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Research Article

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Influence of Formulation Factors on the Aerosol Performance and Stability of Lysozyme Powders: a Systematic Approach

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Abstract. With the growing interest in developing biologics for pulmonary delivery, systematic fast screening methods are needed for rapid development of formulations. Due to the labile nature of macromolecules, the development of stable, biologically active formulations with desired aerosol performance imposes several challenges both from a formulation and processing perspective. In this study, spray-freeze-drying was used to develop respirable protein powders. In order to systematically map the selected design space, lysozyme aqueous pre-formulations were prepared based on a constrained mixture design of experiment. The physicochemical properties of the resulting powders were characterized and the effects of formulation factors on aerosol performance and protein stability were systematically screened using a logic flow chart. Our results elucidated several relevant formulation attributes (density, total solid content, protein:sugars ratio) required to achieve a stable lysozyme powder with desirable characteristics for pulmonary delivery. A similar logical fast screening strategy could be used to delineate the appropriate design space for different types of proteins and guide the development of powders with pre-determined aerodynamic properties.

KEY WORDS: dry powder biologics; spray-freeze-drying; pulmonary delivery; dry powder inhaler.

INTRODUCTION

With increasing numbers of biologics in development pipelines and some products being already approved by FDA (such as the DPI insulin formulations Afrezza by MannKind and Exubera by Pfizer, and Pulmozyme® by Genentech), pulmonary delivery is emerging as a valuable alternative to parenteral administration [1–4]. In particular, the use of dry powders is an appealing formulation strategy for this class of labile therapeutic agents, as it carries the potential for extended shelf-life, and can be used to deliver a wide range of doses in a time-efficient manner [1].

For the injectable route, protein dry powder formulations have been prepared traditionally by lyophilization. However, for pulmonary applications, the dry powder formulation must possess additional aerodynamic characteristics in order to be successfully delivered to the lungs. Particle engineering through spray-freeze-drying (SFD) is a method for producing biopharmaceutical powders with tailored characteristics (including particle's size, surface area, powder density) [5] and therefore could offer additional benefits for the formulation of an inhalable protein powder as compared to traditional lyophilization. In SFD, a solution containing dissolved protein is sprayed with an atomization nozzle into a cold vapor phase of a cryogenic liquid (liquid nitrogen) to form frozen droplets. The frozen slurry is subsequently lyophilized to obtain a dry powder. Processing parameters such as atomization (*e.g.*, type of nozzle, nozzle size, flow rate, back pressure, distance to the liquid nitrogen (LN_2) surface), freeze-drying conditions, and formulations can be optimized to engineer the desired powder aerodynamic characteristics. During atomization, proteins tend to adsorb at the increased

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Some of the findings of this study have been accepted as an abstract at the Respiratory Drug Delivery (RDD) annual conference, Tucson, NM, and will be partially published as an abstract as part of conference proceedings [38].

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air-water interface resulting in potential degradation *via* structural unfolding with subsequent aggregate formation [6]. When sprayed into the vapor over LN_2 in SFD, droplets start freezing as they pass through the cryogenic vapor and freeze completely upon contact with the LN_2 surface [7–9]. This rapid cooling and freezing during SFD process inhibits molecular mobility and, therefore, surface access and potential aggregation of protein molecules [10]. The additional stresses posed by the freezing and drying steps, which may still damage the protein leading to structural denaturation, aggregation, and loss of protein activity, may be mitigated by judicious process and formulation approaches. Excipients including sugars, polyols, surfactants, and buffers are commonly added to SFD formulations [11–13]. Disaccharides (such as sucrose or trehalose) are among the preferred cryoprotectants and lyoprotectants in freeze-drying [11, 13]. They stabilize proteins *via* two main mechanisms: the vitrification theory and water replacement theory. The first mechanism relies on the immobilization of the protein in the glassy sugar matrix which dramatically slows down its degradation (kinetic stabilization). The second mechanism instead relies on the formation of hydrogen bonds between the sugar hydroxyl groups and the protein which replace the hydrogen bonds between water and protein otherwise lost during the drying step. The native conformation is therefore protected during processing ((thermodynamic stabilization) and its functionality can be restored as soon as the hydration and temperature conditions return to normal [12–14]. Polyols (such as mannitol) can be used as stabilizing and/or bulking agents [11]. However, due to the tendency of mannitol to crystallize during lyophilization, it is used in smaller amounts compared to sucrose [8]. Surfactants (such as polysorbate 80) are commonly added to reduce aggregation upon rehydration and to reduce adsorption of proteins at the air-liquid or ice interface. Lastly, buffers are used to adjust the pH of the formulations since many proteins are stable only in a narrow pH range. Because freezing a buffered solution may cause pH changes [15] (the case of sodium phosphate buffer), buffers that lead to minimal pH variations during freezing (such as Tris, histidine, or citrate) are usually preferred [11, 13, 16]. However, the type of buffer selected strongly depends on the specific protein used.

SFD has been tested with several different proteins including BSA [7, 17], lysozyme [18], darbepoetin alfa [19], trypsinogen [20], and human growth hormone (hGH) [21], and has shown promising results in terms of protein stability. However, few studies have investigated the feasibility of using the SFD powders for pulmonary delivery of biologics. In 1999, Maa *et al*. patented and published on SFD of inhaled biologics, preparing rhDNase and anti-IgE powders [8]. Subsequent studies included those of Bi *et al*. [22], who used SDF to prepare dry powder of insulin-loaded liposomes, Murugappan *et al*. [23] and Amorij *et al*. [24], who produced spray-freeze-dried influenza vaccine powder, and Zijlstra *et al*. [25], who prepared formulations of the decapeptide cetrorelix acetate. Collectively, these studies demonstrate the feasibility of developing inhaled biologic powders. However, to date, a systematic screening approach has not been published to evaluate the effect of formulation composition on the aerosolization performance and protein stability of SFD powders.

The goal of this work was to systematically evaluate the influence of formulation factors on the aerosol performance and stability of protein powders. A constrained mixture design of experiment approach was used to create an array of 10 formulations using a model protein, lysozyme. Initially, an analysis of FDA-approved commercially available biologic powders was screened to identify excipients and excipient concentration ranges. From this data, different concentrations of protein, sucrose, and mannitol, and different total solid contents of the aqueous pre-formulations (1 and 10%) were used within the design of experimental matrix. The pre-formulations were processed with SFD to obtain lysozyme lyophilized powders, which were systematically screened following a logic flow chart to assess their suitability for pulmonary delivery. The characterization included physico-chemical characterization, aerosolization performance, and protein stability.

METHODS

Materials

Lysozyme, polysorbate 80, mannitol, sucrose, and histidine used to prepare the formulations were purchased from Sigma-Aldrich (St. Louis, MO). Sodium hydroxide, sodium phosphate monobasic monohydrate, and sodium phosphate dibasic anhydrous used to prepare the SEC-HPLC mobile phase were purchased from Fisher Chemical (Pittsburgh, PA).

