Evaluation of the stability of a spray-dried tuberculosis vaccine candidate designed for dry powder respiratory delivery

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Particle engineering via spray drying was used to develop a dry powder presentation of an adjuvanted tuberculosis vaccine candidate. This presentation utilizing a trileucine-trehalose excipient system was designed to be both thermostable and suitable for respiratory delivery. The stability of the spray-dried vaccine powder was assessed over one year at various storage temperatures (-20, 5, 25, 40, 50 °C) in terms of powder stability, adjuvant stability, and antigen stability. A formulation without trileucine was included as a control. The results showed that the interior particle structure and exterior particle morphology of the powder was maintained for one year at 40 °C, while the control case exhibited a small extent of particle fusing under the same storage conditions. Moisture content was maintained, and powder solid state remained amorphous for all storage temperatures. Aerosol performance was assessed with a commercial dry powder inhaler in combination with a human mouth-throat model. The emitted dose and lung dose were maintained for all samples after one year at temperatures up to 40 °C. Nanoemulsion size and oil content of the adjuvant system were maintained after one year at temperatures up to 40 °C, and the agonist content was maintained after one year at temperatures up to 25 °C. The antigen was completely degraded in the control formulation at seven months of storage at 40 °C; by contrast, 45% of the antigen was still present in the trehalose-trileucine formulation after one year of storage at 50 °C. Comparatively, the antigen was completely degraded in a liquid sample of the vaccine candidate after only one month of

storage at 37 ℃. The spray-dried trehalose-trileucine vaccine powder clearly maintained its inhalable properties after one year's storage at high temperatures and improved overall thermostability of the vaccine.

Key words: spray drying, tuberculosis vaccine, trileucine, respiratory delivery, particle engineering, thermostability

Introduction

Improvement of pharmaceutical product thermostability is of particular concern for global vaccination campaigns as many vaccines must be transported and stored under refrigeration or even colder temperatures. Maintaining refrigeration throughout distribution, however, is not feasible in countries with limited electrical and transportation infrastructure. Overall vaccine wastage has been reported to be approximately 50% globally [1], and exposure to heat or unintended freezing contributes to this wastage. The inability to maintain refrigeration has also led to ineffective distribution of vaccines, contributing to an estimated 19.4 million infants not receiving basic vaccines in 2018 [2]. Therefore, improved thermostability may greatly improve a vaccine's availability. One method of improving thermostability of a liquid vaccine product is through stabilization of a formulation within a dry powder form via spray drying [3, 4, 5].

Spray drying is a desiccation method wherein feedstock droplets atomized into a drying gas medium evaporate to form a powder composed of many microparticles. A benefit of spray drying is that it allows for the design of a powder that is suitable for nasal or pulmonary administration. Administration of a dry powder to the nasal passages or lungs bypasses issues with needle delivery, such as transfer of blood-borne illness, lower compliance due to invasiveness, and increased systemic side effects. Additionally, there is evidence that the direct delivery of a vaccine to the primary site of infection to induce mucosal immunity improves the protective immune response against respiratory illnesses [6, 7]. Therefore, the use of spray drying as a method to improve thermostability and impart inhalable properties to a vaccine dosage form is especially useful for intervention in highly infectious respiratory illnesses, such as tuberculosis (TB).

The Bacille Calmette-Guerin vaccine is the only approved TB vaccine currently available. However, despite its widespread use, this vaccine is not without significant drawbacks [8, 9]. For this reason, many alternative vaccines are being developed, such as the TB vaccine candidate ID93+GLA-SE, which is formulated as a subunit vaccine consisting of an antigen, ID93, and an adjuvant system, GLA-SE. ID93 is

a recombinant fusion protein formed from four *Mycobacterium tuberculosis* antigens [10]. The GLA-SE adjuvant system is a nanoemulsion composed primarily of squalene nanodroplets with DMPC as an emulsifier and glucopyranosyl lipid A (GLA), a TLR4 agonist, at the interface [11]. This vaccine candidate has entered Phase II clinical trials in a liquid injectable presentation [12, 13] and Phase I clinical trials as a lyophilized injectable presentation [14]; however, the liquid formulation needs to be kept refrigerated in order to maintain its potency.

Thermostability of a spray-dried version of ID93+GLA-SE with large particle size designed for eventual reconstitution and subsequent administration by injection has been reported [15]. The powder showed adjuvant stability for 26 months at room-temperature storage, with evidence of antigen presence after 26 months at 40 °C. Following this success, an inhalable spray-dried version of the ID93+GLA-SE vaccine candidate was investigated [16]. Several excipient combinations were characterized to assess both the preservation of the vaccine components after spray drying and the aerosol performance of the dry powder. The best performing candidates used the disaccharide trehalose as a stabilizer and low concentrations of the tripeptide trileucine as a dispersibility enhancing agent.

Trehalose is an excellent stabilizing excipient due to its high glass transition temperature of approximately 110 °C when dry [17] and its great effectiveness as a glass stabilizer for biologics such as proteins [18], phospholipid membranes [19, 20], and bacteriophages [21]. Trileucine has been shown to improve the dispersibility of dry powders for pulmonary delivery and to protect against moisture-related degradation and aerosol performance losses [22, 23]. An especially attractive feature of trileucine is that, because of its low solubility, only small amounts are required to achieve surface enrichment for the production of highly rugose particles. Trileucine also has the potential to improve the stability of protein-based formulations. A study on spray drying bacteriophages found that a trileucine-trehalose excipient system showed fewer losses than a trehalose-only system [21]. Similarly, a patent on spray-dried oxytocin demonstrated that a trileucine-trehalose excipient system improved formulation stability over the more commonly used leucine-trehalose excipient system [24].

