Inhalable Microparticle Platform Based on a Novel Shell-Forming Lipid Excipient and its Feasibility for Respirable Delivery of Biologics

Hui Wang[1], Patrick Connaughton[2], Kellisa Lachacz[2], Nicholas Carrigy[2], Mani Ordoubadi[1], David Lechuga-Ballesteros[2], Reinhard Vehring[1]

[1] Department of Mechanical Engineering, University of Alberta, Alberta, Canada

[2] Inhalation Product Development, Pharmaceutical Technology & Development, Operations, AstraZeneca, South San Francisco, CA, USA

ABSTRACT

Administration of biologics such as proteins, vaccines, and phages via the respiratory route is becoming increasingly popular. Inhalable powder formulations for the successful delivery of biologics must first ensure both powder dispersibility and physicochemical stability. A lipid-based inhalable microparticle platform combining the stability advantages offered by dry powder formulations and high dispersibility afforded by a rugose morphology was spray dried and tested. A new simplified spray drying method requiring no organic solvents or complicated feedstock preparation processes was introduced for the manufacture of the microparticles. Trehalose was selected to form the amorphous particle core, because of its well-known ability to stabilize biologics, and also because of its ability to serve as a surrogate for small molecule actives. Phospholipid distearoyl phosphatidylcholine (DSPC), the lipid component in this formulation, was used as a shell former to improve powder dispersibility. Effectiveness of the lipid excipient in modifying trehalose particle morphology and enhancing powder dispersibility was evaluated at different lipid mass fractions (5%, 10%, 25%, 50%) and compared with that of several previously published shell-forming excipients at their effective mass fractions, i.e., 5% trileucine, 20% leucine, and 40% pullulan. A strong dependence of particle morphology on the lipid mass fraction was observed. Particles transitioned from typical smooth spherical trehalose particles without lipid to highly rugose microparticles at higher lipid mass fractions (> 5%). *In vitro* aerosol performance testing demonstrated a significant improvement of powder dispersibility even at lipid mass fractions as low as 5%. Powder formulations with excellent aerosol performance comparable to those modified with leucine and trileucine were achieved at higher lipid mass fractions (> 25%). A model biologic-containing formulation with 35% myoglobin, 35% glass stabilizer (trehalose), and 30% lipid shell former was shown to produce highly rugose particle structure as designed and excellent aerosol performance for efficient pulmonary delivery. A short-term stability at 40 °C proved that this protein-containing formulation had good thermal stability as designed. The results demonstrated great potential for the new lipid microparticle as a platform for the delivery of both small-molecule APIs and large-molecule biologics to the lung.

KEYWORDS

lipid microparticle; excipient; inhalable biologics; dispersibility enhancement; spray drying; particle engineering

INTRODUCTION

Spray drying is one of the most widely used methods of converting liquid solution, emulsion, or suspension feedstocks into solid microparticle-based powders in the food and pharmaceutical industry [1, 2]. A typical

spray drying process involves liquid feedstock preparation, atomization of the feedstock to form droplets, controlled desiccation of the atomized droplets in a drying chamber, and subsequent collection of dried particles [3]. In comparison to other drying technologies, such as tray drying, rotary drying, and freeze drying, spray drying is a highly-customizable technology that enables more accurate control of both the drying processes and the final particle properties, including the inlet and outlet temperatures, powder moisture, solid phase, particle structure and morphology, and size distribution [4]. Spray drying finds a perfect fit in the field of respiratory drug delivery of solid dosage forms, where drugs are delivered through the human airway in the form of microparticles. Critical product attributes such as particle size distribution, physical and chemical stability, and aerosol performance, which need to be carefully designed and characterized to ensure successful drug delivery to the targeted sites, can all be modified and fine-tuned via particle engineering in the spray drying process [5].

Dry powder formulations offer several advantages over liquid dosage forms, including reduced cost for global distribution in comparison with refrigerated storage and transportation, ease of application using simpler devices such as dry powder inhalers (DPI), and most importantly, improved thermostability. Dispersibility and stability are two of the most important attributes when developing inhalable dry powder formulations, especially with biologics [11]. For dry powder delivery of actives to targeted sites in the respiratory tract to be efficient, suitable excipients are typically used to ensure powder dispersibility. Excipients capable of enhancing the dispersibility of powders have attracted significant research attention [12]. Cohesive micronized drug particles are traditionally blended with external excipients in the form of coarse carrier particles, e.g., consisting of lactose, for improved handling, dispersing, and metering of the powder mixture [13]. In addition to providing bulk to the drugs in the form of carrier particles, various new internal excipients with specific functionalities have been developed in modern formulation development strategies to directly modify the particle and powder properties during particle formation using particle engineering technologies; these excipients are usually formulated in the feedstock in the form of a solution, emulsion, or suspension together with the actives during spray drying [5]. Final powder products of such formulations with internal excipients can usually be produced through simplified manufacturing processes and are therefore more cost-effective for large-scale production [14]; they can be delivered by means of simpler devices without requiring any separation of the excipients and actives, thus also leading to better dose uniformity [15]. Improving the stability of inhalable dry powder formulations is another critical aspect of successful formulation development because the formulated components are subject to various physical and chemical degradation mechanisms. Maintaining stability becomes more challenging when biologics are involved because the biological components are known to be sensitive to the different stresses—for instance, temperature, mechanical, and chemical—frequently encountered in the manufacturing and delivery processes for respirable microparticles [11]. Therefore, excipients capable of stabilizing formulated complex biological components are also being actively investigated.

The types of excipients suitable for formulation in respiratory dosage forms are inherently limited by safety concerns. Amino acids, sugars, and lipids are three of the most commonly used excipient types, with some of these ‘generally recognized as safe’ (GRAS) for pulmonary drug delivery by the Food and Drug Administration Agency (FDA) [8]. Different amino acids have been explored as excipients for powder dispersibility enhancement [16-18]. In addition, surface-active amino acids have also been used to provide protection to the formulated biologics by competing with the biological components to enrich the air-

water droplet interface during spray drying and thereby minimize degradation [11]. As an essential amino acid with moderate surface activity, leucine has been studied extensively as a dispersibility- and stability-enhancing excipient in the spray drying of inhalable pharmaceutical aerosols [19-21]. Trileucine is a highly surface-active tripeptide composed of three leucine residues. It has been demonstrated to be very effective in modifying particle morphology and improving powder dispersibility and stability at relatively low mass fractions [22-25]. Sugar excipients such as disaccharides, polyols, and polysaccharides (lactose, trehalose, sucrose, mannitol, maltodextrin, dextran, pullulan, etc.), all of which are able to embed formulated biologics in an glassy matrix, have been proven to be especially effective in protecting biologics from degradation during both manufacture and storage, thereby significantly improving powder stability [11, 26, 27].

Compared to other types of excipients, lipid-based excipients are more extensively used in carrier systems for the controlled delivery of various drug modalities from small-molecule actives to large-molecule biologics because of their easy processibility, broad accessibility, and excellent biocompatibility [28-30]. Phospholipids, including dipalmitoyl phosphatidylcholine (DPPC) and distearoyl phosphatidylcholine (DSPC), are endogenous to the lungs and represent a significant fraction of the total lipids in the lung tissue (65%) and lung surfactant (90%) [31]. This inherent endogeneity is valuable for efficient pulmonary drug transportation because inhaled drug particles deposited deep in the lungs are first confronted by a thin (10–20 nm) phospholipid-rich surfactant-lining fluid layer before going through either absorptive or non-absorptive clearances [32]. Naturally, various lipid-based excipient systems, especially phospholipid-based ones, have been developed to assist the delivery of drugs through the airways to the lung [33]. Lipid-based pulmonary delivery systems have been engineered into different formats including, but not limited to, micelles, nano or microemulsions, lipid nanoparticles, liposomes, and lipid microparticles [29, 34, 35]. With the exception of lipid microparticles, almost all such delivery systems are preferentially formulated in liquid and aerosolized by nebulization when targeting the lung [34, 36].

Cipolla et al. summarized the various lipids and methods used in the literature for the preparation of lipid microparticles [34]. Of these, DPPC was the lipid that initially attracted the most attention, and spray drying was the most widely used manufacturing method. Vanbever et al. prepared large inhalable porous DPPC-based microparticles by spray drying and observed a strong dependence of particle morphology on lipid content, feed solution pH, and spray drying temperature [37]. Also using spray drying, Corzo et al. produced corrugated lipid microparticles loaded with ibuprofen from solution feedstocks containing lipids with high melting temperatures and reported a strong dependence of production yield on the spray drying temperature [38]. Pilcer et al. spray dried cholesterol and DSPC with tobramycin to form a lipid coating around solid particles that demonstrated a significantly improved dispersibility [39]. Different lipid excipients, including DPPC and DSPC, were co-spray dried with a cohesive small molecule active by Shetty et al. to improve powder dispersibility [40]. DSPC was found to be the most effective of the studied lipids in improving aerosol performance, despite the smooth spherical particles obtained, and improved dispersibility was believed to be related to the high surface enrichment and low cohesiveness of DSPC. Despite the different levels of aerosol performance improvement achieved by these lipid microparticles, almost all of them require the use of organic solvent due to the low solubility of these lipids in water, making them less favorable for preserving biologics and causing issues with residual solvents in the resulting powder.

To circumvent the use of organic solvents, emulsion feedstocks incorporating various lipids have been used [34]. The PulmoSphere™ platform consisting of small, highly porous sponge-like DSPC particles is one

of the lipid microparticle systems that stands out [41]. Its porous lipid microparticles are manufactured by spray drying a perfluorooctyl bromide (PFOB)-in-water emulsion prepared by multiple passes of high-pressure homogenization and stabilized by DSPC and $CaCl_2$. The PFOB oil droplets contained in the larger atomized feedstock droplets are then removed during the subsequent drying process, leaving numerous open pores in the final particles with the foam-like structure of DSPC. This porous particle platform has been applied not only in DPI formulations [42, 43] but also in modified form in pressurized metered dose inhalers [44, 45] with excellent adaptability and performance, leading to the successfully commercialized products Tobi® Podhaler® (Novartis), Bevespi Aerosphere®, and Breztri Aerosphere® (AstraZeneca).

Combining the advantages offered by dry powder formulations and lipid-based excipients, lipid microparticles show great potential for the pulmonary delivery of not only traditional small-molecule APIs but also larger molecule biologics. Targeting the pulmonary delivery of biologics, the formulation strategies explored in this study include using lipid as the shell former and adding a sugar excipient to form an amorphous glass matrix. The lipid aims both to improve the particle dispersibility as a shell former and also to compete with large-molecule biologics to enrich the particle surface, while the sugar core is intended to provide a stabilizing matrix for the biologics. Trehalose has been used in various research applications for the stabilization of a wide range of biological components such as proteins [46], vaccines [47], monoclonal antibodies [48], and bacteriophages [49] and was therefore selected to form the amorphous stabilizing core. As a small-molecule disaccharide of glucose, trehalose also acts as a surrogate for cohesive small-molecule APIs and thus demonstrates a potential for use in the delivery of such actives. Utilizing a novel suspension-based lipid spray drying method developed recently by Wang et al [50], the phospholipid DSPC, as the lipid component in this formulation, was evaluated at different mass fractions, and its effectiveness in modifying the final particle morphology and enhancing powder dispersibility was compared with that of excipients of various types, including amino acids (leucine, trileucine) and a polysaccharide (pullulan). Myoglobin was formulated as a model protein to demonstrate the applicability of the developed lipid microparticles to delivering large-molecule biologics.

MATERIALS AND METHODS

Materials

D-(+)-trehalose dihydrate (CAS 6138-23-4, Fisher Scientific Co., ON, Canada) with a purity of >98% was used as the glass stabilizer for biologics or as a surrogate for cohesive small-molecule APIs in this study. Different dispersibility-enhancing excipients, including leucine, trileucine, pullulan, and a phospholipid, were compared in terms of their effectiveness in modifying particle surfaces and enhancing dispersibility.

A saturated phosphatidylcholine (PC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC or 18:0 PC; CAS 816-94-4, Avanti Polar Lipids Inc., AL, USA) was investigated in this study as the new lipid dispersibility-enhancing excipient. Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$, CAS 10035-0408, Fisher Sci., ON, Canada) was added to the lipid at a lipid-to-salt molar ratio of 2:1 during feedstock preparation to stabilize the liquid dispersion against flocculation [41]. L-leucine with a purity of 99% (CAS 61-90-5, Fisher Scientific Co., ON, Canada), trileucine with a purity of > 90% (CAS 10329-75-6, Sigma Aldrich, ON, Canada), and pullulan (CAS 9057-02-7, Alfa Aesar, MA, USA), as three well-established shell-forming excipients, were also investigated and their respective results compared with those of the new lipid dispersibility enhancer.