Excipient Selection

Formulation excipients were selected based on a literature search which screened the composition of 18 approved lyophilized protein products and 2 protein dry powder formulations for pulmonary delivery (Suppl. Table 1) [26, 27]. The frequency of use of the major excipients (sugars, surfactants, and buffers) in the products was evaluated. Within each major category, we found that the most used excipients were sucrose as the disaccharide, mannitol as the polyol, histidine as the buffer, and polysorbate 80 as the surfactant (Fig. 1). These excipients were therefore selected for our study, while lysozyme was used as protein model. The ranges of concentrations for each component, expressed as a % in the mixture defining our design space, were protein 10–60%, sucrose 20–90%, and mannitol 0–20%.

In order to systematically map the selected design space and screen for formulation variables, aqueous pre-formulations of lysozyme were prepared based on a constrained mixture design of experiment (DoE) (for more details about the DoE, see the "Statistical Analysis" section). Concentrations of lysozyme, sucrose, and mannitol in the aqueous pre-formulations were varied within the pre-selected ranges, while polysorbate 80 and histidine buffer concentrations remained fixed. Total solid content in the pre-formulation was designated as a discrete numeric factor of 1 or 10. Table I summarizes the target composition of the 10 powder formulations (F1–10) investigated.

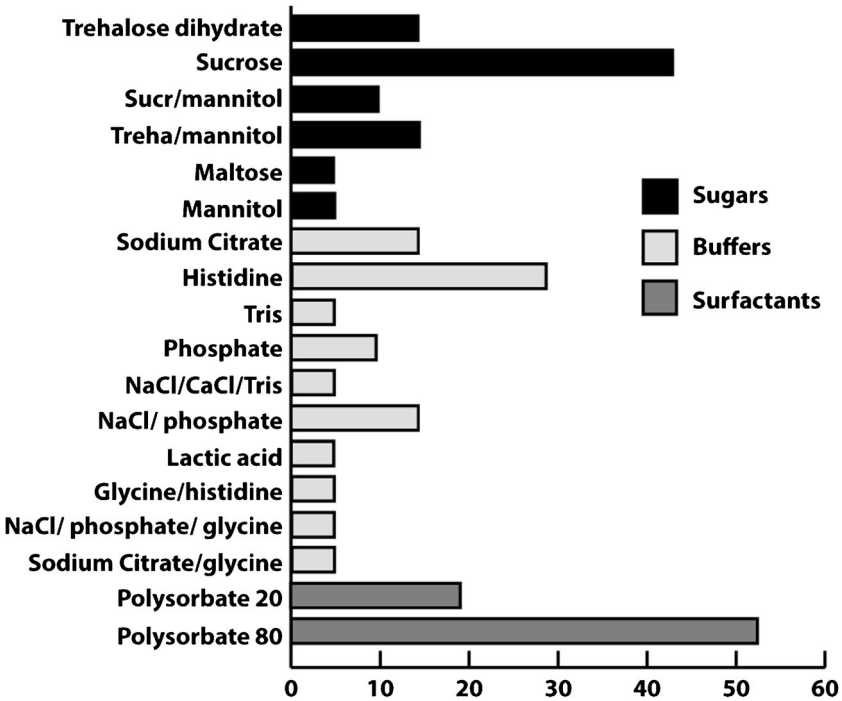


Fig. 1. Excipient selection. Frequency of use of the different excipients in the examined marketed products

Powder Processing: Spray Freezing into Liquid Nitrogen

Spray freezing into liquid nitrogen followed by lyophilization was used to generate lysozyme powder. Liquid pre-formulations of lysozyme containing sucrose, mannitol, histidine as buffer, and polysorbate 80 were prepared by dissolving the protein and the excipients in the desired concentrations in 40–100 mL of double-distilled water. The pH of the formulations was assessed. Solutions were sprayed into the vapor over a cryogenic dewar filled with approximately 500 mL of liquid nitrogen (LN_2), using a ISCO syringe pump and a single fluid nozzle (see experimental setup in Fig. 2). The nozzle consisted in a 5-cm-long, 127-μm internal diameter polyetheretherketone (PEEK) crimped tube. The nozzle was positioned approximately 4.5 cm above the surface of the LN_2. Solutions were sprayed at a flow rate of 20–30 mL/min with a back pressure of approximately 2000–3000 psi. As a quality control measure, droplet size distributions of the sprays generated were monitored using a laser diffraction instrument (Sympatec GmbH, Clausthal-Zellerfeld, Germany) to ensure reproducibility between different sprayed batches. The nozzle was mounted directly over the pathway of the laser beam at a distance of 4.5 cm from the lens (see Suppl. Figure 1). After spraying, the frozen particles were collected and lyophilized for 4 days using an automatic Labconco lyophilizer (Labconco Corporation, Kansas City, MO), at −44°C and 145×10^{-3} mbar.

Powder Characterization

Following lyophilization, powders were weighed and systematically analyzed following the characterization screening flow chart reported in Fig. 3.

Table I. List of DoE Formulations

Formulation	% of the component in the final powder					Total solid % in the pre-formulation
	Tween	Histidine	Lysozyme	Sucrose	Mannitol	
1	0.2	1.7	34.3	63.7	0	1
2	0.2	1.7	58.8	39.2	0	10
3	0.2	1.7	9.8	68.6	19.6	10
4	0.2	1.7	58.8	19.6	19.6	10
5	0.2	1.7	34.3	44.1	19.6	1
6	0.2	1.7	58.8	29.4	9.8	1
7	0.2	1.7	9.8	88.2	0	10
8	0.2	1.7	34.3	53.9	9.8	10
9	0.2	1.7	9.8	78.4	9.8	1
10	0.2	1.7	58.8	39.2	0	1

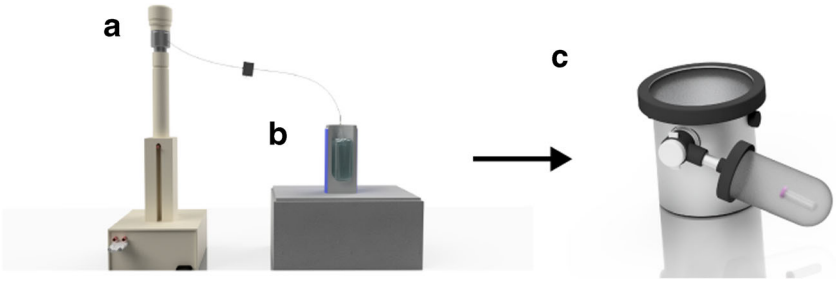


Fig. 2. Experimental setup for spray-freeze-drying. (A) ISCO syringe pump equipped with a PEEK single-fluid nozzle (flow rate of 20–30 mL/min with a back pressure of approximately 2000–3000 psi), (B) cryogenic dewar filled with approximately 500 mL of liquid nitrogen, and (C) automatic lyophilizer (−44°C and 145×10^{-3} mbar)

Bulk Density and Residual Water Content

After a preliminary visual inspection, powders were sieved through a 2-mm filter and tested for bulk density. Powder bulk density measurements (run in triplicates) were performed according to method I-616 of the US Pharmacopoeia [28] with the exception of using a 5-mL volumetric cylinder due to the small sample volumes available. Residual moisture content of all powders post-processing was determined using coulometric Karl-Fischer titration (C20, Mettler Toledo, Columbus, OH). A 10-mg sample was used for each analysis. Powders were then stored at −20°C in sealed glass vials.

