with 24 mm Teflon® Faced Silicone septa (Cat.#W240588, Wheaton, Millville, NJ) which were held in place by open-top screw cap lids (VWR Scientific Products, Austin, TX). Vials were purged with dry nitrogen for 2 min by inserting a needle from a dry nitrogen source through the septa with an additional needle for the gas effluent.

2.9. Surface area measurement

Surface areas of dried powders were measured with a Quantachrome Nova 2000 (Quantachrome Corporation, Boynton Beach, FL) BET apparatus. Dried powders were transferred to the glass BET sample cells in a dry box. Samples were then degassed under vacuum for a minimum of 12 h. The Brunauer, Emmett, and Teller (BET) equation [48] was used to fit adsorption data of nitrogen at 77 K over a relative pressure range of 0.05–0.30. The samples were measured two times.

2.10. Residual moisture content

Aliquots of methanol were dispensed through the septum of the scintillation vials to form a suspension concentration of 10–100 mg/mL. Vials were then placed in a tub sonicator (Mettler Electronics, Anaheim, CA) for 5 min at maximum power to insure complete suspension of the powder. Moisture content was measured for a 200 μL aliquot with an Aquatest 8 Karl-Fischer Titrator (Photovolt Instruments, Indianapolis, IN). The moisture values were corrected with a 200 μL methanol blank. All samples had a moisture content between 2% and 3% (w/w) after drying which compared well to the residual moisture contents of 2–7% (w/w) for BSA prepared by SFD as presented by Costantino et al. [18].

2.11. Scanning electron microscopy

SEM images were collected on a Hitachi Model S-4500 scanning electron microscope (Hitachi Ltd., Tokyo, Japan). The samples were prepared in a dry-box. Aluminum stages fitted with double adhesive carbon conducting tape were gently dipped into sample vials until covered by powder. Stages were then placed in septum capped vials and purged with nitrogen for transfer. To minimize the time samples were exposed to atmospheric moisture the stages were rapidly transferred to a Pelco Model 3 sputter-coater. A conductive gold layer was applied and the samples were then quickly transferred to the SEM. Total exposure to the atmosphere was less than 1 min.

3. Results

In SFL, the exposure of protein to the gas–liquid interface is minimized by the insertion of the nozzle below the meniscus of the cryogenic liquid. Recently, the characteristics of the spray in SFL were observed visually in a transparent insulated dewar. It was shown [47] that the nozzle tip in the SFL spray was hidden by a large expanding nitrogen gas cone produced by the spray, as shown in Fig. 2. The formation of the gaseous N_2 cone was due to the Leidenfrost effect that is an insulating layer of vaporized LN2 which is facilitated by nitrogen's small heat of vaporization. The heat of vaporization is supplied by the liquid aqueous jet. The narrow liquid aqueous jet was observed to penetrate at least 10 cm below the liquid nitrogen surface into the gaseous cone, as shown in Fig. 2. The jet breakup is delayed by the low density and viscosity of the nitrogen gas, relative to the case of a non-evaporating cryogen such as isopentane [47,49,50]. The delay in jet breakup is evident in the appearance of ice crystals emanating from the bottom of the jet, which were subsequently circulated in the flask by the large gas flux of evaporating LN2. Following the spray, large, dense frozen particles were suspended in the quiescent LN2 [47].

The activities for LDH with 100 mg/mL trehalose frozen by lyophilization, SFL, and falling droplets were