Myoglobin from equine skeletal muscle with a purity of > 95% (CAS 100684-32-0, Sigma-Aldrich, ON, Canada) and a molecular size of approximately 17 kDa was formulated as a model biologic.

Methods

Spray drying

A custom-built lab-scale spray dryer [51] in combination with a twin-fluid atomizer was used for the spray drying of formulations listed in **Table 1**. All spray-dried powder batches targeted the same particle size within the respirable range of 1 – 5 μm in order to eliminate the effect of particle size on powder dispersibility [52]. Likewise, all feedstocks were prepared to have the same solids concentration (c_0) at 20 mg/mL and were spray dried at the same settings. For batches #1-4 and #10, consisting of only the water-soluble components trehalose, trileucine, leucine, pullulan, and myoglobin, solution feedstocks were prepared at room temperature by directly dissolving the needed quantities of raw material. The mass fractions for trileucine (5%), leucine (20%), and pullulan (40%) were selected according to the literature [21, 24, 26] with the aim of achieving substantial surface modification to the particle morphology. Prepared feedstocks were supplied to a twin-fluid atomizer for spray drying using a peristaltic pump (Masterflex® L/S®, Cole-Parmer, QC, Canada).

For the lipid-containing formulations (#5-9, 11), pure water was heated to 55°C on a hotplate equipped with a feedback temperature control. Trehalose was first added to the heated water until completely dissolved. Lipid DSPC and calcium chloride were then added at a lipid-to-salt molar ratio of 2:1 or mass ratio of 14:1, followed by gentle agitation of the dispersion to fully immerse the added solids. For simplicity, the combination of DSPC lipid and calcium chloride is hereafter referred to as the lipid or phospholipid excipient. The resulting translucent feedstocks were kept on the hotplate for another 30 minutes, followed by a high shear mixing process in which the dispersing probe (S18N-19G, IKA, NC, USA) of a high shear mixer (T-18 Ultra-Turrax, IKA, NC, USA) was immersed into the liquid for 3 minutes at 25,000 rpm to disperse the feedstocks. For batch #11, a concentrated myoglobin solution of 100 mg/mL was added to the feedstock as an annex solution after the high shear mixing. The feedstocks were then supplied to a twin-fluid atomizer using the peristaltic pump with the dispersing probe still immersed in the liquid running at approximately 5000 rpm to prevent the sample from flocculating. Small aliquots of the feedstocks for batches #5-9 were extracted during spray drying for dynamic light scattering analysis. A more detailed description of the spray drying processes has been reported previously [50].

All spray drying runs used the same processing parameters, i.e., a total feedstock volume of 100 mL, a fixed feed flow rate of 2.5 mL/min, a drying glass flow rate of 600 SLPM, an inlet temperature of 55 °C that corresponded to outlet temperatures of about 36.0 °C, and a dispersing gas flow rate of ~20 L/min that corresponded to an air-liquid ratio of 10 for the atomizer. Based on droplet size calibration data presented previously [53], the twin-fluid atomizer was operated at an air-liquid ratio of 10 and a 2.5 mL/min feed flow rate corresponding to an initial droplet mass median diameter (d_0) of about 9 μm. According to Eqn. (1), in which ρ^* stands for unit density (1 g/cm^3), the aerodynamic diameter of the final particles (d_a) was expected to be mainly dependent on the initial droplet diameter (d_0) and the feedstock solids concentration (c_0), and only weakly dependent on the particle density (ρ_p). Assuming a final particle density close to 1 g/cm^3, the dried particles were expected to be within the respirable range at about 2 μm.

$$d_a = \sqrt[6]{\frac{\rho_p}{\rho^*}} \bullet \sqrt[3]{\frac{c_0}{\rho^*}} d_0 \quad (1)$$

Primary particle size distributions of the dried particles were measured in-process during spray drying using the combination of a custom-designed isokinetic sampler [51], an aerosol diluter (3302A, TSI, MN, USA), and a time-of-flight aerodynamic particle sizer (APS, 3321, TSI, MN, USA). Powders collected by a stainless-steel cyclone into collection bottles were transferred to glass vials and dried for more than 24 h in a vacuum desiccator at room temperature to further remove residual moisture content.

Table 1 Experimental matrix for spray-dried formulations with different excipients. T = trehalose, Tri = trileucine, L = leucine, P = pullulan, LC = lipid and calcium chloride, M = myoglobin. All numbers are expressed in mass fraction of the respective component.

Batch #	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Formulation	95T5Tri	80T20L	60T40P	**100T**	95T05LC	90T10LC	75T25LC	50T50LC	100LC	50T50M	35T35M30LC
Trileucine (%)	5	-	-	**-**	-	-	-	-	-	-	-
Leucine (%)	-	20	-	**-**	-	-	-	-	-	-	-
Pullulan (%)	-	-	40	**-**	-	-	-	-	-	-	-
Trehalose (%)	95	80	60	**100**	95	90	75	50	-	50	35
Myoglobin (%)	-	-	-	**-**	-	-	-	-	-	50	35
Lipid-$CaCl_2$ (%)	-	-	-	**-**	5	10	25	50	100	-	30

Dynamic light scattering

To better understand the particle formation process of the lipid-containing formulations (#5-9 in **Table 1**), the hydrodynamic diameter and polydispersity of the dispersed DSPC in the respective feedstocks after high shear mixing were measured using a dynamic light scattering technique (ZetaSizer Nano, Malvern, UK). Approximately 1 mL of liquid feedstock for each sample was extracted from the feedstock during spray drying, transferred to disposable cuvettes (ZEN0040, Malvern, UK), and measured at a 173° backscattering angle. All samples were measured at a temperature of 25°C from a fixed position of 3 mm from the bottom of the cuvette, with each measurement taking approximately 1-3 minutes. The reported average and standard deviations of the size distribution statistics for each formulation are from the same sample analyzed three times. The polydispersity index, as a measure of the broadness of the size distribution, was calculated as the squared value of the standard deviation of the measured size distribution divided by the mean hydrodynamic diameter.

Particle morphology analysis

Micrographs of the collected particles were obtained using a field emission scanning electron microscope (Zeiss Sigma, Carl Zeiss, Germany). Particles of each sample were first loaded onto standard SEM pin mounts (16111, Ted Pella Inc., CA, USA) covered with double-sided adhesive carbon tapes and further coated with gold nanoparticles to a thickness of approximately 16nm using a sputter coater (Desk II, Denton Vacuum, NJ, USA) to remove any charging effects. A low accelerating voltage of 5 kV was used for all cases to prevent potential electron beam damage to the particles.

Powder moisture content measurement

Residual moisture content is known to affect the aerosol performance of powder formulations [54]. A coulometric Karl Fischer titrator (C30, Mettler Toledo, OH, USA) equipped with an oven autosampler (Stromboli, Mettler Toledo, OH, USA) was used to determine the water content of powders before *in vitro* aerosol performance characterization. All instrument accessories used for the measurement, including sample vials, drift vial aluminum insert, rubber seals and aluminum foils, were pre-conditioned in a dry glove box (<1 % RH) for more than 30 minutes. For each sample, about 80 ± 20 mg of powder was used for the measurement. The heating temperature of the oven was set to 130 °C to vaporize any residual water in the powder samples. Clean and dry nitrogen gas was supplied to the sample vials at 80 – 100 mL/min to bring the water vapor to the cell filled with titration reagent (Hydranal™, Coulomat AG, Honeywell, ON, Canada) for coulometric determination of the water content.

Solid state analysis

A custom-designed Raman spectroscopy instrument [55] was used to determine the solid phases of different components in each of the formulations. The Raman instrument uses a 671nm diode laser (Ventus 671, Laser Quantum, UK) with a maximum output power of 500mW. Powder samples loaded into a 0.2 µL conical cavity in an aluminum sample holder were exposed to the laser in a sample chamber flushed with clean, dry nitrogen (< 3% RH) at room temperature. Pure trehalose, trileucine, and L-leucine were measured directly to obtain their respective crystalline Raman reference spectra. The pullulan raw material, as a polysaccharide polymer, was found to be amorphous when received and was measured to obtain its amorphous reference spectrum. No crystalline pullulan was produced or measured in this study. Spray-dried pure trehalose and trileucine measured and found to be amorphous were used as their respective amorphous reference spectra [24]. Spray-dried pure leucine generally leads to its crystalline form [19]. Therefore, a low mass fraction of leucine (10%) was co-spray dried with trehalose to inhibit its crystallization. Extra-fine particles recovered from the exhaust gas post-cyclone were measured according to the size-dependent crystallinity of leucine reported previously [21]. An amorphous L-leucine reference spectrum was then obtained by subtracting trehalose from the spectrum of this mixture. A deconvolution method for multi-component systems [55, 56] was used to determine the contribution of each component. The deconvoluted spectroscopic contribution of each component was then compared with its reference spectrum for solid phase determination. Briefly, the deconvolution process can be summarized using the following Eq. (1), where the normalized reference Raman spectra of each component, $S_{i,N}$, was subtracted gradually from the raw spectrum of the multi-component system, *S*. *B* is the spectral background and I_i is the intensity factor for component *i*. A small frequency shift adjustment, Δv, was added to the reference spectra as needed to minimize the residual spectrum.

$$S = B + \sum I_i S_{i,\mathrm{N}}(\Delta\nu + b_i) \quad (1)$$

Powder crystallinity was further verified using an X-ray diffractometer (D8 Discover, Bruker AXS, Germany) under ambient conditions. Samples were subjected to Cu-sourced X-ray radiation at 30mA and 40kV. Powders were loaded onto microscope slides (12-550C, Fisher Sci., ON, Canada) and scanned at a rate of 5°/min from 5° to 50°.

Dynamic vapor sorption analysis

To investigate whether the various shell-forming excipients added to the microparticles affected the interaction of water with the trehalose core, a gravimetric water vapor sorption analyzer (DVS Intrinsic

Plus, Surface Measurement Systems, PA, USA) was used to measure the water sorption behavior of the different powders. For each measurement, 15.0 ± 1.3 mg of powder was loaded and loosely dispersed onto the sample pan and then equilibrated for at least 2 hours at 0% RH to remove residual water in the powders. Trehalose has been reported to have a critical crystallization relative humidity of roughly 50% at room temperature, above which amorphous trehalose starts to crystalize quickly [57]. Therefore, powders produced in this study were exposed to a constant 60% RH under 25 °C to assess the level of protection against moisture provided by the different excipients. A drying step at 0% RH was added to the end of each measurement to determine the net amount of water absorbed during the moisture exposure process. For each stage, the equilibrium criterion was set to <0.002 %/min, only after which was the powder deemed to have reached equilibration at the set relative humidity and the testing ready to proceed to the next step.

Aerosol performance characterization

To compare the effectiveness of the different excipients in enhancing powder dispersibility, the aerosol performance of all the spray-dried powders was tested *in vitro* using the Alberta Idealized Throat (8511, Copley Sci. Ltd., Nottingham, UK). Two main parameters are typically determined during *in vitro* aerosol performance characterization measurements: the emitted dose (ED), sometimes also called the delivered dose or emitted fraction, which is defined as the percent of the loaded powder emitted from the DPI device following actuation; and the total lung dose (TLD), which usually represents the respirable fraction of the loaded capsule dose that bypasses deposition in the idealized throat model. Rather than a traditional *in vitro* approach of estimating aerosol performance, which relies on cascade impaction, a simplified approach combining the Alberta Idealized Throat with a downstream filter was employed to estimate the total lung dose (TLD) of the produced powders in this study. This simplified approach has been demonstrated to correlate well with estimates from cascade impactor-based measurements [58].

In this study, a commercial capsule-based DPI device (Seebri Breezhaler®, Novartis) was used to disperse the powders. A 3D-printed mouthpiece adaptor was attached to the inlet of the model throat to ensure an airtight seal during device actuation. Before each experiment, both halves of the Alberta Idealized Throat were coated with silicone oil spray (Molykote 316, Dow Corning Corp., MI, USA) and given at least 20 minutes for the solvent to evaporate. A downstream bacterial filter (RT020, Fisher & Paykel Healthcare Ltd., New Zealand) was connected to the exit of the idealized throat, followed by a mass flowmeter (4040, TSI, MN, USA) for airflow control and monitoring. A standard square wave flow profile was generated using a critical flow controller (TPK 2000, Copley Sci. Ltd., UK) connected to a vacuum pump (Maxima M16C, Fisher Sci., ON, Canada). Powders were first filled into hydroxypropyl methylcellulose DPI capsules (Size 3, Quali-V-I, Qualicaps Inc., IN, USA) in a dry environment (< 0.1% RH); these were then pierced on both ends by the DPI device before actuation. Each capsule contained 37 ± 7 mg powder, and three capsules were actuated per test to obtain sufficient mass on the filter for subsequent gravimetric analysis. Experiments were performed under ambient conditions in triplicate for each set of samples.