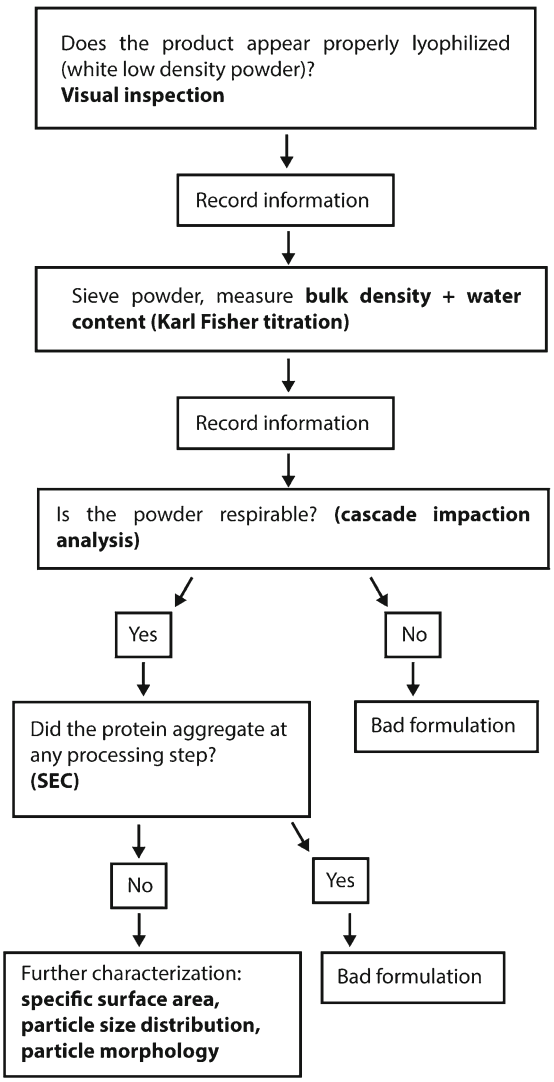


Fig. 3. Flow chart describing the powder characterization process

Determination of Aerosol Performance

Following the flow chart (Fig. 3), we next analyzed the aerodynamic performance of the powders. This was assessed utilizing a Monodose RS01 DPI (device resistance 0.1 mbar), a gift from Plastiape S.p.a (Osnago, Italy). Size 3 hydroxypropyl methylcellulose (HPMC) capsules (Capsugel Inc., Morristwon, NJ) were loaded with a known amount of powder (corresponding to an average protein loading of approximately 10 mg). Experiments were performed using a next-generation impactor (NGI) (MSP Corporation, Shoreview, MN). To reduce particle bounce, NGI plates were coated with 1% (*v*/*v*) silicon oil in hexane and allowed to dry. Following USP specifications, experiments were performed at a 4-kPa pressure drop (equivalent to 60 L/min on the RS01 device) for a duration of time of 4 s to draw 4 L of air through the apparatus [29]. The resultant dispersed powder was collected from the inhaler, the pre-separator, the adapter, the induction port, stages 1–7, and the micro-orifice collector (MOC) by rinsing with DI water. The amount of powder left in the capsule was evaluated by weight. Mass of the active ingredient (lysozyme) in each sample was assessed by UV-absorbance at a wavelength of 280 nm using a Tecan1 Infinite1 200 PRO multimode microplate reader (Tecan Systems, Inc., San Jose, CA).

The respirable fraction (RF) was calculated as the percentage of the total powder load predicted to have an aerodynamic diameter below 5 μm. Powder mass with aerodynamic diameter below 5 μm was calculated by summing the mass of the powder deposited in the plates having a cutoff <5 μm. The mass median aerodynamic diameter (MMAD), which represents the mass-based median point of the aerodynamic particle size distribution, was determined by plotting the cumulative percentage of mass less than the stated aerodynamic size cut (expressed as probits) against aerodynamic size cutoff (log scale). A linear regression was performed to determine the aerodynamic diameters corresponding to the 50% percentile by mass, otherwise referred to as the MMAD.

Determination of Protein Stability Post-processing

Potential formation of protein aggregates was evaluated with size exclusion chromatography (SEC-HPLC) using a Dionex UltiMate 3000 HPLC System (Thermo Scientific, Sunnyvale, CA), equipped with a G3000SWXL column. An Ultimate 3000 Autosampler was utilized to inject 75-μL samples. The sample was eluted isocratically with a 200-mM sodium phosphate (pH 6.8) mobile phase at a flow rate of 0.5 mL/min. The eluent was detected using UV absorbance at 280 nm. Reconstituted powders (underwent both spray and lyophilization processing), pre-formulations sprayed only, and unprocessed pre-formulations (no spray) were analyzed and compared to a freshly prepared lysozyme control solution. For the spray-only samples, the formulations were sprayed into an empty vial and then stored at 4°C until further analysis. SEC was also performed on the optimized formulation samples recovered after NGI test. Samples recollected from stage 1 and combined stages 2–8 (corresponding to the respirable fraction) were analyzed. Qualitative analysis of sample aggregation was performed by comparing the retention time of the peak in the samples with the one in the known standard. Relative retention time (RT) was calculated by dividing the lysozyme standard main peak retention time by the sample main peak retention time. % peak area reported the area of each peak in the chromatogram as a percentage of the total area of all peaks. Chromeleon Version 6.80 software (Thermo Scientific, Sunnyvale, CA) was used to process all chromatography data.

Further Characterization

Finally, as represented in the flow chart, formulation(s) that possessed medium/good aerosolization performance and no signs of aggregation were further characterized. Specific surface area of the optimized formulation was measured using a BET gas adsorption method. A known amount of powder was loaded into the sample cell and degassed at 37°C overnight prior to analyzing the samples with a monosorb BET analyzer (Quantachrome instruments, Boynton Beach, FL).

To assess the dispersibility of the powders and the geometric particle size distribution of the powder, samples were analyzed with a HELOS laser diffractor (Sympatec GmbH, Clausthal-Zellerfeld, Germany) using RODOS dispersion. A pressure of 4 bar was used to ensure full dispersion (see Suppl. Figure 1). Measurements were taken every 1 ms following powder dispersion. Measurements that were between 5 and 25% optical concentration were averaged to determine particle size distribution.