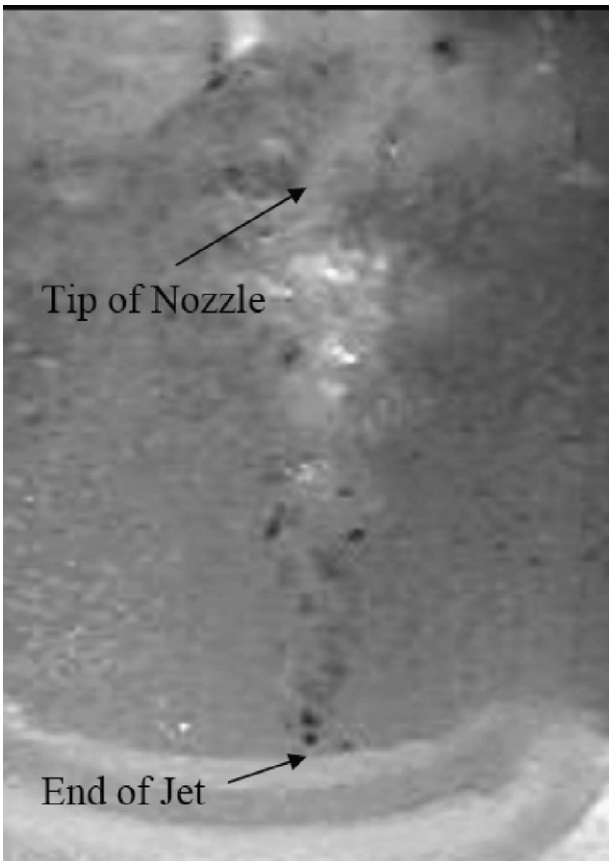

Fig. 2. SFL of dye solution sprayed at 10 mL/min as seen through double glass dewar in LN2.

Table 1
Activities for 0.25 mg/mL LDH, 100 mg/mL trehalose formulations prepared in pH 7.5, 10 mM KPO_4 buffer

Freeze process	%Activity	Replicates (n)
Lyophilization	107 ± 9.7	3
Falling droplet	114 ± 6.7	3
SFL	114 ± 6.9	4
SFD-130 μm	97 ± 9.3	5
SFD-40 μm	87 ± 6.8	5
SFD-10 μm	84 ± 6.1	5
SA-10 μm	85 ± 7.7	3

Table 2
Activities for 0.25 mg/mL LDH, 30 mg/mL trehalose formulations prepared in pH 7.5, 10 mM KPO_4 buffer

Freeze process	%Activity	Replicates (n)
Lyophilization	97 ± 5.4	3
Falling droplet	92 ± 6.4	3
SFL	98 ± 5.3	4
SFD-130 μm	85 ± 8.2	3
SFD-40 μm	74 ± 6.7	5
SFD-10 μm	80 ± 5.4	4

extremely high (Table 1) and not significantly different ($p < 0.05$) from each other according to a Student's t test where the number of replicates (n) is shown in Table 1. The activities are even higher than the measured activities of the original dialyzed formulation. The large trehalose concentration present during drying may have altered the protein conformation resulting in higher activity after drying and reconstitution [41,51]. The very high LDH activities for these three processes demonstrate that the protein did not lose activity from the combined stresses of the freezing, drying and reconstitution steps. As calculated previously [47], the 3–4 orders of magnitude increase in cooling rate provided by the SFL process compared to lyophilization suggests that the greater ice–liquid interfacial area created from a faster cooling rate does not result in LDH activity loss.

The SFL activities were significantly higher than those produced for all three droplet sizes in SFD (Table 1). For SFD, the LDH activity was significantly higher for a droplet diameter of 130 μm than for the smaller 40 and 10 μm diameter droplets. As shown in Table 1, for the SA process with 10 μm diameter droplets, the LDH activity was almost equivalent to the LDH activity of the SFD-10 μm process. These results suggest that the activity loss in SFD is from the spraying step and that additional activity loss from the freezing and drying steps was insignificant.

The SEM micrographs in Fig. 3 show the effect of increasing the cooling rate on the morphology of the dried powder prepared with 100 mg/mL trehalose without any protein. Since the mass ratio of trehalose:LDH in the LDH formulation was 400:1, it was observed that LDH did not affect the morphology (not shown). For lyophilization, which was the slowest cooling process, large plates were observed that were greater than 15 μm in diameter (Fig. 3A). For the SFL process with a much faster cooling rate, the particles were characterized by approximately 1 μm features (Fig. 3B), consistent with a measured SSA of 13 m^2/g (Table 3). The SSA of the SFL powder was over an order of magnitude higher than for powders prepared by lyophilization [47]. The SEM for the SFD-10 μm process, which had the highest cooling rate [47], revealed spherical particles that ranged 40-100 nm in diameter (Fig. 3C). The calculated SSA range between 60 and 150 m^2/g for 100 and 40 nm spheres, respectively, is consistent with the measured lysozyme SSA value of 126 m^2/g in Table 3. Therefore, the particles produced by SFD relative to SFL had an approximate 4-fold larger powder SSA and finer features in the SEMs.

Upon lowering the trehalose concentration to 30 mg/mL in the LDH formulation, the LDH activities for the lyophilization, SFL, and falling droplet processes (Table 2) were still very high and not significantly different ($p < 0.05$) from each other. The SFL activities with the low trehalose concentration were also significantly higher ($p < 0.05$) than those for samples prepared by SFD, as reported above for the high 100 mg/mL trehalose concentration. The SFD-130 μm activity was higher than for the two SFD sprays with smaller droplet sizes.