As a DPI device with a low device resistance of about 0.02 $kPa^{0.5}$/(L/min) [59], the Breezhaler® is usually tested at the standard flow rate of 100 L/min recommended by the US Pharmacopeia [60], since the flow rate required to generate a 4 kPa pressure drop across the device is typically higher than this. To better demonstrate the differences in the dispersibility of powders modified by different shell formers, a lower flow rate at 60 L/min was intentionally used for the initial testing of all the powders. A 4.0 s inhalation profile was chosen such that 4.0 L of air was withdrawn from the mouthpiece of the inhaler [60]. In

addition, the formulations with varying mass fractions of lipid excipient (#5-8 listed in **Table 1**) were also tested at the standard 100 L/min for 2.4 s to evaluate their aerosol performance under standard testing conditions.

Stability study

To evaluate the thermal stability of the trehalose-lipid microparticle platform, the protein-containing formulation #11 (35T35M30LC) from **Table 1** with 35% trehalose, 35% myoglobin, and 30% lipid excipient was selected for an accelerated short-term stability study. Packaging of the powder sample for stability study took place within a glovebox set to 0% RH. The powder sample was first transferred to glass vials and sealed with screw caps and parafilm sealing tape. Samples vials were then placed in an aluminum bag along with silica gel pouches that had been equilibrated at 0% RH. This bag was then double heat-sealed and placed into an incubator set to 40 °C for a stability study. After 1 month of aging at 40 °C, the powder sample was taken out and characterized for particle morphology, solid state, moisture content, and aerosol performance.

RESULTS AND DISCUSSION

Feedstock characterization for lipid-containing formulations

Hydrated DSPC lipid has a main phase transition temperature of about 55°C [61], above which the lipid can transition from the gel phase to a more mobile liquid crystalline phase. The liquid feedstock was therefore intentionally heated to close to the main phase transition temperature in order to induce the lipid transition [50]. It was likely that a considerable fraction of the raw lipids was able to go through the main phase transition and form the more mobile liquid crystalline phase, which was then further disrupted into smaller lipid particles by the high shear mixing process. The DLS results shown in **Fig. 1** indicate a predominance of lipid nanoparticles in the range of 60 – 100 nm for all the cases. The consistent size distributions (solid, dot, and dash) measured for each sample indicated relatively stable feedstocks. With increasing amounts of lipid added to the formulation, the feedstock transitioned from a slightly cloudy to an opaque, milk-like state.

Dispersed mobile lipid molecules in water can rearrange into different formats, including lipid bilayers, micelles, and liposomes covering a wide size range [33]. Dissolved free lipid molecules can be neglected here because of the extremely low solubility of DSPC in water [61]. However, the exact forms of the lipids in the feedstock could not be determined simply from the size distribution; rather, a mixture of multiple phases was likely. Considering that the feedstock preparation temperature was intentionally set close to the main phase transition temperature of DSPC for this case, some lipid may have remained in the gel phase and only been micronized to the micrometer range by the high shear mixer. Volume-based size distributions (data not shown) proved the presence of larger lipid particles higher in the micrometer range. All the lipid particles dispersed in the feedstock were expected to have high Péclet numbers during particle formation and were therefore expected to enrich near the surface of the final particles as a shell former [5].

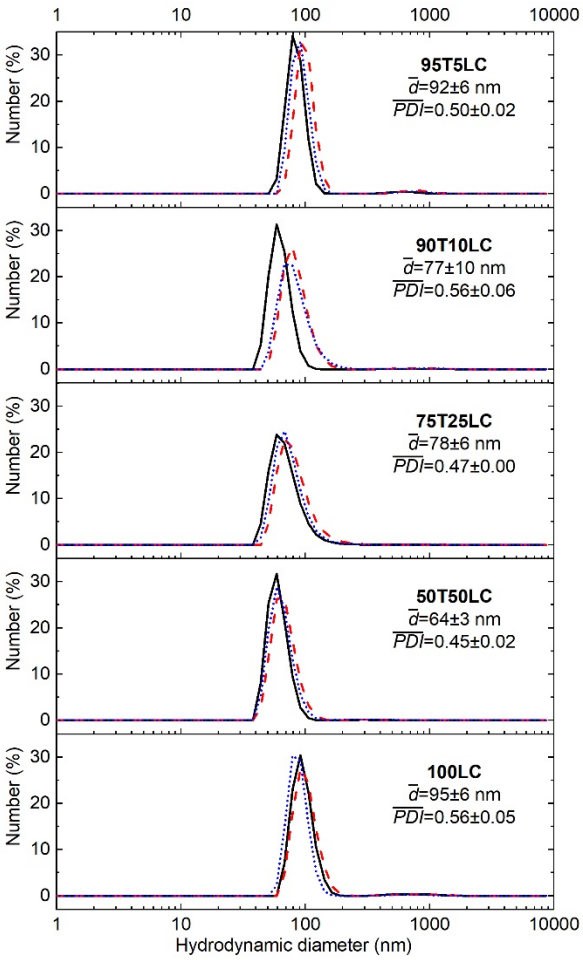

Fig. 1 Number particle size distributions of dispersed DSPC lipids in the feedstock after high shear mixing show a predominance of lipid nanoparticles in the hydrodynamic diameter range of 60 – 100 nm. Different lines for each sample stand for three consecutive measurements (solid to dot to dash), with each measurement taking 1 – 3 minutes. The insert $\overline{PDI}$ stands for the average polydispersity index, and $\overline{d}$ stands for the average z-average diameter of the three measurements.

Particle size distribution and moisture content

Primary particle size distributions isokinetically measured by the aerodynamic particle sizer and listed in **Table 2** agreed well with the theoretical prediction. All spray-dried powder batches had similar mass median aerodynamic diameters in the 1.5 – 2.0 µm range as designed and close geometric standard deviations all within the 1.4 – 1.6 range such that any effect of particle size on powder dispersibility was excluded. Results from Karl Fischer titration proved all powders had residual moisture contents lower than 2% prior to the *in vitro* aerosol performance characterization such that the effect of powder water content on powder dispersibility could be neglected as well [54].

Table 2 Isokinetically measured primary particle size distribution, spray drying yield (for a batch size of 2 g), and powder moisture content measured by Karl Fischer titration. T = trehalose, Tri = trileucine, L =

leucine, P = pullulan, LC = lipid and calcium chloride, M = myoglobin, MMAD = mass median aerodynamic diameter, and GSD = geometric standard deviation.

Batch #	Formulation	MMAD	GSD	Yield (%)	Moisture content (%)
1	**95T5Tri**	1.56±0.06	1.47±0.02	72.5	1.05
2	**80T20L**	1.73±0.03	1.55±0.03	83.5	1.47
3	**60T40P**	1.50±0.02	1.57±0.03	83.5	1.74
4	**100T**	1.64±0.01	1.51±0.01	72.0	1.37
5	**95T5LC**	1.60±0.01	1.50±0.02	71.0	1.60
6	**90T10LC**	1.67±0.04	1.50±0.01	71.0	1.61
7	**75T25LC**	1.84±0.07	1.57±0.02	70.5	1.53
8	**50T50LC**	1.59±0.03	1.60±0.05	77.5	1.31
9	**100LC**	1.81±0.03	1.52±0.00	80.0	0.91
10	**50T50M**	1.66±0.02	1.46±0.02	86.4	2.33
11	**35T35M30LC**	1.53±0.02	1.47±0.01	83.3	3.21

Surface modification

Particle formation during spray drying

Particle formation models [5, 62] describing the radial distribution of components within the atomized droplets during the spray drying process were used to interpret the structure and morphology of particles with different excipients. During the drying process of solution droplets or droplets with solid dispersions, the dimensionless Péclet number—defined as the ratio of the solvent evaporation rate, κ, to the diffusion coefficient of solute or dispersed solid, D—was used to predict the final particle morphology. The Péclet number is a parameter affected by both the material properties of the components and their processing conditions and can be directly correlated to the surface enrichment of each component, which plays a critical role in determining the final particle morphology [62].

$$Pe_i = \frac{\kappa}{8D_i} \quad (2)$$

Briefly, according to simplified particle formation theory, higher Péclet numbers usually lead to higher surface enrichment and earlier shell formation. Depending on the time difference between shell formation and solidification of the core, hollow particles or solid particles with layered core-shell structures may be formed. Depending on the properties of the shell, hollow particles with shells lacking sufficient mechanical strength may further collapse into wrinkled particles. The final particle structure for low-Péclet number systems also depends on the solubility of the components in the solvent. Components with a low Péclet number and a high solubility will exhibit low surface enrichment throughout the entire droplet lifetime, producing homogeneous solid particles. However, for low Péclet number systems with low solubility, the droplet surface becomes supersaturated early due to solvent evaporation, leading to

the nucleation or separation of a secondary solid phase and formation of a shell, which may also collapse, and form wrinkled particles later in the particle drying process. The use of more complex models for a more substantial discussion of particle formation during spray drying [5, 62, 63] is beyond the scope of this study.

Surface modification by established shell formers

As a non-reducing disaccharide, trehalose has been widely used as a glass stabilizer to protect biologics such as proteins, phages, and vaccines in spray-dried microparticles [64]. However pure trehalose particles have been found to be very cohesive. Therefore, trehalose can be viewed in this study not only as a typical glass former for biologics but also as a surrogate for cohesive small-molecule APIs to demonstrate the effectiveness of surface modification by different shell formers. Having a low Péclet number ($Pe \approx 1$) and a high solubility in water (690 mg/mL), trehalose is expected to have a negligible concentration gradient within the droplet during drying and will solidify almost simultaneously across the entire droplet once a critical saturation is reached. Therefore, trehalose particles were expected to have a spherical shape with little or no internal void space, and with particle density close to the true density of trehalose (1.58 g/cm^3). This morphology was indeed observed, as shown in **Fig. 2**(a-b).

Low trileucine mass fractions between 2% and 10% have led to significant modifications to particle morphology [23] and improvements to the resultant powder dispersibility [22]. Therefore, a 5% trileucine mass fraction was selected as a comparator for improving powder dispersibility. Typical morphologies of trileucine-modified low-density, rugose particles resembling those reported in the literature are presented in **Fig. 2**(c-d). An in-depth discussion of the formation process for trileucine-containing particles has been provided by Ordoubadi et al. [23].

The particle formation process for leucine-containing formulations, which is controlled by crystallization, has been previously described in detail [19]. Briefly, precipitated leucine crystals accumulate quickly on the droplet surface and merge to form a shell that subsequently deforms, leading to corrugated particles covered with crystalline leucine patches. Their typical morphology is presented in **Fig. 2**(e-f). In comparison to trileucine, a higher mass fraction of leucine—between 5% - 40%—is usually needed for sufficient surface modification to achieve a considerable improvement in aerosol performance [20]; therefore, the formulation with 20% leucine was evaluated in this study.

Pullulan is a water-soluble non-reducing polysaccharide with a very high glass transition temperature (~261°C) that has been widely explored as a stabilizer for biopharmaceuticals in freeze-dried formulations [65]. Recently, it has been demonstrated that pullulan can be used as a surface modifier in spray drying to improve thermal stability and powder dispersibility of microparticles for respiratory drug delivery applications [26, 66, 67]. During particle formation, pullulan molecules will enrich near the droplet surface and form a shell. Surface folding is expected to occur while the particles are still large. Notable surface modification was observed when the mass fraction of pullulan exceeded 30% [26]. Spray-dried trehalose particles formulated with 40% pullulan are presented in **Fig. 2**(g-h). These show irregular shapes and surface folding as expected.