A scanning electron microscope (SEM) Zeiss Supra 40VP SEM (Carl Zeiss Microscopy GmbH, Jena, Germany) was used to analyze the morphology of powders. The powder samples were mounted on aluminum SEM stubs using double adhesive carbon conductive tape and sputter coated with 12 nm of platinum/palladium (Pt/Pd) in an argon atmosphere using a Cressington sputter coater 208 HR (Cressington Scientific Instruments Ltd., Watford, UK). Undispersed particles and particles dispersed using the RODOS disperser at 4 bar were examined.

Polarized light microscopy (PLM) was used to investigate the presence of crystalline material in our optimized formulation. Images were acquired using an Olympus BX-53 (Olympus, Waltham, MA) polarizing light microscope with a first-order red compensator. Images were acquired with a QICAM digital camera (QImaging, BC, Canada) with Qcapture, v 2.0.13 (QImaging, BC, Canada) using a ×20 objective.

Thermal properties of optimized formulation were analyzed using differential scanning calorimetry (DSC) with a DSC Q20 (TA Instruments, New Castle, DE) equipped with a refrigerated cooling system. Approximately 3 mg of sample was loaded into a Tzero aluminum pan press-sealed with an aluminum lid (PerkinElmer, Waltham, MA). A crimped empty pan was used as a reference. Sucrose and lysozyme alone and a physical mixture of lysozyme-sucrose (60:40 ratio; corresponding to the same ratio used in the formulation) were run as controls. Experiments were performed at a heating ramp rate of 10°C/min in the range of 25–205°C, under a dynamic flow rate of nitrogen at 40 mL/min. Data was analyzed using TA Universal Analysis 2000 software (TA Instruments, New Castle, DE).

X-ray diffraction (XRD) analysis of the optimized formulation was conducted using a Rigaku Miniflex600 (Rigaku Americas, The Woodlands, TX) instrument equipped with a Cu-Kα radiation source generated at 40 kV and 15 mA. Samples were scanned in continuous mode with a step size of 1° over a 2θ range of 10°–30° at a rate of 1°/min. Unprocessed crystalline sucrose was used as control.

Determination of optimized formulation water sorption isotherm was carried out by dynamic vapor sorption (DVS) (Surface Measurement Systems Ltd., London, UK) using a gravimetric method to determine the humidity-dependent increase of mass. Approximately 10 mg processed lysozyme powder was loaded into the pan. Two full sorption/desorption cycles were performed from 0 to 90% RH in steps of 10% RH at 25°C. The equilibrium criterion for each step was reached when dm/dt was less than 0.01% within an interval of 10 min. Percentage of change in mass was calculated and plotted as a sorption isotherm.

Lysozyme is an enzyme that naturally digests bacterial cell walls. To evaluate its biological activity, lysozyme was incubated with a suspension of *Micrococcus lysodeikticus* bacteria (Sigma Chemical Co., St. Louis, MO) prepared in potassium phosphate buffer (66 mM; pH 6.2). The digestion of bacterial membrane causes a decrease in turbidity. The turbidity of the solution was monitored over time measuring the absorption at 450 nm with a Tecan1 Infinite1 200 PRO multimode microplate reader (Tecan Systems, Inc., San Jose, CA) every minute during a total incubation period of 6 min at 25°C [30]. The activity of the lysozyme was estimated calculating the slope of the absorption curve. Unprocessed lysosome (control), lysozyme in aqueous pre-formulation, lysozyme sprayed only, and lysozyme fully processed (including spray into liquid nitrogen and lyophilization) were tested and compared to thermally denatured lysozyme.

Statistical Analysis

An array of 10 formulations was designed using the software JMP (SAS Institute Inc., Cary, NC) and a

constrained mixture design of experiments (DoE), with secondary interactions. In a mixture experiment, the independent factors of the DoE are proportions of different components of the formulation and the measured response is assumed to depend only on these relative proportions. The mixture components were subject to the constraint that they must sum to one. In addition, each component was subjected to a maximum and minimum constraint value. Protein, sucrose, and mannitol were designated as continuous independent factors in the DoE. The ranges of concentrations for each component, expressed as a % in the mixture, were based on literature data. The following ranges were applied: protein 10–60%, sucrose 20–90%, mannitol 0–20%. Polysorbate 80 and histidine were kept constant, while total solid content in the pre-formulation was designated as a discrete numeric factor of 1 or 10. Table I reports the list of the formulations tested based on the DoE, expressed as theoretical % in the final dry powder formulations, including histidine and polysorbate 80.

The screening characterization of the formulations was performed as a single run for each composition, with the exception of bulk density and biological activity of the optimized formulation which were run in triplicates and reported as an average. For the biological activity test, the statistical significance of experimental results was assessed using analysis of variance (ANOVA) in Excel (Microsoft Corporation). Alpha level was set at 0.05.

RESULTS

Processing of Powders and Initial Characterization

Spray-freeze-drying (SFD) was used as a processing technique to generate lysozyme powders. Aqueous pre-formulations of lysozyme, prepared according to Table I, possessed a pH of approximately pH 5.2. Formulations were sprayed into liquid nitrogen (LN_2) using a syringe pump and a crimped PEEK nozzle, at a flow rate of 20–30 mL with back pressures ranging ~ 2000–3000 PSI.

The spray droplet size was monitored with laser diffraction to ensure reproducible spray characteristics between different batches. Across multiple days and multiple batches, the droplet size distribution was $D10 = 7.3 \pm 3.1$ μm, $D50 = 20.2 \pm 5.8$ μm, and $D90 = 38.7 \pm 10.1$ μm. The experimental setup for spray quality and an example of spray size distribution can be found in Suppl. Figure 2. After spraying, the frozen particles were collected and lyophilized for 4 days using an automatic single cycle Labconco lyophilizer.

Lyophilized powders were weighed and analyzed following the characterization screening flow chart reported in Fig. 3. Powders were sieved and their bulk density and percentage of residual moisture were measured. All powders presented a residual water content between 7 and 12%, comparable with the moisture content of raw unprocessed lysozyme (which was approximately 10%).

Bulk density positively correlated with sugar content, with formulations possessing higher % of sugars (and therefore lower protein/sugars ratio) having higher density (Table II). This was also confirmed by simple visual inspection of the powders. Higher bulk density powders appeared sticky, while the low-density powders appeared cotton-like and brittle.

Aerosol Performance

Following the initial physical-chemical characterization, the formulations were screened for aerosol performance using a RS01 Monodose DPI. Formulation F10 containing ~ 60% lysozyme and ~ 40% sucrose displayed the best performance with a RF of 57%. It was followed by formulation F6 containing ~ 60% lysozyme, ~ 30% sucrose, and ~ 10% mannitol, with a RF of 48%. The MMAD of each powder was 4.5 and 4.9 μm, respectively. NGI stage deposition patterns for F2, 4, 6, and 10 are reported in Fig. 4 while Table III summarizes the NGI results. F2 and F4 had an intermediate performance with RF of 9.5 and 16%, respectively, and an MMAD of 8.6 and 7.5 μm. Based upon cascade impaction results, the remaining formulations were found to be non-suitable for pulmonary delivery due to low RF (Table III).