The effect of trehalose concentration on powder morphology for a given cooling rate is shown in the SEMs for the SFL powders (Fig. 3B and D). For 30 mg/mL trehalose (Fig. 3D), large platelets approximately 1–2 μm in diameter were mixed with spheres as small as 50 nm and threads 200–300 nm in diameter. The 1–2 μm features were comparable to those in Fig. 3B for 100 mg/mL trehalose, but submicron particles were not seen. The measured specific surface area (SSA) of trehalose powder sprayed at 50 mg/mL feed concentration was 14 m^2/g which is nearly identical to the value of 13 m^2/g measured for the formulation with 100 mg/mL trehalose (Table 3) [47]. The submicron particles seen for the dilute 30 mg/mL trehalose formulation in Fig. 3D suggest that the measured SSA should have been significantly higher than for the more concentrated 100 mg/mL trehalose formulation shown in Fig. 3B. However, the measured SSAs (Table 3) were nearly identical. The discrepancy may have been caused by different exposure times of trehalose, which is hygroscopic, to moisture during each analysis. For the SEM analysis the trehalose was exposed to atmospheric moisture for less than 1 min during transfer to the SEM. For the BET analysis, loading the trehalose powder in BET glass cells took 10–15 min to accomplish in the dry box with 14% RH. Even with the relatively low RH in the dry box significant moisture may have been adsorbed by the trehalose resulting in a significant decrease in the expected SSA. Although moisture control was problematic for measuring SSAs, the SEM micrographs indicate that the SFD and SFL processes generate a sufficient degree of atomization and rapid cooling to produce much higher surface areas than in lyophilization, even for concentrated feed solutions.

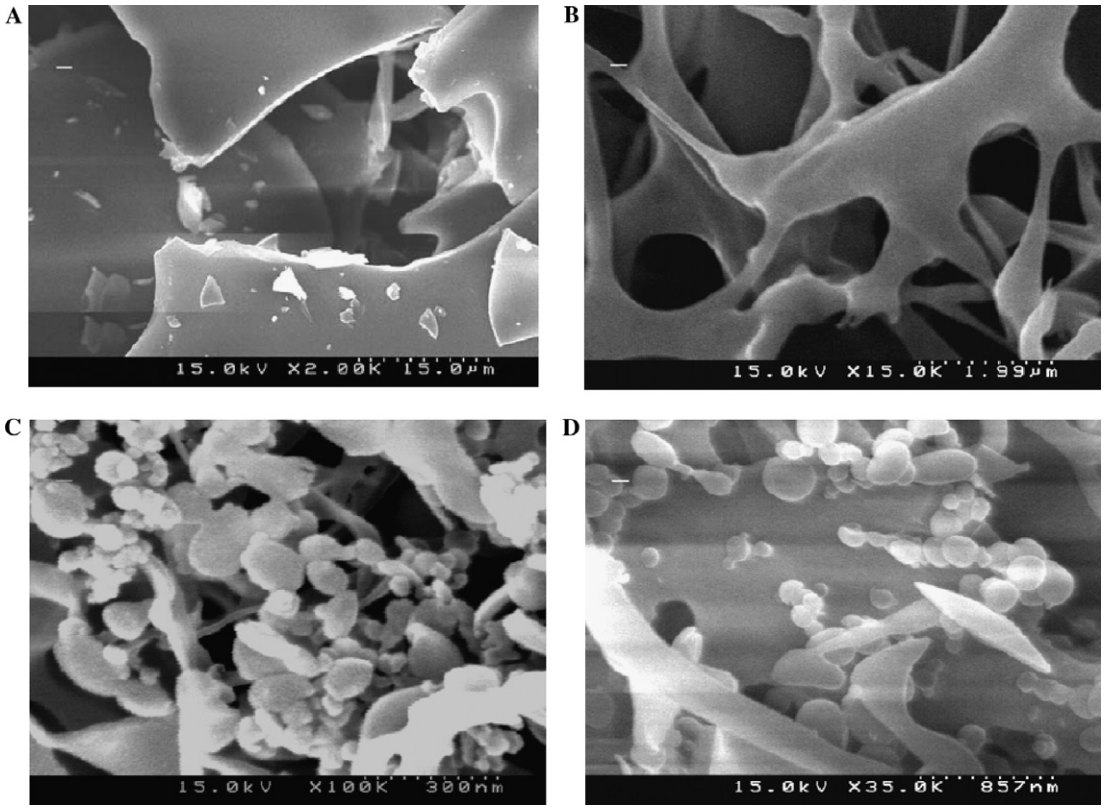

Fig. 3. 100 mg/mL trehalose formulations processed by lyophilization (A), SFL (B), and SFD-10 μm (C) and 30 mg/mL trehalose processed by SFL (D).