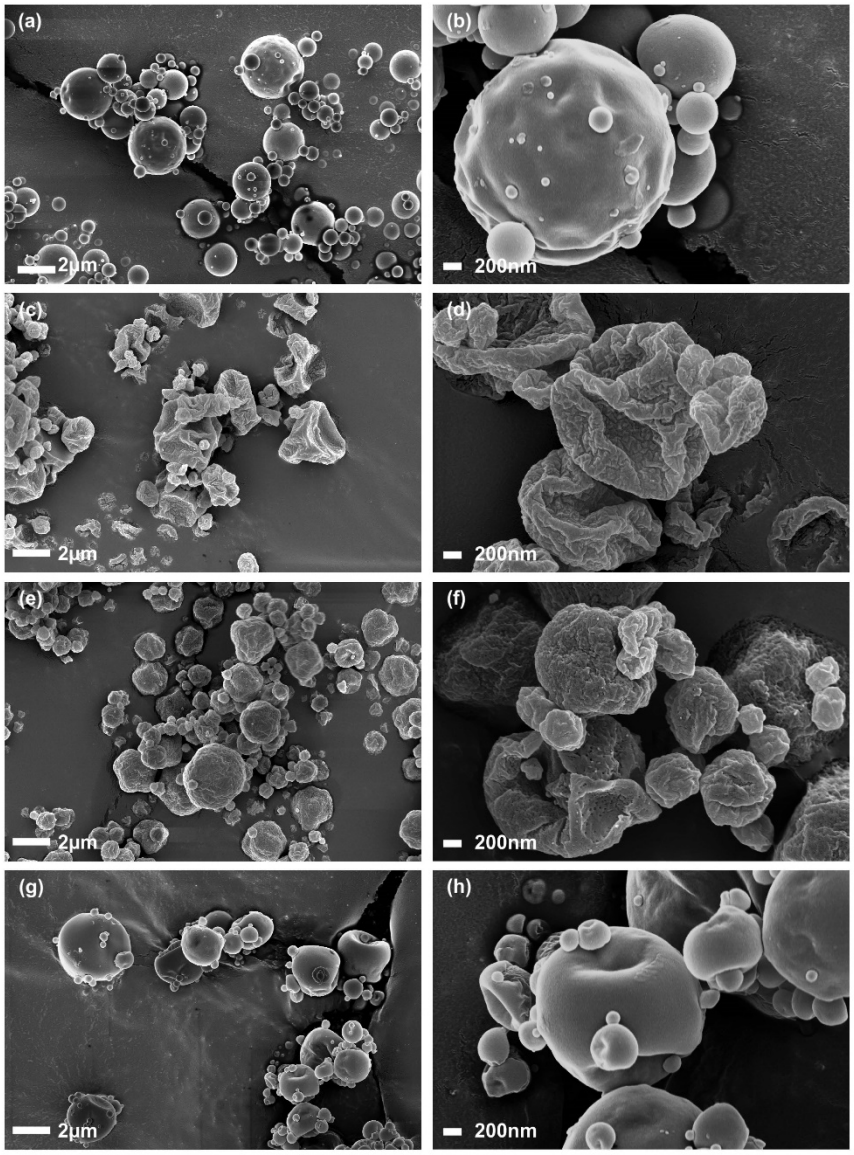

Fig. 2 Morphology of pure trehalose particles (a-b) and trehalose particles modified by different mass fractions of shell formers: (c-d) 5% trileucine, (e-f) 20% leucine, and (g-h) 40% pullulan.

Surface modification by phospholipid shell former

The phospholipid in combination with calcium chloride at a molar ratio of 2:1 was investigated as a new shell-forming excipient, and its effectiveness was compared with the well-established ones. Different mass fractions of the lipid excipient from 5% to 50% were first formulated to evaluate their effectiveness in surface modification. From the particle morphologies presented in **Fig. 3**, a clear trend of increasing rugosity and surface folding can be observed with an increase in the mass fraction of lipid. Overall, all batches showed polydisperse particles in the respirable range, a result that is in good agreement with the primary particle size distributions listed in **Table 1**.

Notable morphological differences can already be observed between the pure trehalose particles shown in **Fig. 2**(a-b) and the particles modified by 5% lipid shown in **Fig. 3**(a-b). That is, some of the particles still

exhibit smooth surfaces inherited from trehalose, but many particles have started to display surface folding and indentations, especially for the larger particles. However, with higher mass fractions of lipid, the folding became more prominent, and more rugose particles were obtained. The corrugated surface feature became more consistent throughout the powder, including the smaller particles, when the lipid mass fractions were higher than 10%. The formulation with 50% lipid led to extremely rugose particles, shown in **Fig. 3** (g-h), which resemble the trehalose-free lipid particles presented in **Fig. 3**(i-j) and reported recently by Wang et at [50]. Increased rugosity and more surface folding are usually indicators of good aerosol performance. Compared to trileucine, more lipid is needed for a significant particle morphology modification, while the required amount is about the same for leucine (~20%) and compares favorably with pullulan, leaving plenty of payload in the formulation for actives and stabilizers.

A possible explanation for the particle formation process is as follows: during the high shear mixing process, most of the lipid was dispersed into lipid nanoparticles in the 60 - 100 nm range, as indicated by the DLS measurements shown in **Fig. 1**. During droplet drying, the lipid nanoparticles accumulated on the droplet surface due to their very high Péclet numbers and formed a shell before the trehalose solidified. The lipid shells likely rearranged and folded later in the particle formation process, leading to the highly rugose surface structure shown in **Fig. 3**. With increasing initial lipid concentration, the shell formation occurred earlier while the droplets were still larger, leaving more time and space for the lipid to rearrange and fold and thus causing the more rugose particle morphology.

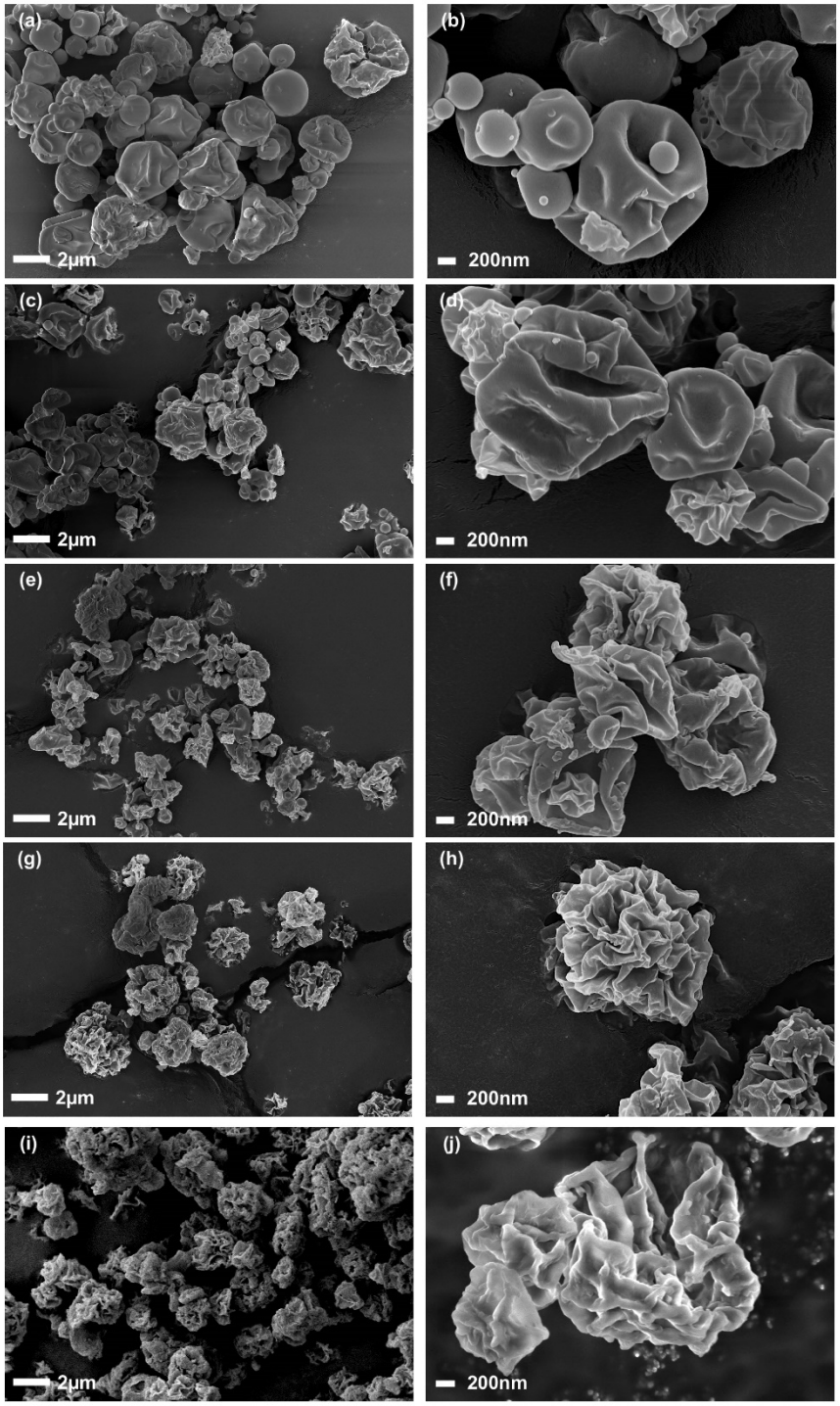

Fig. 3 Morphology of trehalose particles modified by different mass fractions of the DSPC lipid excipient: (a-b) 5%, (c-d) 10%, (e-f) 25%, (g-h) 50%, and (i-j) 100% lipid.

Solid phases of shell formers in the microparticles

To confirm the solid phases of the formulated components in the particles after spray drying, reference spectra for amorphous trehalose (a-Tre), crystalline trehalose (c-Tre), amorphous trileucine (a-Tri),

amorphous leucine (a-Leu), crystalline leucine (c-Leu), and pullulan (a-P) were measured and are shown in **Fig. 4**. In general, different crystalline spectra showed distinct differences in the fingerprint regions (> 200 cm^{-1}). In comparison, peaks also showed significant line-broadening in the spectra of the amorphous samples relative to the sharp peaks in the spectra of corresponding crystalline materials, a result due mainly to the greater disorder of molecules in the amorphous state [56].

Trehalose was found to be amorphous in all the spray-dried formulations as expected [64]. In agreement with the solid phases previously reported in the literature [19, 24, 26] the residual spectra shown in **Fig. 4** after the subtraction of amorphous trehalose from the raw spectra of 90T5Tri, 80T20L, and 60T40P also demonstrate the presence of amorphous trileucine, crystalline leucine, and amorphous pullulan in the respective formulations. Phospholipids are known to exist in numerous solid phases [61]. A clear identification of different lipid phases by Raman spectroscopy is challenging as the spectral differences between different phases can be minor [68]. In general, the C-C stretching region (1000 – 1150 cm^{-1}) and CH_2 bending region (1450 cm^{-1}) are believed to be more sensitive to different solid phases, and the change in the ratios of peaks in these regions have been used for qualitative solid state analysis [69, 70]. As shown in **Fig. 4**, no marked spectral differences can be observed between the spectra of raw DSPC material, 100% spray dried DSPC, and the residual spectrum after subtracting amorphous trehalose from the spectrum of 50T50LC. However, the intensity ratio of the peaks marked '1' and '2' at the wavenumbers of 1128 cm^{-1} and 1062 cm^{-1} increased from 0.79 for raw DSPC material to 1.00 for 100LC and 0.96 for 50T50LC, respectively, indicating changes in the intrachain disorder after feedstock preparation and spray drying [69].

Results of the solid-state analysis by Raman spectroscopy were further verified by powder X-ray diffraction. As expected, no crystalline content was detected in the formulations with 5% trileucine, 40% pullulan, or 50% myoglobin. For powder matrices containing 80% trehalose and 20% leucine, the appearance of both crystalline peaks and a broad halo pattern indicated the presence of crystalline and amorphous materials. Positions of the peaks aligned well with those of raw crystalline leucine, suggesting that the crystallinity of the powder matrices was due to the leucine component, while trehalose stayed fully amorphous. X-ray diffraction patterns of lipid-containing samples all featured a single peak at about 21°, which is typical for phospholipids and indicates the regular packing of the DSPC hydrocarbon chains [71]. Similarly, no crystalline content was detected in any of the lipid-containing formulations, even for the protein-containing formulation (#11, 35T35M30LC) after storage at 40°C for 1 month, demonstrating the successful formation of a thermally stable glass matrix as designed.