Protein Stability

Formulations that demonstrated medium/good aerosolization efficiency (formulations F2, 4, 6, and 10) were next assayed for protein stability. Presence of aggregation (as determined by size exclusion chromatography) was used as the main parameter for this initial stability screening. In order to test the influence of atomization on protein aggregation, the atomization step was separated from the freezing drying steps by sampling liquid pre-formulations during spray. Reconstituted powders (exposed to both spray and lyophilization processing, and then reconstituted in the HPLC eluent buffer), pre-formulations exposed to spray processing only, and unprocessed pre-formulations were analyzed and compared to a freshly prepared lysozyme standard control solution. Qualitative results are reported in Table IV. None of the samples showed signs of aggregation, with chromatograms presenting a major peak at relative retention time ~ 1 as compared to standard lysozyme solution and % peak area > 98%.

Table II. Physical-Chemical Characterization Powders. Table Summarizing % Residual Water and Density of the Tested Formulations

Formulations	Physicochemical characterization	
	% residual water	Bulk density (g/cm^3)
1	10	0.133
2	7	0.043
3	10.7	0.190
4	7.3	0.034
5	10	0.051
6	8.9	0.01
7	7.2	0.325
8	6.9	0.077
9	7.9	–
10	12.6	0.031

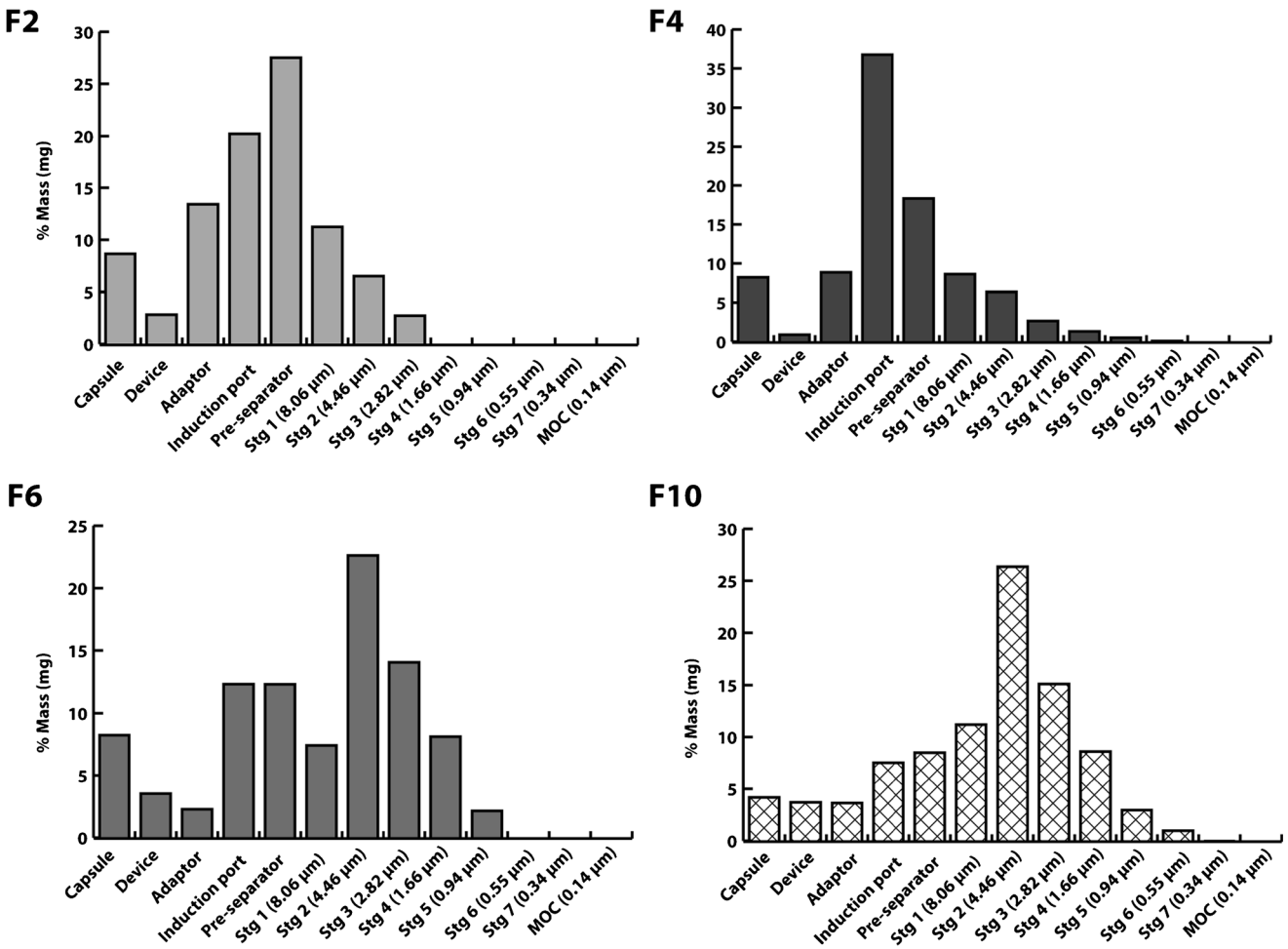


Fig. 4. Screening study of powders aerosolization performance. NGI stage deposition pattern for F2, 4, 6, and 10 ($n = 1$)

Full Characterization of Optimized SFD Lysozyme Formulation

Based on the screening process, F10 (~ 60% lysozyme and ~ 40% sucrose) appeared to be the most promising formulation in terms of aerosolization performance and lack of aggregation, followed by F6 (~ 60% lysozyme, ~ 30% sucrose, ~ 10% mannitol). Therefore, these two formulations were further characterized using laser diffraction with RODOS disperser, BET surface analysis, DSC, DVS, and XRD.

SEM photographs of F10 revealed a highly porous powder structure with a large, irregular, and rough surface (Fig. 4, top left). Morphology of F10 was compared to formulations F6 (characterized by the same protein to sugars ratio, 60:40, with 30% sucrose and 10% mannitol, and same pre-formulation total solid content, 1%) and F2 (characterized by the same protein to sugars ratio, 60:40, but 10% pre-formulation solid content). While F6 revealed a similar brittle porous matrix structure as F10 (Suppl. Figure 3), F2 powder possessed a more sphere-like shape (Fig. 5b). F10 powder dispersed into smaller particles upon dispersion with RODOS at 4 bar pressures (Fig. 5c), leading to a geometric particle size distribution of D10 = 0.9 µm, D50 = 3.1 µm, and D90 = 10 µm. The high porosity of F10 was confirmed by BET surface area analysis, which measured a specific surface area of 12.6 m^2/g. Similar particle dispersion and surface area results were obtained for formulation F6 with D10 = 0.9 µm, D50 = 3 µm, D90 = 11.9 µm, and 11.3 m^2/g. When dispersed by RODOS at 4 bar pressures, formulation F2 resulted in larger particles, with a geometric particle size distribution of D10 = 1.2 µm, D50 = 8.9 µm, and D90 = 55.1 µm, which was qualitatively confirmed by SEM imaging (see Fig. 5d).