Table 3
Specific surface areas measured on lysozyme and trehalose powders [47]

Process	Component	Feed concentration (mg/mL)	SSA (m^2/g)
SFL	Lysozyme	50	34 ± 2
SFL	Lysozyme	100	38 ± 2
SFL	Trehalose	50	14 ± 2
SFL	Trehalose	100	13 ± 1
Falling droplet	Lysozyme	50	4.2 ± 0.02
SFD-130 μm	Lysozyme	50	26 ± 1
SFD-40 μm	Lysozyme	50	87 ± 3
SFD-10 μm	Lysozyme	50	126 ± 5

For the SFD process the intense splashing of the LN2 during the spray did not permit observation of the spray pattern of the liquid droplets. The spray pattern of the droplets was observed by spraying water into air through the SFD nozzle. The atomized liquid droplets for each air flow rate were observed to be well mixed within the air stream plume. When the spray was directed over a sheet of paper 10 cm below the nozzle a solid wet circle was formed on the paper indicating that the atomized droplets were dispersed throughout the cross-sectional area of the plume.

4. Discussion

4.1. Slow cooling with low surface area: lyophilization and falling droplet

The purpose of the lyophilization and falling droplet experiments was to investigate the effects of a low gas–liquid droplet surface area/volume ratio and a slow cooling rate on LDH activity. The differences in cooling rates for these two processes have been described in detail previously [47]. The lyophilization process had the slowest cooling rate in this study; it took 1 mL samples approximately 20 min to freeze when placed on a −80 °C shelf. For the falling droplet process with the next fastest cooling rate, the 3.6 mm diameter liquid droplets impacted the LN2 and floated for 30 s on the LN2 surface before sinking. The aqueous droplets floated due to the boiling of the cryogen in contact with the droplets as a result of the Leidenfrost effect. As the droplets floated on the LN2 during freezing, two droplets occasionally coalesced into a larger droplet. Therefore, ice formation appeared to require at least 30 s after the liquid droplet impacted the LN2 surface. Once the frozen droplets had sufficiently cooled the boiling ceased and the droplets descended to the bottom of the beaker [47]. Although the falling droplet process had a relatively long exposure time to the gas–liquid interface during freezing, the surface area/volume ratio of the droplet was sufficiently low that the LDH activity loss was not significant. Relative to tray lyophilization, the slightly faster cooling rate (∼1 K/s) in the falling droplet experiment does not affect LDH activity.

The high LDH activities for each of these processes at each trehalose concentration in Tables 1 and 2 indicate that the trehalose concentration was sufficient to protect LDH from denaturing in the freeze concentrate during slow cooling (∼1 K/min) and even led to an LDH activity increase.