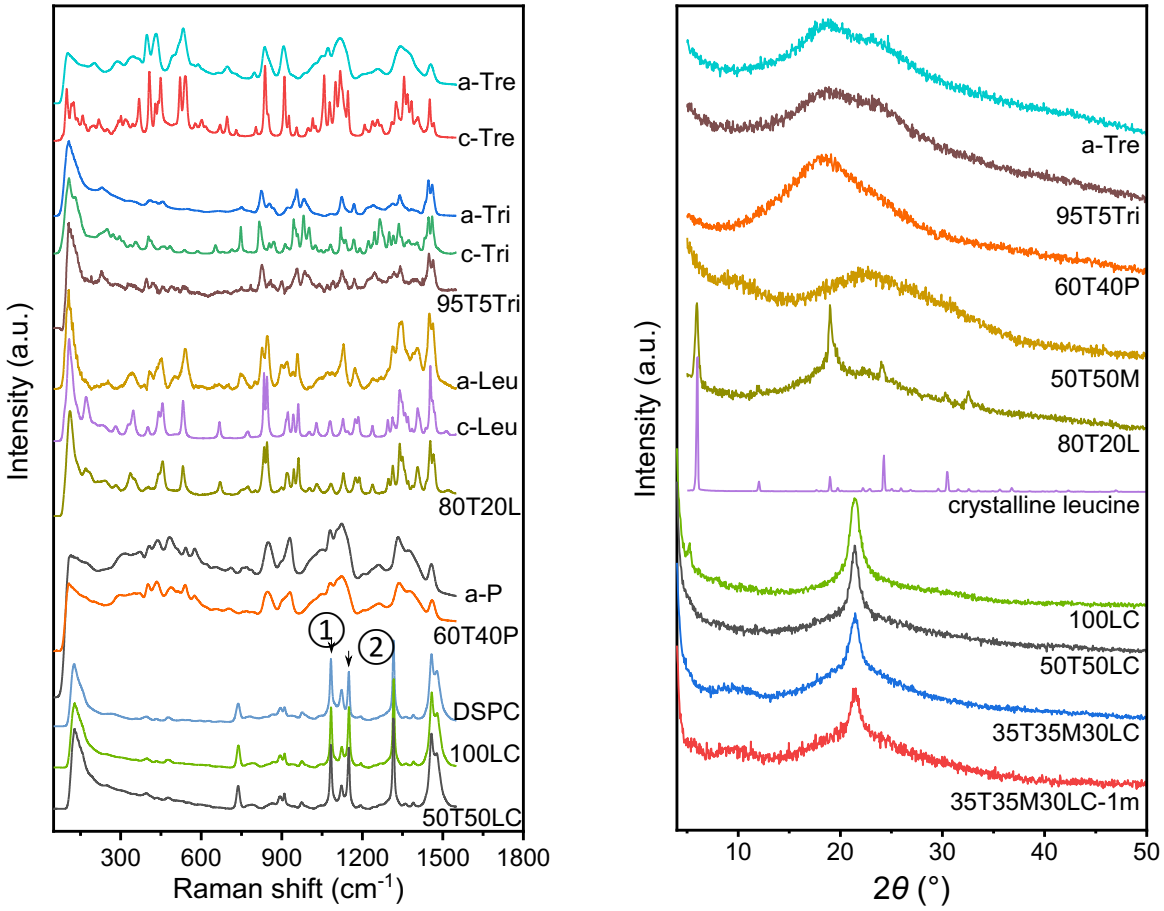

Fig. 4 Reference Raman spectra (left) for amorphous trehalose (a-Tre), crystalline trehalose (c-Tre), amorphous trileucine (a-Tri), amorphous leucine (a-Leu), crystalline leucine (c-Leu), and pullulan (a-P). Residual spectra after subtracting amorphous trehalose from the raw spectra of 90T5Tri, 80T20L, and 60T40P show the presence of amorphous trileucine, crystalline leucine, and amorphous pullulan in the respective formulations. Differences between the spectra of raw DSPC, 100% spray-dried DSPC (100LC), and the residual spectrum after subtracting amorphous trehalose from that of 50T50LC indicate changes in the lipid intrachain disorder. X-ray diffraction patterns (right) indicated that no crystalline content was detected in any of the formulations except for the case with 20% leucine.

Interaction of surface-modified particles with moisture

An advantage of using trehalose as a glass stabilizer in pharmaceutical formulations is its relatively high dry glass transition temperature of >110°C. This, however, decreases quickly with increasing powder moisture content due to the plasticizing effect of water [64]. It is known that amorphous trehalose can crystallize into its dihydrate form when stored at relative humidities higher than 44% at 25°C [72]. The black trace shown in **Fig. 5** indicates that the amorphous trehalose powder sample pre-conditioned at 0% for more than 2 hours started to adsorb water immediately when exposed to 60% relative humidity at 25°C. Up to 11.3% water was adsorbed after about 20 min, followed by a gradual decrease of moisture content to 9.2%, with the latter due mainly to the crystallization of amorphous trehalose into trehalose dihydrate and concomitant release of excess water. Raman spectroscopy measurement of the powder

sample after the DVS measurement (data not shown) revealed fully crystalline trehalose with no remaining amorphous trehalose. This water expulsion phenomenon observed during conditioning at 60% relative humidity for the other powder samples including 95T5Tri, 80T20L, 95T5LC, 75T25LC, and 50T50LC indicated the crystallization of trehalose in those formulations, while no crystallization was observed for formulations with pullulan (60T40P) or myoglobin (50T50M, 35T35M30LC). Quantitatively speaking, for a completely dried amorphous trehalose powder to fully crystallize into the dihydrate form and then be dried to remove any excess water, a 10.5% mass change is expected stoichiometrically. However, at the end of the final drying step, only a 9.2% change in mass was observed. This was likely due to incomplete drying of the powder sample during pre-conditioning. Assuming an initial 1.37% water content measured by Karl Fischer titration as listed in **Table 2**, full crystallization of this amorphous trehalose powder sample would lead to a 9.0% net change in mass, which agrees well with the actual measurement.

As listed in **Table 3**, the predicted net water adsorbed stands for the expected amount of mass change assuming the following conditions were all met: the powders had initial water contents as listed in **Table 2**, trehalose was fully crystallized, and the non-trehalose contents in the powders were dried to their respective initial moisture contents at the end. The measured net adsorbed water at the end of the sorption analysis all agreed with the predictions except for the pullulan and myoglobin-containing formulations. This means that, despite the addition of different shell formers, amorphous trehalose still almost fully crystallized in all cases except that of the formulations with pullulan or myoglobin. Pullulan has a very high glass transition temperature (~260°C) and is commonly used as a film-forming agent [65]. For the pullulan-containing microparticles, a pullulan-enriched shell and a higher glass transition temperature of the trehalose-pullulan mixture were achieved, which prevented the amorphous trehalose from crystallizing at room temperature despite the exposure to high humidity [26, 73]. Similarly, myoglobin also prevented amorphous trehalose from crystallizing in the two formulations that contained 50% (#10, 50T50M) and 35% (#11, 35T35M30LC) myoglobin, respectively.

With respect to the other shell formers, leucine is known to form crystalline patches on the particle surface. These appear not to be effective in slowing down moisture ingress or trehalose crystallization, since the trehalose in the leucine-containing formulation crystallized at the same time as the pure trehalose powder, as shown in **Fig. 5**. However, high concentrations of leucine have been reported to maintain powder dispersibility for some time even when exposed to high humidities [25, 74]. The thin amorphous trileucine shell did not prevent trehalose crystallization either; however, for pure trehalose, the crystallization time was slowed to about 40 min from the original 20 min. No crystallization of amorphous trileucine was detected by Raman spectroscopy. Low mass fractions of trileucine have also been demonstrated to be able to maintain the dispersibility of spray-dried powders for a brief period upon exposure to high humidities [25]. Trehalose in the lipid-modified particles also crystallized after about 20 min exposure. These results indicate that the lipid nanoparticles comprising the shells for these microparticles did not prevent moisture from diffusing into the amorphous core, a situation similar to that for leucine.

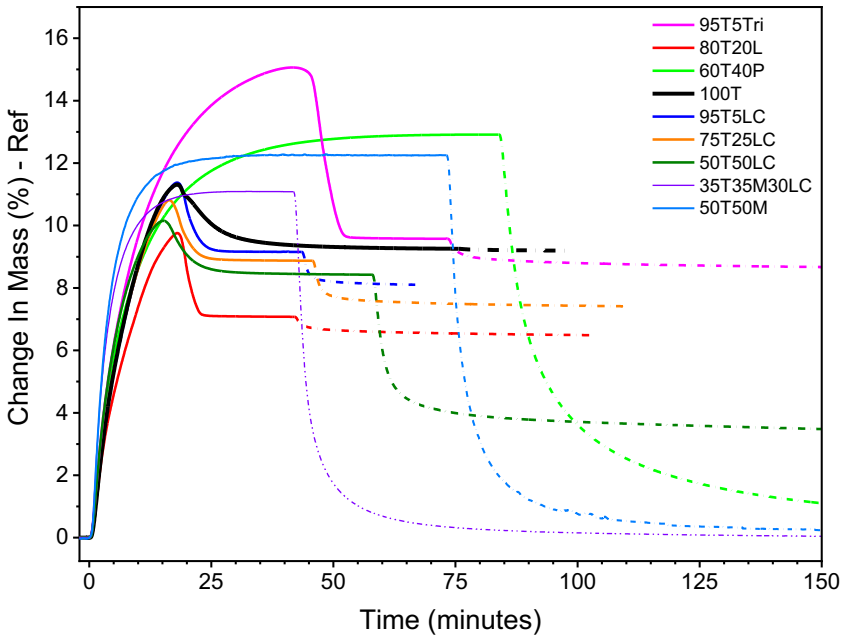

Fig. 5 Water sorption behavior of the spray-dried powders with different shell formers when exposed to 60% relative humidity. The vertical axis stands for the relative change in mass compared to the initial sample mass (Ref) after pre-conditioning at 0% RH. Amorphous trehalose crystallized in all formulations except the cases with pullulan or myoglobin. Dashed lines stand for the drying process at 0% added to the end of each measurement. Data for 95T5Tri, 60T40P, 50T50LC, 50T50M, and 35T35M30LC were truncated for clarity and the respective final mass changes recorded in **Table 3**.

Table 3 Mass change of powders with different shell formers during the gravimetric water vapor sorption analysis. Maximum water adsorbed represents the greatest mass change during the water sorption analysis. Predicted retained moisture content stands for the expected amount of mass change after completion of the cycle, assuming the powders had initial water contents as listed in **Table 2**, trehalose was fully crystallized, and the non-trehalose contents in the powders were dried to their initial moisture contents at the end. Actual retained moisture content stands for the mass change detected at the end of the water sorption analysis.

Formulation	Maximum water adsorbed (%)	Predicted moisture content retained (%)	Actual moisture content retained (%)
95T5Tri	15.1	8.9	8.7
80T20L	9.8	7.1	7.1
60T40P	12.9	5.2	0.1
100T	11.3	9.0	9.2
95T5LC	11.4	8.3	8.1
75T25LC	10.8	6.6	7.4

50T50LC	10.2	4.5	3.1
50T50M	12.3	4.0	0.2
35T35M30LC	11.1	2.5	<0.1

Dispersibility enhancement

To better demonstrate the effectiveness of different excipients in enhancing powder dispersibility, a low flow rate of 60 L/min was intentionally used for the testing of all the powders, with the results shown in **Fig. 6**. Of all the powders tested, pure trehalose had the worst dispersibility with a 30.6% emitted dose and only 19.9% total lung dose despite its small primary particle size (< 2 μm); this relatively poor performance was due to the cohesive nature of the particles. By comparison, all shell formers improved the powder dispersibility to some extent. Formulations with trileucine or leucine showed highly improved emitted doses (~ 90%) and total lung doses (> 60%). The dispersibility enhancement provided by pullulan was less effective, with the emitted dose and total lung dose moderately improved to 43.6% and 23.9%, respectively.