DSC analysis of F10 showed a broad band between 30

Table III. Powder Aerosolization Performance Summary Table

Formulations	Powder performance-NGI	
	Respirable fraction (RF)	MMAD (µm)
1	0.52	Not calculable
2	9.5	8.6
3	0	Not calculable
4	12.38	7.5
5	2.78	Not calculable
6	48	4.9
7	0	Not calculable
8	0	Not calculable
9	0	Not calculable
10	57	4.5

Table IV. Protein Stability (SEC-HPLC). Table Summary for Formulations F2, 4, 6, and 10

Formulation	Stability SEC		
	SEC (liquid pre-formulation)	SEC (spray)	SEC (reconstituted powder)
Standard	RT = 22.56, % peak area 98.9%		
2	Relative RT = 0.99, % peak area = 100	Relative RT = 0.99, % peak area = 100	Relative RT = 0.99, % peak area = 100
4	Relative RT = 0.99, % peak area = 100	Relative RT = 0.99, % peak area = 100	Relative RT = 0.99, % peak area = 100
6	Relative RT = 0.99, % peak area = 100	Relative RT = 0.99, % peak area = 98.1	Relative RT = 0.99, % peak area = 100
10	Relative RT = 0.99, % peak area = 98.1	Relative RT = 0.99, % peak area = 100	Relative RT = 0.99, % peak area = 100

Relative retention time (RT) = lysozyme standard main peak RT/sample main peak RT. % peak area reports the area of each peak in the chromatogram as a percentage of the total area of all peaks

and 120°C, likely due to water removal and an endothermic transition at ~150°C (Fig. 6b). Unprocessed sucrose alone and lysozyme alone were run as controls. Sucrose thermogram confirmed the crystalline sugar melting point at approximately 190°C, while lysozyme thermogram presented a previously reported typical shape for amorphous lysozyme with a broad band of water evaporation [31]. The physical mixture of sucrose-lysozyme showed a slight depression of sucrose melting point to approximately 185°C, suggesting potential interaction between these two components. Similar DSC results were obtained for F6 (Suppl. Figure 4).

Polarized light microscopy analysis of F10 confirmed the presence of crystals of ≤1 μm in size, dispersed in an amorphous matrix (Fig. 6c). Moisture sorption isotherms of F10 were typical of a hygroscopic material, primarily non-crystalline (Suppl. Figure 5). Similarly, F10 XRD analysis did not reveal any crystalline material (Suppl. Figure 5).

SEC analysis of F10 samples collected from NGI following dispersion from the RS01 device was performed to ensure that the process of aerosolization through the device did not induce aggregation. As reported in Fig. 6a table, no sign of aggregation was detected.

Retention of lysozyme biological activity after processing was confirmed by incubating the reconstituted powder with a suspension of *Micrococcus lysodeikticus* bacteria and assessing bacterial digestion by the enzyme over time. Similarly to SEC analysis, activity of the reconstituted powder was compared to a lysozyme standard control as well as to pre-formulation and sprayed pre-formulation. A denatured lysozyme stock was used as positive control for reduced activity. Figure 6d shows that the denatured lysozyme stock presented a significant decrease in activity as compared to control lysozyme (P value = 0.0006, < 0.05), while both spray-only and reconstituted powder were not significantly different (P value = 0.5 and 0.8, > 0.05), confirming that SFD processing did not significantly affect the lysozyme biological activity.

Finally, moisture content, agglomeration, and DSC analysis were conducted after 6 months of storage at −20°C and showed no significant changes in powder characteristics.

DISCUSSION

Analysis of the 10 lysozyme formulations showed that composition and total solid concentration of the pre-formulation influenced powder aerosolization performance and morphology. In particular, an increase in protein concentration, and concurrent decrease in sugar amount (sucrose + mannitol), positively impacted aerosolization performance (Fig. 7). Among the formulations tested, powder formulations containing approximately 60% lysozyme and 40% sugar amount (F6 and F10) performed better than other formulations, with a RF of 48 and 57%, respectively. This correlated with a decrease in bulk density as the concentration of sugars decreased (0.03 and 0.01 g/cm^3 for F10 and F6 presenting 40% sugar *vs* 0.325 and 0.133 g/cm^3 for F7 and F1, presenting 88 and 64% sugar, respectively; see Table II).

A highly porous powder structure with a large, irregular, and rough surface was clearly visible in the SEM photographs for F10 and F6 (Fig. 5a and Suppl. Figure 3), which correlated with the formulations' high specific surface area (SSA) of 11 and 12.6 m^2/g, respectively. Figure 5c, d and Suppl. Figure 3 bottom showed that this porous structure is likely broken down into smaller particles upon turbulent dispersion with the RODOS powder disperser instrument, with particles having a geometrical size distribution characterized by a d50 = 3.1 μm. The presence (F6) or absence (F10) of mannitol in the formulation did not affect powder morphology, but may impact powder performance (RF decreased from 57 to 48% when mannitol is present).

Overall, formulations that contained a higher percentage of sugar appeared visually denser and tacky and did not produce a powder suitable for inhalation.

Pre-formulation total solid content (1 *vs* 10%) affected powder morphology and performance, with formulations obtained from 10% solid content solutions generally presenting a reduced aerodynamic performance (Fig. 7). SEM images in Fig. 5 compared the morphology of formulations F10 (a) and F2 (b), possessing the same protein sucrose ratio (60:40), but solid content of 1 and 10%, respectively. Spherical particles with a lower degree of porosity were obtained for the formulation with higher total solid content (F2) which also displayed a decreased RF of 12% as compared to RF of 57% of F10.

Our SEM observations are consistent with previously reported data showing similar SFD protein porous powder structures (7, 9, 18), with comparable SSA (19.2 m^2/g) [32].