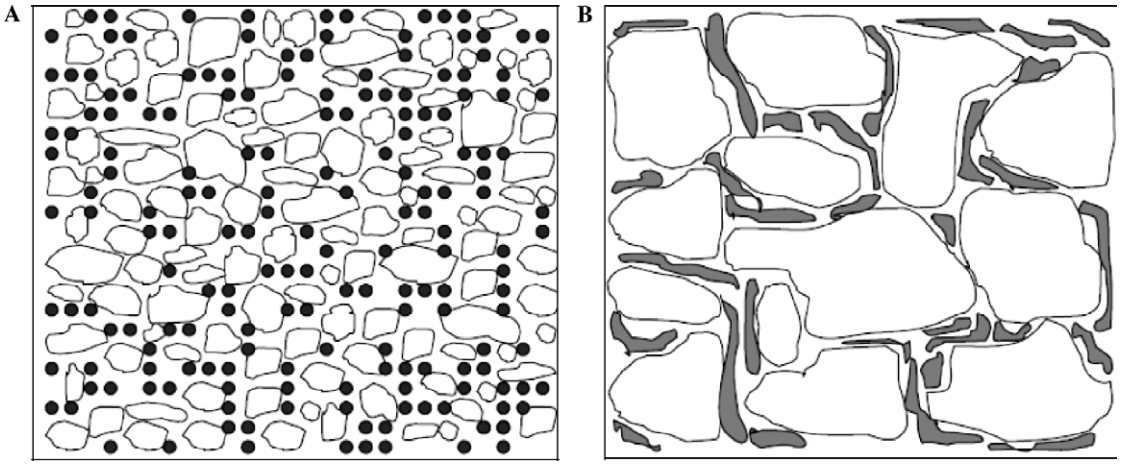

Fig. 4. Frozen morphologies of concentrated solutions with high supercooling (A) and low supercooling (B). Amorphous ice particles are represented as white domains and solute precipitate as solid dots or gray regions.

The >100% LDH activities achieved in Table 1 have also been achieved in other studies with LDH [52] and the tetramer β-galactosidase [51]. The unfavorable free energy of preferential exclusion of the trehalose stabilizes the lower surface area folded state to a greater extent than the greater surface area denatured state [1,43,53,54]. For multimeric proteins that form a tetramer such as LDH, the stability of the tetramer is increased in the presence of a preferentially excluded solute since the surface area of the protein is much smaller in the tetramer form [43,54]. It has been suggested that freeze concentration of LDH in the presence of trehalose or sucrose favors the association of dimers to form the active LDH tetramer [52]. Upon reconstitution of the dried powder followed by the enzyme assay, the protein activity is increased from the increased formation of the LDH tetramer [51,52]. At trehalose concentrations lower than 30 mg/mL in buffer solution at 25 °C, LDH tetramers are known to dissociate. As already mentioned, the LDH formulation with 30 mg/mL trehalose would begin to lose activity in buffer solution after 1 h. Consequently, lower trehalose concentrations were not investigated in this study. These experiments establish that the concentrations of trehalose studied were sufficient to maintain cryoprotection due to preferential exclusion during slow cooling when the solute concentrations increase in the unfrozen liquid, which is exposed to an ice–liquid interface. In the SFD and SFL processes, the time of exposure to the ice–liquid interface will be much shorter, but the interfacial area will be much larger.

4.2. Rapid cooling with large exposure to gas–liquid interface: spray freeze-drying (SFD)

The effect of the gas–liquid and ice–liquid interfaces on protein stability has been the subject of numerous studies [5,18–20,23,25–27,36,55,56]; however the mechanisms are not fully understood. In the SFD process, both the area of the gas–liquid interface and cooling rate increase as the droplet diameter is decreased [5]. The faster cooling rates can increase the surface area of the ice–liquid interface (Fig. 4) [47]. High cooling rates of concentrated feed solutions (>50 mg/mL) produced with small 10 μm diameter droplets lead to rapid nucleation of amorphous water surrounded by thin channels containing growing protein/excipient clusters that have nucleated from the freeze concentrate (Fig. 4A). For larger droplets, lower cooling rates decrease the nucleation rate of amorphous water domains leading to fewer, larger frozen water domains with a smaller area of ice–liquid interface (Fig. 4B). Only a few previous studies have separated the effects of the gas–liquid and ice–liquid interfaces on protein stability by analyzing protein stability after the spraying (spray into air), freezing (spray freeze–thaw), and drying steps (spray freeze-drying) [19,26]. It was shown that protein destabilization occurred during the spray into air process and that the freezing and drying steps contributed very little additional destabilization; therefore, exposure to the ice–liquid interface during freezing played a minor role [19,26]. Webb et al. reported that the protein would not have sufficient time to diffuse to the ice–liquid interface to denature before the droplet would be completely frozen [19]. Furthermore, X-ray photoelectron spectroscopy (XPS) measurements indicated that the amount of protein within a monolayer of the surface of the dried powder was consistent with the theoretical amount of protein adsorption at the gas–liquid interface [19]. For the very large trehalose:LDH ratio used in this study, XPS analysis would not provide sufficient sensitivity to determine the surface LDH concentration. For 10 μm diameter droplets, a comparison of the spray into air and SFD processes in Table 1 indicates that the large ice–liquid interface in the rapid SFD freezing process had a negligible effect on LDH activity loss. The loss was caused by the large gas–liquid interface, in agreement with Webb et al.'s conclusion [19], as was also observed for lysozyme [26].