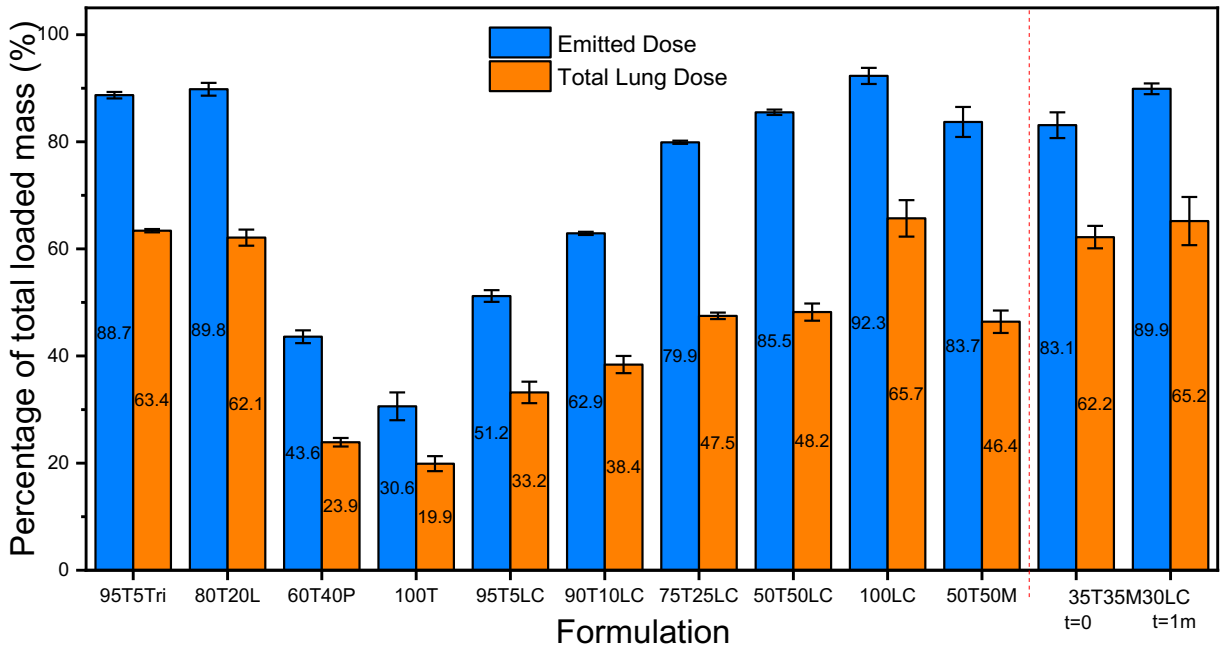

Fig. 6 *In vitro* aerosol performance of powders with different shell formers tested with the low-resistance dry powder inhaler device Seebri Breezhaler® at an intentionally reduced flow rate of 60 L/min.

Particles modified by the lipid showed a clear dependence of aerosol performance on the mass fraction of lipid in the formulations, corresponding to the increasing rugosity, as shown in **Fig. 3**. Using lipid as a shell former at a low mass fraction of 5% in itself demonstrated a better dispersibility enhancement than the 40% pullulan, bringing the emitted dose and total lung dose to 51.2% and 33.2%, respectively. Despite the low testing flow rate of 60 L/min, formulations with lipid mass fractions higher than 25%

demonstrated excellent aerosol performance with ≥ 80% emitted dose and > 45% total lung dose. Not surprisingly, the best-performing formulation was the 100LC case without any trehalose, in which only extremely rugose lipid particles were detected, as shown in **Fig. 3**(i-j).

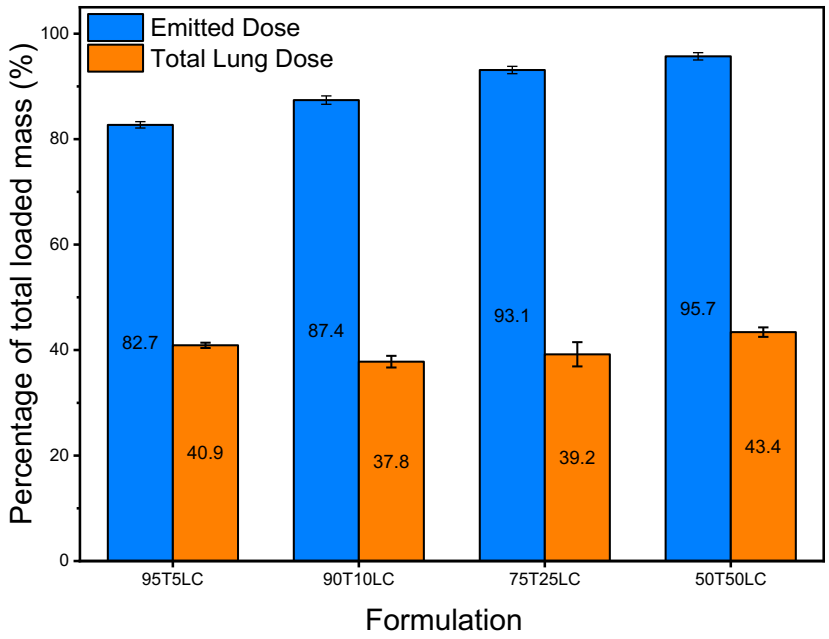

Fig. 7 *In vitro* aerosol performance of lipid-modified particles measured at a standard flow rate of 100 L/min.

The lipid-modified trehalose particles were also tested at the standard flow rate of 100 L/min to evaluate their aerosol performance under more realistic testing conditions. A significant improvement of the emitted dose for all the tested formulations was observed at this higher testing flow rate. Total lung doses increased at low lipid mass fractions but decreased slightly for the formulations with 25% and 50% lipid when compared to the results of testing at 60 L/min. This is likely because the extent of particle deposition in the upper respiratory tract depends on the trade-off between the increased degree of powder deagglomeration and increased inertial impaction with increasing flow rates [75]. Therefore, different drug-device combinations may show different degrees of dependence on the flow rate for the total lung dose. It has been reported that the Breezhaler® device used in this study, in combination with different powder formulations, can exhibit an inverse dependence of the total lung dose on the flow rate [76]. Overall, all formulations tested showed > 80% emitted doses and ≥ 40% total lung doses, results that already outperform most commercially available DPI products [77, 78]. Treating the trehalose component as a surrogate for small-molecule APIs, the new lipid excipient successfully improved the powder dispersibility by covering the final particles with highly rugose lipid structures via a shell-forming mechanism. Some biologics require a high dose to be administered. The excellent dispersibility enhancement achieved even by low mass fractions of the lipid shows great potential for its use in formulation platforms designed to deliver biologics with a high payload.

Formulation with large-molecule biologics

Relatively low drying temperatures are required for the production of rugose lipid particles. However, biologics are also typically spray dried at low temperatures [79], so this formulation platform may be a good match for actives that are sensitive to thermal stress. When stabilizing proteins in spray-dried particle formulations, the weight ratio of stabilizer to protein has been reported to be important in affecting the formulation stability, with a weight ratio of at least 1:1 being recommended [48]. A 1:1 stabilizer-to-protein weight ratio was therefore used for batch #10, which consisted of 50% trehalose and 50% myoglobin. To demonstrate the effect of additional lipid excipient in formulations loaded with large-molecule biologics, 30% lipid excipient was added to formulation #11 while maintaining the 1:1 weight ratio of trehalose and myoglobin. Both spray drying runs produced particles in the respirable range, as shown by the in-line measured particle size distributions in **Table 2**. Both batches also had a good yield, especially batch #10 (86.4%). Batch #11 had a relatively lower yield (83.3%) because mild foaming occurred when annex myoglobin solution was added to the feedstock during mixing. The foaming was not unexpected since myoglobin is a surface-active protein [80]. Some loss of the feedstock occurred because the foamed liquid could not be entirely pumped to the atomizer.

As the micrographs in **Fig. 8**(a-b) show, the formulation with 50% trehalose and 50% myoglobin produced particles with dimpled surfaces; these particle morphologies are similar to those reported in the literature [81]. The wrinkled particle surfaces were directly related to the surface activity and high molar mass of myoglobin, which led to a high Péclet number, high surface enrichment, early shell formation, and subsequent surface deformation. With the addition of 30% lipid excipient, a clear morphological transition to highly rugose microparticles was observed. In comparison to the lipid-free particles, the morphology of these lipid-containing particles was dominated by the added lipid excipient, since the surface structures resembled the lipid-modified trehalose particles at similar mass fractions shown in **Fig. 3**. This morphological transition after the addition of lipid indicated that the lipid excipient likely replaced much of the myoglobin on the surface during particle formation despite the surface activity and large molar mass of the myoglobin. The highly rugose surface structure of the particles was retained after 1 month of storage at 40 °C as shown in **Fig. 8**(e-f), demonstrating good thermal stability of the trehalose-lipid stabilizer platform. This unchanged particle morphology is in good agreement with the amorphous solid phase confirmed by XRD and shown in **Fig. 4**.

Trehalose-myoglobin particles in themselves already demonstrated good dispersibility, as shown in **Fig. 6**, despite being tested at 60 L/min with the low-resistance Breezhaler®. A high emitted dose (83.7%) and total lung dose (46.4%) were achieved. By comparison, the formulation with an additional 30% lipid excipient maintained a high emitted dose at 83.1%. Furthermore, the total lung dose increased to 62.2%, demonstrating much-improved powder dispersibility. The improvement was likely caused by the much higher rugosity of the lipid-modified particles. After 1 month of storage at 40 °C, the powder still presented a very good dispersibility with a high emitted dose (89.9%) and total lung dose (65.2%). The aerosol performance even improved slightly after the stability study, perhaps because of the decreased powder moisture content to 2.36% after dry condition storage at 40 °C for 1 month. The retained amorphous solid state, rugose particle structure, and good aerosol performance of protein-containing microparticles after the short-term stability study show that trehalose in combination with the novel lipid excipient can provide a promising formulation approach for the high-efficiency delivery of biologics through the respiratory route.

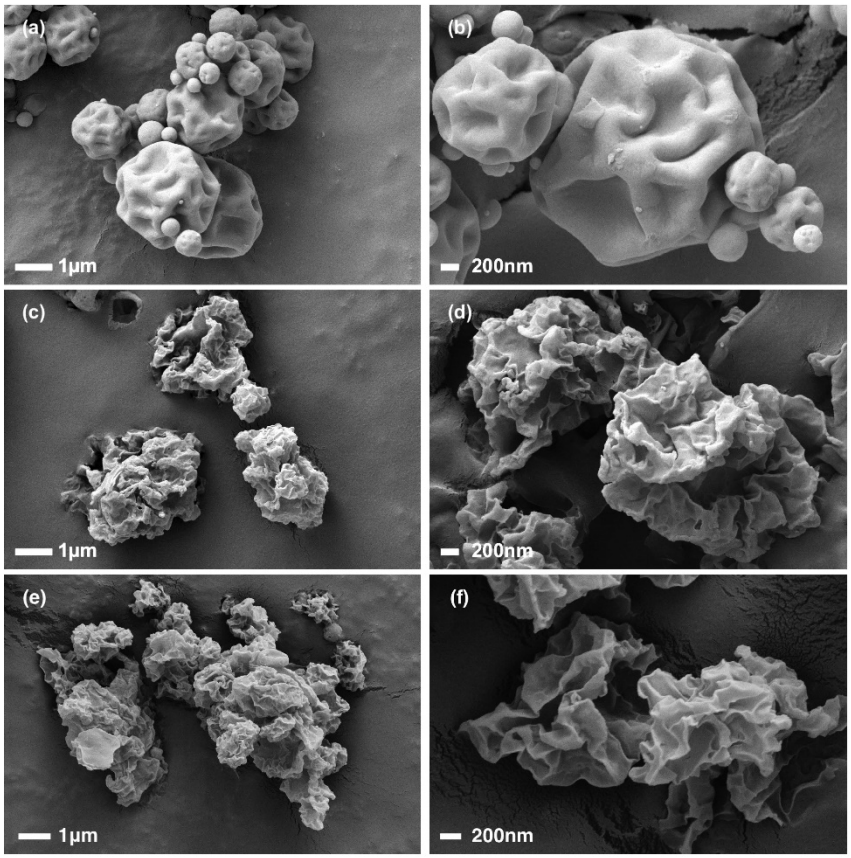

Fig. 8 Morphology of myoglobin protein particles formulated with 50% trehalose as glass stabilizer (a-b) and formulated with 35% trehalose stabilizer and 30% phospholipid excipient before (c-d) and after (e-f) stability study.

CONCLUSIONS

Combining the advantages offered by dry powder formulations and lipid-based excipients, a highly rugose lipid-based microparticle platform manufactured with a simplified spray drying method was presented. Compared to traditional methods of manufacturing solid lipid microparticles, this new method requires no organic solvent or complicated feedstock preparation processes. The phospholipid DSPC was demonstrated to be an effective shell former that can significantly modify particle morphology while maintaining compatibility with an amorphous trehalose core that can provide protection to biologics during manufacturing, storage, and application. The benefits of adding lipid excipient include improving powder dispersibility, forming a lipid shell to improve the bioavailability of embedded active ingredients, and excluding large-molecule biologics from particle surfaces, which may be helpful in reducing surface-mediated denaturation. The proposed lipid microparticle platform is promising for carrying biologics that are cohesive in nature and biologics like bacteriophages, vaccines, and monoclonal antibodies that are sensitive to various thermal, mechanical, or chemical stresses. Demonstrated thermal stability of the proposed trehalose-lipid stabilizer system offers a promising formulation approach for the respiratory delivery of the above-mentioned large-molecule biologics.