Porous particles are designed to enhance powder fluidization and dispersibility, as particle morphology strongly influences the aerodynamic diameter (D_a), which governs particle deposition in the respiratory tract. D_a is defined as

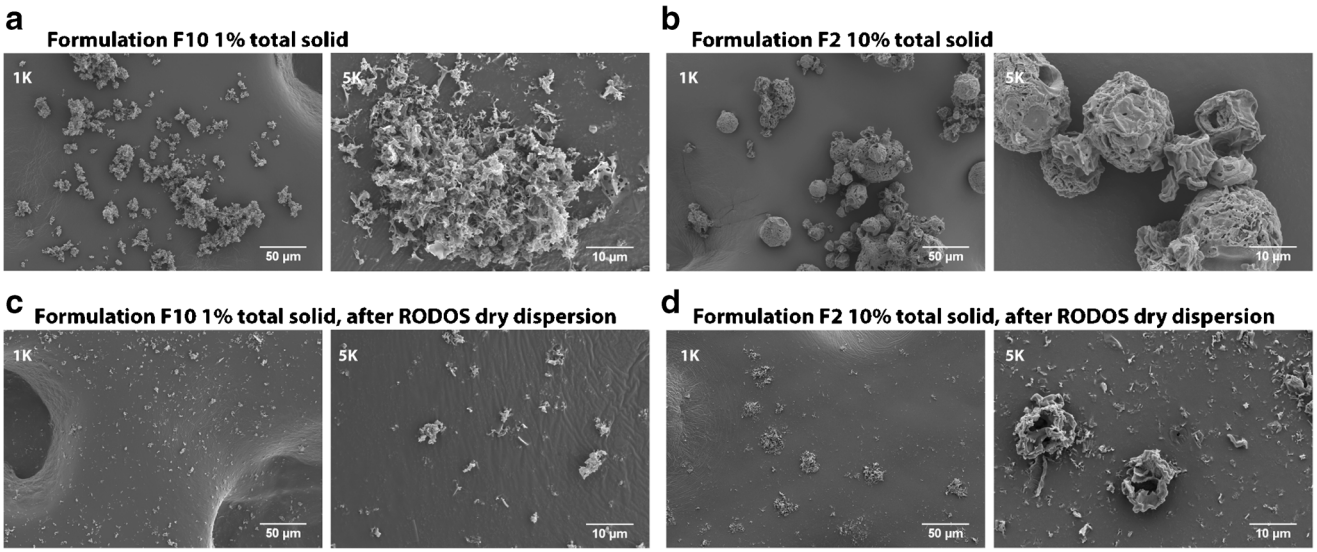
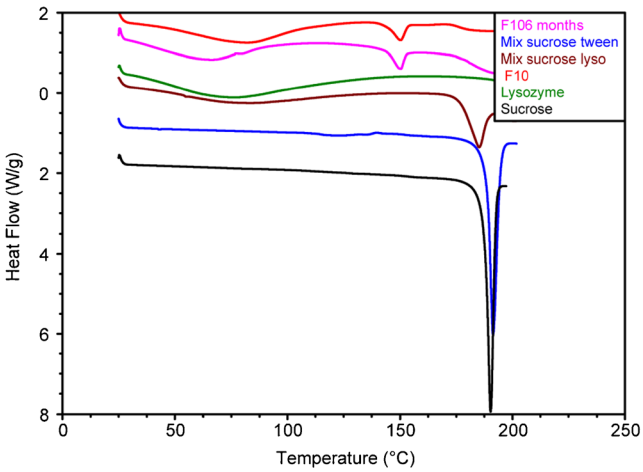
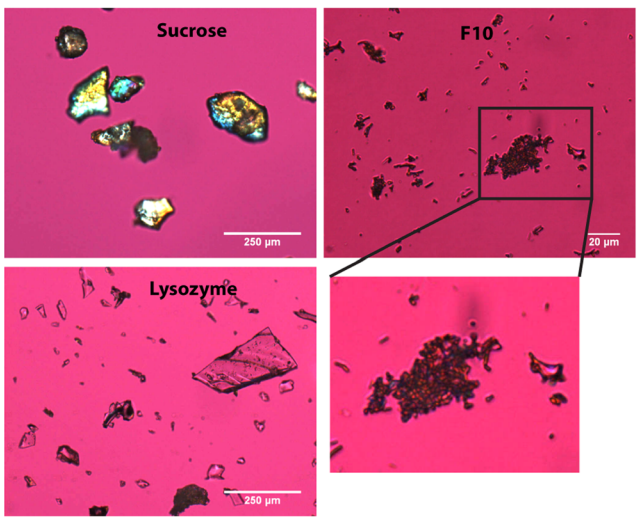


Fig. 5. Powder dispersibility: SEM images of F10 and F2 powders. **a**, **b** Undispersed powder and **c**, **d** dispersed powder upon application of 4 bar air pressure with RODOS

a

F10 Characterization	
Geometric particle size (LD-RODOS dry dispersion) (µm)	d_{10}=0.93, d_{50}=3.09, d_{90}=10.01, at 4 bar
Specific surface area (BET) (m^2/g)	12.56 (degassed at 37C)
Aggregation after aerosolization (SEC)	Relative RT=0.99, % peak area=100
Biological activity (Enzymatic assay)	Active
Crystallinity (DVS and PLM)	Amorphous with micro-crystals
Phase transitions (DSC)	Endothermic peak at ~150C





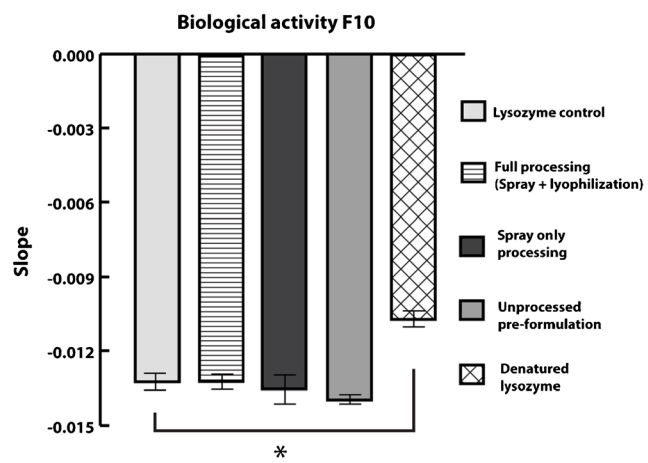


Fig. 6. Characterization of F10. **a** Summary table of analytical methods used. **b** DSC thermograms. **c** PLM images. **d** Biological activity test results ($n = 3$, results reported as average ± SD). * expresses the statistical significance of experimental results as assessed by ANOVA with an alpha level set at 0.05

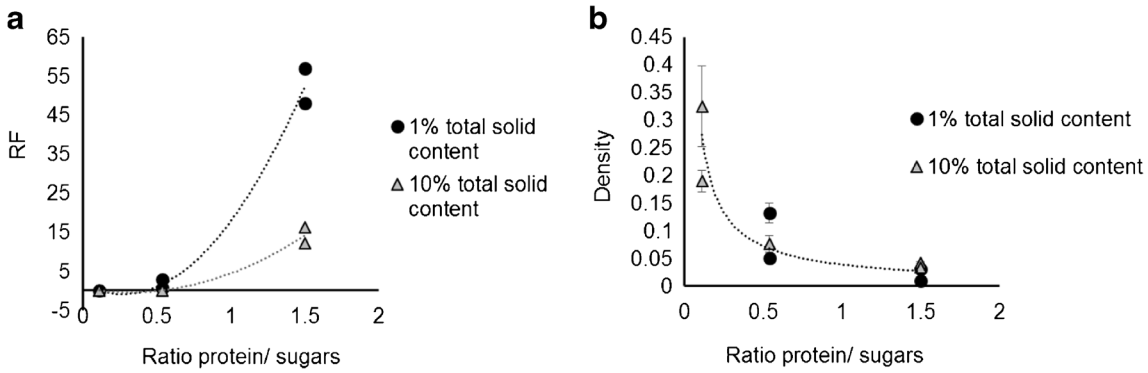


Fig. 7. Effect of formulation factors on SFD powders. Effect of protein and sugar concentration, expressed as a ratio, **a** on density and **b** on respirable fraction (RF). Each marker represents one of the formulations investigated. Density measurements were performed in triplicates and the data are reported as average ± SD