The time of flight of the atomized droplets through the gas phase above the cryogen may be calculated assuming the droplets move with the same velocity as the air. From

the continuity equation, the product of velocity and area of the conical air jet is constant. The velocity of the air 10 cm below the nozzle exit was 0.80 m/s for the 10 μm diameter droplets and 0.16 m/s for the 130 μm diameter droplets. The reciprocal of this velocity times this distance, 10 cm, gives time of flight values of ~0.1 s (SFD-10 μm) and ~0.6 s (SFD-130 μm) for the droplets. A greater air flow rate was utilized to atomize the liquid jet into smaller droplets resulting in larger droplet velocities [47]. Although the cold LN2 gas above the LN2 meniscus potentially would be able to freeze the surface of the droplets during the time of flight through the gas phase, the warm atomizing air in the conical jet will delay freezing. The similar activity losses in the spray into air and SFD processes suggest that the LDH adsorbed at the gas–liquid interface before the droplet surface froze. Webb et al. explained that adsorption of rhINF-γ, and globular proteins in general, to the gas–liquid interface occurs of the order of milliseconds, which is much shorter than the ~1 s time of flight through the gas phase [19].

For the slowest cooling process (lyophilization) and the fastest cooling process (SFD-10 μm) it was shown that ice–liquid interface had a negligible effect on LDH activity loss. For any intermediate cooling rate it therefore may be expected that the ice–liquid interface would also not affect LDH stability. Thus the higher enzyme activities for the 130 μm droplets versus the smaller droplets may be expected to be caused by the smaller surface area of the gas–liquid interface. The limit of low surface area/volume in the falling droplet experiment produced an even higher enzyme activity.

4.3. Rapid cooling with low exposure to nitrogen gas–liquid interface: spray freezing into liquid (SFL)

The stabilities for the SFL powders were very high indicating that none of the steps, spraying, freezing and drying, caused much loss in enzyme activity. Thus the SFL process overcame the key limitation observed in SFD, the destabilization of protein in the spraying step. The exposure time to gas–liquid interface is much shorter for the SFL process relative to SFD according to the following calculation. For SFL, the liquid jet flowed through the boiling LN2 cryogen at 53 m/s upon exiting the nozzle. At this velocity the jet would travel 10 cm below the LN2 surface (Fig. 2) in 2 ms before breaking apart into frozen particles. Therefore, the extremely short time of exposure to the nitrogen gas–liquid interface is a result of the very small nozzle I.D. of 63 μm forming a small diameter jet with a high velocity. As already mentioned, for the SFD sprays the time of flight of the droplets through the gas phase above the cryogen varied from ~0.1 to ~0.6 s. Therefore, the time of exposure for the SFD process to the gas–liquid interface was 2 orders of magnitude longer than for the SFL process. Even though the droplets in the SFD-130 μm spray may have similar or even lower surface area/volume ratios than in the SFL spray, the longer time of exposure to the nitrogen gas–liquid interface resulted in lower LDH activities in SFD.

Even if some protein diffused to the gas–liquid interface during its very short exposure of 2 ms, additional factors could have helped prevent unfolding. The very low temperature of the nitrogen gas in the funnel surrounding the jet in SFL may have raised the water viscosity. The high viscosity of the water and the low thermal energy would then slow down the dynamics of protein unfolding and aggregation at the outer surface of the jet. Furthermore, the outer surface of the water may have vitrified preventing the protein from reaching the nitrogen gas–liquid interface. Finally, relative to the SFD process, warm atomizing air was not present, which would otherwise have delayed freezing.

Although the cooling rate in the SFL process (7200 K/s) was reduced by the Leidenfrost effect, it was still 3–4 orders of magnitude greater than for lyophilization (~1 K/min)

Table 4
Activities for porcine LDH (PLDH) or rabbit LDH (RLDH) processed with various freezing and drying processes

Process	Protein	Formulation composition	Activity (%)
SFD [44]	RLDH	0.015 mg/mL LDH	
		+100 mg/mL trehalose	58
		+250 mg/mL trehalose	56
		0.03 mg/mL LDH	
		+250 mg/mL trehalose and 1 mg/mL Tween 80	83
		+250 mg/mL trehalose and 5 mg/mL Tween 80	92
SD [23]	PLDH	0.300 mg/mL LDH	
		+100 mg/mL trehalose	90
		+100 mg/mL trehalose and 0.1 mg/mL Tween 80	93
		5 mg/mL LDH	
		+95 mg/mL trehalose	90
		+95 mg/mL trehalose and 0.1 mg/mL Tween 80	98
$scCO_2$A-BD [24]	RLDH	0.1 mg/mL LDH	15
		+100 mg/mL mannitol	40
		+100 mg/mL sucrose	66
		+100 mg/mL sucrose and 0.1 mg/mL Tween 20	95