The effectiveness of surface modification and dispersibility enhancement was demonstrated to be dependent on the formulated lipid mass fractions, and substantial improvement of aerosol performance was achieved even at low lipid mass fractions, indicating a potentially high payload for this formulation platform. A high-payload formulation can significantly reduce the total mass of powder that needs to be inhaled, improving patient acceptance and compliance. This new lipid microparticle platform shows good potential for delivering both small-molecule APIs and large-molecule biologics to the lung. However, it seems to be most suited for situations where high processing temperatures have already been ruled out due to the thermo-sensitivity of the actives.

DECLARATION

Funding Statement

This work was supported by the Natural Sciences and Engineering Research Council of Canada through its Collaborative Research & Development program (Grant CRDPJ 543336-19).

Conflicts of Interest Statement

The authors declare no conflict of interest.

Acknowledgments and Disclosures

The authors acknowledge language editing provided by Luba Slabyj. PC, KL, NC, DLB are employees of AstraZeneca and may own stock or stock options.

REFERENCES

[1] R. Murugesan, V. Orsat, Spray drying for the production of nutraceutical ingredients—a review, Food Bioproc. Tech., 5 (2012) 3-14. https://doi.org/10.1007/s11947-011-0638-z

[2] K. Sollohub, K. Cal, Spray drying technique: II. Current applications in pharmaceutical technology, J. Pharm. Sci., 99 (2010) 587-597. https://doi.org/10.1002/jps.21963

[3] K. Cal, K. Sollohub, Spray drying technique. I: Hardware and process parameters, J. Pharm. Sci., 99 (2010) 575-586. https://doi.org/10.1002/jps.21886

[4] T. Kudra, A.S. Mujumdar, Advanced drying technologies, CRC press, 2009. https://doi.org/10.1201/9781420073898

[5] R. Vehring, Pharmaceutical particle engineering via spray drying, Pharm. Res., 25 (2008) 999-1022. https://dx.doi.org/10.1007/s11095-007-9475-1

[6] G. Tiwari, R. Tiwari, B. Sriwastawa, L. Bhati, S. Pandey, P. Pandey, S.K. Bannerjee, Drug delivery systems: An updated review, Int. J. Pharm. Investig., 2 (2012) 2. https://doi.org/10.4103/2230-973X.96920

[7] W.H. Finlay, The mechanics of inhaled pharmaceutical aerosols: an introduction, Academic Press, 2001. https://dx.doi.org/10.1016/B978-012256971-5/50008-0

[8] E. Fröhlich, S. Salar-Behzadi, Oral inhalation for delivery of proteins and peptides to the lungs, Eur. J. Pharm. Biopharm., 163 (2021) 198-211. https://doi.org/10.1016/j.ejpb.2021.04.003

[9] R.Y.K. Chang, M. Wallin, Y. Lin, S.S.Y. Leung, H. Wang, S. Morales, H.-K. Chan, Phage therapy for respiratory infections, Adv. Drug Del. Rev., 133 (2018) 76-86. https://doi.org/10.1016/j.addr.2018.08.001

[10] E. Bodier-Montagutelli, A. Mayor, L. Vecellio, R. Respaud, N. Heuzé-Vourc'h, Designing inhaled protein therapeutics for topical lung delivery: what are the next steps?, Expert Opin. Drug Deliv., 15 (2018) 729-736. https://doi.org/10.1080/17425247.2018.1503251

[11] R.Y.K. Chang, M.Y. Chow, D. Khanal, D. Chen, H.-K. Chan, Dry powder pharmaceutical biologics for inhalation therapy, Adv. Drug Del. Rev., (2021). https://doi.org/10.1016/j.addr.2021.02.017

[12] G. Pilcer, K. Amighi, Formulation strategy and use of excipients in pulmonary drug delivery, Int. J. Pharm., 392 (2010) 1-19. https://doi.org/10.1016/j.ijpharm.2010.03.017

[13] M.J. Telko, A.J. Hickey, Dry powder inhaler formulation, Respir. Care, 50 (2005) 1209-1227.

[14] S. Ohtake, K.-i. Izutsu, D. Lechuga-Ballesteros, Drying Technologies for Biotechnology and Pharmaceutical Applications, John Wiley & Sons, 2020. https://doi.org/10.1002/9783527802104

[15] J.G. Weers, D.P. Miller, Formulation design of dry powders for inhalation, J. Pharm. Sci., 104 (2015) 3259-3288. https://doi.org/10.1002/jps.24574

[16] P.C. Seville, T.P. Learoyd, H.-Y. Li, I. Williamson, J.C. Birchall, Amino acid-modified spray-dried powders with enhanced aerosolisation properties for pulmonary drug delivery, Powder Technol., 178 (2007) 40-50. https://doi.org/10.1016/j.powtec.2007.03.046

[17] A. Minne, H. Boireau, M.J. Horta, R. Vanbever, Optimization of the aerosolization properties of an inhalation dry powder based on selection of excipients, Eur. J. Pharm. Biopharm., 70 (2008) 839-844. https://doi.org/10.1016/j.ejpb.2008.06.013

[18] T. Sou, L.M. Kaminskas, T.-H. Nguyen, R. Carlberg, M.P. McIntosh, D.A. Morton, The effect of amino acid excipients on morphology and solid-state properties of multi-component spray-dried formulations for pulmonary delivery of biomacromolecules, Eur. J. Pharm. Biopharm., 83 (2013) 234-243. https://dx.doi.org/10.1016/j.ejpb.2012.10.015

[19] A.L. Feng, M.A. Boraey, M.A. Gwin, P.R. Finlay, P.J. Kuehl, R. Vehring, Mechanistic models facilitate efficient development of leucine containing microparticles for pulmonary drug delivery, Int. J. Pharm., 409 (2011) 156-163. https://dx.doi.org/10.1016/j.ijpharm.2011.02.049

[20] N. Alhajj, N.J. O'Reilly, H. Cathcart, Leucine as an excipient in spray dried powder for inhalation, Drug Discov. Today, (2021). https://doi.org/10.1016/j.drudis.2021.04.009

[21] M. Ordoubadi, F.K.A. Gregson, H. Wang, M. Nicholas, S. Gracin, D. Lechuga-Ballesteros, J.P. Reid, W.H. Finlay, R. Vehring, On the particle formation of leucine in spray drying of inhalable microparticles, Int. J. Pharm., 592 (2021) 120102. https://doi.org/10.1016/j.ijpharm.2020.120102

[22] D. Lechuga-Ballesteros, C. Charan, C.L.M. Stults, C.L. Stevenson, D.P. Miller, R. Vehring, V. Tep, M.C. Kuo, Trileucine improves aerosol performance and stability of spray-dried powders for inhalation, J. Pharm. Sci., 97 (2008) 287-302. https://dx.doi.org/10.1002/jps.21078

[23] M. Ordoubadi, F.K.A. Gregson, H. Wang, N.B. Carrigy, M. Nicholas, S. Gracin, D. Lechuga-Ballesteros, J.P. Reid, W.H. Finlay, R. Vehring, Trileucine as a dispersibility enhancer of spray-dried inhalable microparticles, J. Control. Release, 336 (2021) 522-536. https://doi.org/10.1016/j.jconrel.2021.06.045

[24] H. Wang, D.S. Nobes, R. Vehring, Particle surface roughness improves colloidal stability of pressurized pharmaceutical suspensions, Pharm. Res., 36 (2019) 43. https://dx.doi.org/10.1007/s11095-019-2572-0

[25] I. Sibum, P. Hagedoorn, M.P. Kluitman, M. Kloezen, H.W. Frijlink, F. Grasmeijer, Dispersibility and storage stability optimization of high dose isoniazid dry powder inhalation formulations with L-leucine or trileucine, Pharmaceutics, 12 (2020) 24. https://doi.org/10.3390/pharmaceutics12010024

[26] N.B. Carrigy, M. Ordoubadi, Y. Liu, O. Melhem, D. Barona, H. Wang, L. Milburn, C.A. Ruzycki, W.H. Finlay, R. Vehring, Amorphous pullulan trehalose microparticle platform for respiratory delivery, Int. J. Pharm., 563 (2019) 156-168. https://doi.org/10.1016/j.ijpharm.2019.04.004

[27] R.Y. Chang, J. Wong, A. Mathai, S. Morales, E. Kutter, W. Britton, J. Li, H.-K. Chan, Production of highly stable spray dried phage formulations for treatment of Pseudomonas aeruginosa lung infection, Eur. J. Pharm. Biopharm., 121 (2017) 1-13. https://dx.doi.org/10.1016/j.ejpb.2017.09.002

[28] H. Shrestha, R. Bala, S. Arora, Lipid-based drug delivery systems, J. Pharm., 2014 (2014). https://dx.doi.org/10.1155/2014/801820

[29] C.L. Ngan, A.A. Asmawi, Lipid-based pulmonary delivery system: a review and future considerations of formulation strategies and limitations, Drug Deliv. Transl. Res., 8 (2018) 1527-1544. https://doi.org/10.1007/s13346-018-0550-4

[30] J. Li, X. Wang, T. Zhang, C. Wang, Z. Huang, X. Luo, Y. Deng, A review on phospholipids and their main applications in drug delivery systems, Asian J Pharm. Sci., 10 (2015) 81-98. https://dx.doi.org/10.1016/j.ajps.2014.09.004

[31] A.B. Fisher, Lung lipid composition and surfactant biology, in: R.A. Parent (Ed.) Comparative biology of the normal lung, Academic Press, 2015, pp. 423-466. https://doi.org/10.1016/B978-0-12-404577-4.00022-9.

[32] T. Praphawatvet, J.I. Peters, R.O. Williams III, Inhaled nanoparticles - an updated review, Int. J. Pharm., (2020) 119671. https://doi.org/10.1016/j.ijpharm.2020.119671

[33] N. Wauthoz, K. Amighi, Phospholipids in pulmonary drug delivery, Eur. J. Lipid Sci. Technol., 116 (2014) 1114-1128. https://doi.org/10.1002/ejlt.201300368

[34] D. Cipolla, B. Shekunov, J. Blanchard, A. Hickey, Lipid-based carriers for pulmonary products: preclinical development and case studies in humans, Adv. Drug Del. Rev., 75 (2014) 53-80. https://dx.doi.org/10.1016/j.addr.2014.05.001

[35] S. Weber, A. Zimmer, J. Pardeike, Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) for pulmonary application: a review of the state of the art, Eur. J. Pharm. Biopharm., 86 (2014) 7-22. https://dx.doi.org/10.1016/j.ejpb.2013.08.013

[36] S. Scalia, P.M. Young, D. Traini, Solid lipid microparticles as an approach to drug delivery, Expert Opin. Drug Deliv., 12 (2015) 583-599. https://doi.org/10.1517/17425247.2015.980812

[37] R. Vanbever, J.D. Mintzes, J. Wang, J. Nice, D. Chen, R. Batycky, R. Langer, D.A. Edwards, Formulation and physical characterization of large porous particles for inhalation, Pharm. Res., 16 (1999) 1735-1742. https://doi.org/10.1023/A:1018910200420

[38] C. Corzo, A. Fuchsbichler, I. Savencu, J.A. Urich, A. Zimmer, D. Lochmann, S. Reyer, S. Salar-Behzadi, Lipid-microparticles for pulmonary delivery of active pharmaceutical ingredients: impact of lipid crystallization on spray-drying processability, Int. J. Pharm., (2021) 121259. https://doi.org/10.1016/j.ijpharm.2021.121259

[39] G. Pilcer, T. Sebti, K. Amighi, Formulation and characterization of lipid-coated tobramycin particles for dry powder inhalation, Pharm. Res., 23 (2006) 931-940. https://doi.org/10.1007/s11095-006-9789-4

[40] N. Shetty, J. Hou, E. Yanez, J. Shur, J. Cheng, C.C. Sun, K. Nagapudi, A.S. Narang, Effect of lipidic excipients on the particle properties and aerosol performance of high drug load spray dried particles for inhalation, J. Pharm. Sci., (2021). https://doi.org/10.1016/j.xphs.2021.09.004