$D_a = D_s \times \rho_s^{0.5} \times \chi^{-0.5}$, where D_s is the physical diameter of the particle, ρ_s is its density, and χ is the dynamic shape factor. Therefore, assuming same physical diameter, a light (low density) particle has a smaller D_a and therefore higher propensity to travel in the air stream and reach the deep lung for effective deposition. Particles with a D_a of less than around 5 μm are generally considered suitable for inhalation [33, 34]. The MMAD calculated for formulations F10 and F6 from NGI measurements was 4.5 and 4.9 μm, respectively, and therefore, these formulations may be suitable for further development as respirable biologic powders. Furthermore, the use of highly porous particles (SSA ~ 8.6 m^2/g, ~ 70% porosity) has been successfully applied in the marketed product TOBI Podhaler (PulmoSphere technology) for the delivery of tobramycin inhalation powder [35], highlighting the commercialization potential for powders presenting similar physical characteristics.

However, it is important to remember that for biologic powders, both optimization of the aerodynamic characteristics of the powders and protection of the protein structure are essential to achieve an effective formulation. Thus, the above considerations based on the DoE analysis of our NGI and bulk density data need to be integrated with additional protein characterization. The most common protein physical degradation mechanisms are denaturation and noncovalent aggregation. Denaturation is the unfolding of the three-dimensional structure of the protein caused by various stresses including shear stress, exposure to interfaces (air-liquid and solid-liquid), and drying. Generally, in the native conformation, hydrophobic portions of the protein are folded inward, but denaturation can expose these groups. The increased surface area and exposure of hydrophobic groups in unfolded proteins intensify the risk of noncovalent aggregation. Therefore, the presence of lysozyme aggregates by SEC was used as the main screening parameter to assess protein stability in the formulations that demonstrated medium/good aerosolization efficiency (F2, 4, 6, and 10).

Powder preparation in our work consisted of four main operations: (i) preparation of the lysozyme solutions, (ii) atomization of the liquid pre-formulations through the nozzle, (iii) fast freezing of the generated spray in LN_2, and (iv) freeze-drying of the frozen microdroplets. Our results (Table IV) showed no signs of aggregation of lysozyme for the four investigated formulations at any of the processing steps as well as after powder aerosolization with RS01 DPI (Fig. 6a table).

The most promising formulation, F10, was further characterized. The integrity of lysozyme biological activity in F10 was also verified *in vitro* using an enzymatic assay. Previous data reported in literature [36] have shown that preparation of dry powder lysozyme using a different processing approach (spray drying) lead to reduction in bioactivity due to thermal denaturation. From our SEC and bio-activity test results, it appeared that SFD did not affect lysozyme negatively.

In addition, PLM, DSC, DVS, and XRD analyses were performed. PLM images revealed the presence of crystals in the micron size range dispersed into an amorphous matrix (Fig. 6c). As expected, PLM images of sucrose and lysozyme unprocessed showed their respectively crystalline and amorphous nature, suggesting that the micro-crystals should be sucrose. The presence of a mixture of two phases, amorphous dispersion phase (sucrose + lysozyme) and residual crystalline phase (likely sucrose), was confirmed by DSC (Fig. 6b). Analysis of F10 revealed the presence of a broad band between 30 and 120°C and an endothermic peak at ~ 150°C, which was un-changed after 6 months of storage. F10 possesses a residual moisture content of ~ 10%, comparable with unprocessed lysozyme's moisture content. Therefore, the broad band between 30 and 120°C (present in both F10 and unprocessed lysozyme) is due to water evaporation. The endothermic peak at ~ 150°C is instead likely the melting point of a sucrose polymorph pre-existing in the SFD samples [37].

Likely due to the lower sensitivity of XRPD and DVS as compared to DSC and PLM, presence of the crystalline phase could not be detected and F10 appeared mainly amorphous based on these techniques (Suppl. Figure 5). DVS also showed the hygroscopic nature of the powder, which was expected due to high porosity and therefore large surface area. This can explain the high residual moisture measured by Karl Fisher. According to DVS analysis, around 30–40% RH (condition that can be experienced in Texas), the formulation tends to absorb ≥ 5% moisture. Therefore, although longer lyophilization time could potentially help to reduce the residual water, absorption of moisture from the environment during handling would still happen.

Finally, in addition to DSC analysis, F10 moisture content and agglomeration was also determined after 6 months storage at −20°C in sealed vials. No significant changes were observed, supporting stability of the powder over the period of time investigated. Based upon these initial promising results, more rigorous stability testing adhering to ICH standards will be performed in future studies.

These promising data show that the selected excipients as well as the selected processing parameters allowed an active stable lysozyme powder suitable for pulmonary delivery to be obtained.

The critical attributes for the lysozyme formulation in these studies were percentage of sucrose, percentages of total solids, and bulk density. Formulations possessing a 1% total solid content and percentage of sugar < 40% could potentially lead to porous particles with an even higher RF and more favorable API potency (% protein > 60%). The formulation design space will be rationally expanded in future studies to test this hypothesis. In addition, the systematic analysis, which in this work focused on formulation parameters, could be extended to the optimization of processing parameters to further refine the final powder. Due to the extremely large variety of proteins and their labile nature, protein stability will need to be verified case by case. However, the same logical screening mechanism could be used to establish the appropriate design space for each protein.

CONCLUSIONS

A systematic fast screening approach to verify the suitability of SFD lysozyme powders for pulmonary delivery was developed. The effect of formulation factors on aerosolization performance and protein aggregation was systematically investigated. Our results elucidated several relevant attributes (density, total solid content, % sucrose + mannitol) required to achieve a stable lysozyme powder with desirable characteristics for pulmonary delivery. With the growing interest in developing biologics for pulmonary delivery and some products being already approved by FDA, the use of a systematic fast screening method will be beneficial for faster development of protein formulations.

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COMPLIANCE WITH ETHICAL STANDARDS

The terms of this arrangement have been reviewed and approved by the University of Texas at Austin in accordance with its policy on objectivity in research.

Conflict of Interest The author (HDCS) of this paper consults for and has equity ownership in Respira Therapeutics and Nob Hill Therapeutics on inhaled product development. The other authors declare that they have no conflict of interest.

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