and the falling droplet (~1 K/s) processes as calculated previously [47]. The more rapid nucleation and growth during freezing in SFL produced SSAs an order of magnitude larger than in the falling droplet process (Table 3) [47], as was also evident in the SEMs. The LDH activities remained high in spite of the large area of the ice–liquid interface. As seen for SFD [19], the freezing step in SFL also appeared to be too fast for the protein to diffuse to the ice–liquid interface and denature.

Other particle forming processes shown in Table 4 including SFD, spray-drying, and $scCO_2$A-BD all required the use of Tween 20 or Tween 80 to achieve high LDH activities for similar protein and sugar concentrations [23,24,44]. Without a surfactant the LDH activities were significantly lower compared to SFL. Each of these processes exposed the protein to the gas–liquid interface for a sufficiently long time period to produce aggregation. Therefore, the minimization of exposure to the gas–liquid interface in the SFL process removes the need for the addition of a surfactant.

Other reports have indicated that exposure to increased ice–liquid interface significantly destabilizes protein [5,18,55]. With freeze–thaw studies Chang et al. demonstrated that increasing the cooling rate from slow cooling on a shelf to quench freezing in LN2 increases the ice–liquid interfacial area which decreases LDH activity [55]. This study did not use a stabilizing sugar such as trehalose to prevent destabilization during freezing and/or drying. Activity loss was decreased by adding the surfactant Tween 80 to the LDH formulation. From freeze–thaw studies, it was concluded that Tween 80 competes with the protein for ice–liquid interface [55]. In the present study in which sufficient trehalose was present the LDH remained stable through the freezing and the drying steps, in spite of the rapid cooling rate. In another study, it has been shown with the SFD process that BSA monomer loss increases with an increase in powder SSA [18]. As the diameter of the sprayed droplets decreases, the more rapid cooling rate produces particles with a finer microstructure [18]. It is likely that the gas–liquid interface played an important role in the protein aggregation for BSA, given the results for rhIFN-γ [19], lysozyme [26] and LDH (this study) with SFD.

5. Conclusions

In SFD, the activity loss in the spraying step has been shown to be caused by protein adsorption and aggregation at the gas–liquid interface, as reported for rhIFN-γ [19] and lysozyme [26]. In this study, the LDH activity loss was the same as when spraying the protein solution into air without freezing, suggesting that the freezing (ice–liquid interface) and drying steps may not have contributed further to the activity loss. Furthermore, the activity loss increased with a decrease in droplet size, and thus gas–liquid interfacial area, consistent with results for BSA [18], and for rhIFN-γ and lysozyme. Given that long exposure to the gas–liquid interface in SFD and spray-drying can destabilize proteins, the ability to shorten this exposure time is of considerable practical importance.

The SFL process may be used to reduce exposure to the gas–liquid interface. For 0.25 mg/mL LDH with either 30 or 100 mg/mL trehalose, the SFL process produced powders with very high enzyme activities comparable to lyophilization upon reconstitution, despite surface areas that were an order of magnitude higher. The combined spraying, freezing, and drying stresses of the SFL process did not produce any significant loss in LDH activity. The enzyme activities in SFL were higher than for SFD, even with the larger 130 μm droplets, despite the similar powder surface areas in both cases. Although a gas cone was formed about the liquid jet in SFL, as a result of nitrogen evaporation (Leidenfrost effect), the nitrogen gas–liquid contact had a negligible impact on the LDH stability. The exposure time of only 2 ms to the nitrogen gas–liquid interface in this cone was calculated to be 2 orders of magnitude faster than for SFD. This very short exposure time appeared to prevent loss in activity from protein adsorption and unfolding, even without the use of a surfactant. It is likely the primary cause of the higher LDH activities in SFL versus SFD. The ability to produce stable high surface area submicron particles of fragile proteins such as LDH by SFL, in addition to much more stable proteins such as BSA [27] and lysozyme [26], is of practical interest in protein storage and in controlled release applications including encapsulation in bioerodible polymers. The successful scale down of the SFL process for protein formulation volumes as small as 1 mL will be beneficial for therapeutic proteins available only in limited quantities.

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