[41] J. Weers, T. Tarara, The PulmoSphere™ platform for pulmonary drug delivery, Ther. Deliv., 5 (2014) 277-295. https://dx.doi.org/10.4155/tde.14.3

[42] D.E. Geller, J. Weers, S. Heuerding, Development of an inhaled dry-powder formulation of tobramycin using PulmoSphere™ technology, J. Aerosol Med. Pulm. Drug Deliv., 24 (2011) 175-182. https://doi.org/10.1089/jamp.2010.0855

[43] D.P. Miller, T. Tan, T.E. Tarara, J. Nakamura, R.J. Malcolmson, J.G. Weers, Physical characterization of tobramycin inhalation powder: I. rational design of a stable engineered-particle formulation for delivery to the lungs, Mol. Pharm., 12 (2015) 2582-2593. https://doi.org/10.1021/acs.molpharmaceut.5b00147

[44] T.E. Tarara, M.S. Hartman, H. Gill, A.A. Kennedy, J.G. Weers, Characterization of suspension-based metered dose inhaler formulations composed of spray-dried budesonide microcrystals dispersed in HFA-134a, Pharm. Res., 21 (2004) 1607-1614. https://doi.org/10.1023/B:PHAM.0000041455.13980.f1

[45] R. Vehring, D. Lechuga-Ballesteros, V. Joshi, B. Noga, S.K. Dwivedi, Cosuspensions of microcrystals and engineered microparticles for uniform and efficient delivery of respiratory therapeutics from pressurized metered dose inhalers, Langmuir, 28 (2012) 15015-15023. https://dx.doi.org/10.1021/la302281n

[46] M.A. Mensink, H.W. Frijlink, K. van der Voort Maarschalk, W.L. Hinrichs, How sugars protect proteins in the solid state and during drying (review): Mechanisms of stabilization in relation to stress conditions, Eur. J. Pharm. Biopharm., 114 (2017) 288-295. https://dx.doi.org/10.1016/j.ejpb.2017.01.024

[47] M. Gomez, M. Archer, D. Barona, H. Wang, M. Ordoubadi, S.B. Karim, N.B. Carrigy, Z. Wang, J. McCollum, C. Press, A. Gerhardt, C.B. Fox, R.M. Kramer, R. Vehring, Microparticle encapsulation of a tuberculosis subunit vaccine candidate containing a nanoemulsion adjuvant via spray drying, Eur. J. Pharm. Biopharm., (2021). https://doi.org/10.1016/j.ejpb.2021.03.007

[48] J. Massant, S. Fleurime, M. Batens, H. Vanhaerents, G. Van den Mooter, Formulating monoclonal antibodies as powders for reconstitution at high concentration using spray-drying: Trehalose/amino acid combinations as reconstitution time reducing and stability improving formulations, Eur. J. Pharm. Biopharm., 156 (2020) 131-142. https://doi.org/10.1016/j.ejpb.2020.08.019

[49] S.S. Leung, T. Parumasivam, A. Nguyen, T. Gengenbach, E.A. Carter, N.B. Carrigy, H. Wang, R. Vehring, W.H. Finlay, S. Morales, Effect of storage temperature on the stability of spray dried bacteriophage powders, Eur. J. Pharm. Biopharm., (2018) 213–222. https://dx.doi.org/10.1016/j.ejpb.2018.02.033

[50] H. Wang, M. Ordoubadi, P. Connaughton, K. Lachacz, N. Carrigy, S. Tavernini, A.R. Martin, W.H. Finlay, D. Lechuga-Ballesteros, R. Vehring, Spray Dried Rugose Lipid Particle Platform for Respiratory Drug Delivery, Pharm. Res., (2022). https://doi.org/10.1007/s11095-022-03242-w

[51] J. Ivey, Particle formation from evaporating microdroplets for inhaled drug delivery, in: Mech. Eng., University of Alberta, Edmonton, Alberta, 2018. https://doi.org/10.7939/R3RB6WJ3S

[52] H. Adi, D. Traini, H.-K. Chan, P.M. Young, The influence of drug morphology on aerosolisation efficiency of dry powder inhaler formulations, J. Pharm. Sci., 97 (2008) 2780-2788. https://doi.org/10.1002/jps.21195

[53] S. Hoe, J.W. Ivey, M.A. Boraey, A. Shamsaddini-Shahrbabak, E. Javaheri, S. Matinkhoo, W.H. Finlay, R. Vehring, Use of a fundamental approach to spray-drying formulation design to facilitate the development of multi-component dry powder aerosols for respiratory drug delivery, Pharm. Res., 31 (2014) 449-465. https://dx.doi.org/10.1007/s11095-013-1174-5

[54] P.M. Young, A. Sung, D. Traini, P. Kwok, H. Chiou, H.-K. Chan, Influence of humidity on the electrostatic charge and aerosol performance of dry powder inhaler carrier based systems, Pharm. Res., 24 (2007) 963-970. https://doi.org/10.1007/s11095-006-9218-8

[55] H. Wang, D. Barona, S. Oladepo, L. Williams, S. Hoe, D. Lechuga-Ballesteros, R. Vehring, Macro-Raman spectroscopy for bulk composition and homogeneity analysis of multi-component pharmaceutical powders, J. Pharm. Biomed. Anal., 141 (2017) 180-191. http://dx.doi.org/10.1016/j.jpba.2017.04.003

[56] H. Wang, M.A. Boraey, L. Williams, D. Lechuga-Ballesteros, R. Vehring, Low-frequency shift dispersive Raman spectroscopy for the analysis of respirable dosage forms, Int. J. Pharm., 469 (2014) 197-205. https://dx.doi.org/10.1016/j.ijpharm.2014.04.058

[57] H.R. Costantino, J.G. Curley, S. Wu, C.C. Hsu, Water sorption behavior of lyophilized protein–sugar systems and implications for solid-state interactions, Int. J. Pharm., 166 (1998) 211-221. https://doi.org/10.1016/S0378-5173(98)00050-7

[58] K.T. Ung, N. Rao, J.G. Weers, A.R. Clark, H.-K. Chan, In vitro assessment of dose delivery performance of dry powders for inhalation, Aerosol Sci. Technol., 48 (2014) 1099-1110. https://doi.org/10.1080/02786826.2014.962685

[59] M. Molimard, I. Kottakis, J. Jauernig, S. Lederhilger, I. Nikolaev, Performance characteristics of Breezhaler® and Aerolizer® in the real-world setting, Clin. Drug Investig., (2021) 1-10. https://doi.org/10.1007/s40261-021-01021-w

[60] U.S. Pharmacopeia, <601> Aerosols, nasal sprays, metered dose inhalers, and dry powder inhalers, in: US Pharmacopeial Convention, Rockville, MD, USA, 2012.

[61] D. Marsh, Handbook of lipid bilayers, 2nd Edition, CRC press, 2013. https://dx.doi.org/10.1201/b11712

[62] R. Vehring, W.R. Foss, D. Lechuga-Ballesteros, Particle formation in spray drying, J. Aerosol Sci, 38 (2007) 728-746. https://dx.doi.org/10.1016/j.jaerosci.2007.04.005

[63] M.A. Boraey, R. Vehring, Diffusion controlled formation of microparticles, J. Aerosol Sci, 67 (2014) 131-143. https://dx.doi.org/10.1016/j.jaerosci.2013.10.002

[64] S. Ohtake, Y.J. Wang, Trehalose: current use and future applications, J. Pharm. Sci., 100 (2011) 2020-2053. https://doi.org/10.1002/jps.22458

[65] T.D. Leathers, Biotechnological production and applications of pullulan, Appl. Microbiol. Biotechnol., 62 (2003) 468-473. https://doi.org/10.1007/s00253-003-1386-4

[66] N.B. Carrigy, L. Liang, H. Wang, S. Kariuki, T.E. Nagel, I.F. Connerton, R. Vehring, Trileucine and pullulan improve anti-Campylobacter bacteriophage stability in engineered spray-dried microparticles, Ann. Biomed. Eng., (2019). https://doi.org/10.1007/s10439-019-02435-6

[67] E.-Y. Xu, Y. Xu, X.-F. Yang, X.-N. Li, Y. Cai, H.-Y. Li, A novel pullulan excipient for inhalable spray-dried powders, in: Respiratory Drug Delivery Asia, 2014.

[68] C.B. Fox, R.H. Uibel, J.M. Harris, Detecting phase transitions in phosphatidylcholine vesicles by Raman microscopy and self-modeling curve resolution, J. Phys. Chem. B, 111 (2007) 11428-11436. https://doi.org/10.1021/jp0735886

[69] R.P. Goodrich, J.H. Crowe, L.M. Crowe, J.D. Baldeschwieler, Alterations in membrane surfaces induced by attachment of carbohydrates, Biochemistry, 30 (1991) 5313-5318. https://dx.doi.org/doi.org/10.1021/bi00235a026

[70] H. Wang, P. Tan, D. Barona, G. Li, S. Hoe, D. Lechuga-Ballesteros, D.S. Nobes, R. Vehring, Characterization of the suspension stability of pharmaceuticals using a shadowgraphic imaging method, Int. J. Pharm., 548 (2018) 128-138. http://dx.doi.org/10.1016/j.ijpharm.2018.06.053

[71] H. Nagase, H. Ueda, M. Nakagaki, Effect of water on lamellar structure of DPPC/sugar systems, Biochim. Biophys. Acta, 1328 (1997) 197-206. https://doi.org/10.1016/S0005-2736(97)00088-6

[72] H.A. Iglesias, J. Chirife, M.P. Buera, Adsorption isotherm of amorphous trehalose, J. Sci. Food Agric., 75 (1997) 183-186. https://doi.org/10.1002/(SICI)1097-0010(199710)75:2<183::AID-JSFA860>3.0.CO;2-T

[73] N. Teekamp, Y. Tian, J.C. Visser, P. Olinga, H.W. Frijlink, H.J. Woerdenbag, W.L. Hinrichs, Addition of pullulan to trehalose glasses improves the stability of β-galactosidase at high moisture conditions, Carbohydr. Polym., 176 (2017) 374-380. https://dx.doi.org/10.1016/j.carbpol.2017.08.084

[74] Z. Wang, H. Wang, R. Vehring, Leucine enhances the dispersibility of trehalose-containing spray-dried powders on exposure to a high-humidity environment, Int. J. Pharm., (2021) 120561. https://doi.org/10.1016/j.ijpharm.2021.120561

[75] J. Weers, A. Clark, The impact of inspiratory flow rate on drug delivery to the lungs with dry powder inhalers, Pharm. Res., 34 (2017) 507-528. https://doi.org/10.1007/s11095-016-2050-x

[76] J.G. Weers, A.R. Clark, N. Rao, K. Ung, A. Haynes, S.K. Khindri, S.A. Perry, S. Machineni, P. Colthorpe, In vitro–in vivo correlations observed with Indacaterol-based formulations delivered with the Breezhaler®, J. Aerosol Med. Pulm. Drug Deliv., 28 (2015) 268-280. https://doi.org/10.1089/jamp.2014.1178

[77] L. Borgström, B. Olsson, L. Thorsson, Degree of throat deposition can explain the variability in lung deposition of inhaled drugs, J. Aerosol Med., 19 (2006) 473-483. https://doi.org/10.1089/jam.2006.19.473

[78] S. Newman, W. Busse, Evolution of dry powder inhaler design, formulation, and performance, Respir. Med., 96 (2002) 293-304. https://dx.doi.org/10.1053/rmed.2001.1276

[79] M. Gomez, J. McCollum, H. Wang, S. Bachchhav, I. Tetreau, A. Gerhardt, C. Press, R.M. Kramer, C.B. Fox, R. Vehring, Evaluation of the stability of a spray-dried tuberculosis vaccine candidate designed for dry powder respiratory delivery, Vaccine, (2021). https://doi.org/10.1016/j.vaccine.2021.07.002

[80] A.M. D'Souza, Y. Song, D.P. Miller, C.M. Stults, R. Vehring, D. Lechuga-Ballesteros, M. Kuo, Surface Properties Are a Major Factor in Determining the Dispersibility of Protein Dry Powders for Inhalation, in: AAPS PharmSci, 2003.

[81] N.E. Wilson, T.T. Mutukuri, D.Y. Zemlyanov, L.S. Taylor, E.M. Topp, Q.T. Zhou, Surface composition and formulation heterogeneity of protein solids produced by spray drying, Pharm. Res., 37 (2020) 1-11. https://doi.org/10.1007/s11095-019-2738-9