Development of a dry powder TB vaccine that is both thermostable and suitable for pulmonary administration may increase accessibility and possibly improve immune protective response. This study evaluates the stability of such a spray-dried inhalable presentation of the ID93+GLA-SE vaccine candidate utilizing a trehalose-trileucine excipient system.

Materials and Methods

Materials

Manufacture and storage of the ID93+GLA-SE system are described elsewhere [15]. The primary stabilizing excipient used in this study was trehalose dihydrate with a purity of 98% (CAS 6138-23-4; Fisher Scientific Ottawa, ON, Canada). Trileucine with a purity of ≥90% (CAS 10329-75-6; Sigma Aldrich, Oakville, ON, Canada) was used as a dispersibility enhancing agent. A buffer system of Tris(hydroxymethyl)aminomethane (Tris) (CAS 77-86-1; Sigma Aldrich, Oakville, ON, Canada) and hydrochloric acid (CAS 7647-01-0; Sigma Aldrich, Oakville, ON, Canada) was used to adjust the pH of the feedstock prior to spray drying. All formulations were prepared in deionized water.

The excipient screening carried out in Gomez et al. [16], identified the lead inhalable spray-dried candidate as the trehalose-trileucine formulation (T3Tri). A similar formulation without trileucine was also included in the stability study as a control (T). The formulation parameters and designed particle composition of both formulations are detailed in Table 1.

Table 1. Formulation parameters and nominal particle composition of the spray-dried trehalose-trileucine and control formulations.

Component	Control		Trehalose-trileucine	
	Feedstock Composition (mg/mL)	Particle Composition (w/w)	Feedstock Composition (mg/mL)	Particle Composition (w/w)
Trehalose	33.3	81%	33.3	78%
Tris (buffer)	0.81	2%	0.81	2%
Trileucine	-	-	1.30	3%
Squalene	5.73	14%	5.73	14%
DMPC	1.27	3%	1.27	3%
GLA	0.00333	0.01%	0.00333	0.01%

ID93	0.00133	0.003%	0.00133	0.003%

The formulation procedure has been described elsewhere [15, 16]. Briefly, trehalose, Tris, and trileucine were weighed and dissolved in a glass beaker using deionized water as a solvent. Once the chemicals were fully dissolved, the solution was pH-adjusted using hydrochloric acid to a pH of approximately 7.5. After pH adjustment, the solutions were syringe-filtered using a sterile filter with a 0.22 μm pore size (GSWP02500; Sigma Aldrich, Oakville, ON, Canada). GLA-SE was then added to the solution and gently stirred. ID93 was added last to the formulation.

Spray Drying

Appropriate spray drying parameters were developed in a previous study [15]. The parameters were calculated to produce low moisture content powders suitable for long-term room-temperature storage, as well as minimize potential antigen or agonist losses during processing. Briefly, an energy and mass balance model [25] was used iteratively to determine processing conditions that led to an outlet temperature of 36 °C and 7% outlet relative humidity (RH). Spray drying was completed using a custom research spray dryer with a twin fluid atomizer. The feedstock was supplied at a flow rate of 0.6 mL/min, and the atomized droplets were dried in heated air flowing at 200 SLPM, with an inlet temperature of 65 °C. Manufacturing of the control was completed in one batch, while manufacturing of the trehalose-trileucine formulation was completed in two batches due processing time limits. The two batches were characterized (not shown) at the beginning of the study to confirm consistency.

Dry Powder Packaging

Protective packaging of powders for storage has been shown to significantly influence powder stability [3, 26]. Furthermore, several studies [27, 28] have demonstrated that exposure of trehalose-based particles to moisture facilitates particle fusing and possible crystallization of the powder. An intensive packaging process used to protect the spray-dried powder from moisture uptake during the stability study is described in detail elsewhere [15]. Briefly, the powder samples were packaged along with silica gel as a

desiccant to regulate the RH of the enclosed environment. The packaging process took place within a glovebox set to 0% RH. The powder was first transferred into low-bind snap-cap tubes (Product Z768820; Sigma Aldrich, Oakville, ON, Canada). These tubes were then placed in an aluminum bag along with a silica gel pouch that had been equilibrated to 0% RH. This bag was then double heat-sealed and placed into another aluminum bag containing an additional desiccant pouch. This external aluminum bag was then also double heat-sealed.

Stability Study

The preliminary stability target was at least three months at 40 °C or at least one year at 25 °C. Stability of the dry powder was assessed according to the following acceptance criteria: no visible particle fusing, change in moisture content ≤1% (absolute), no crystallization, and no significant decrease in aerosol performance. Stability of the adjuvant system was assessed according to the following acceptance criteria: average nanoemulsion droplet size change ≤50%, polydispersity index ≤0.20, and squalene and GLA content loss ≤20%. Lastly, stability of the antigen system was defined as ID93 content loss ≤20%. Similar criteria were used to assess lyophilized candidates [29] and an earlier spray-dried product candidate [15].

The trehalose-trileucine powders were stored at -20, 5, 25, 40, and 50 °C in the sealed packages as described above. The control powder was stored at 5, 25, and 40 °C. Characterization tests for the physical and chemical stability of the powder were designed to be completed at the same timepoints throughout the twelve-month stability study. The physical powder stability plan excluded a -20 °C storage arm and limited the 50 °C arm to 7 months due to equipment and financial constraints.

Dry Powder Characterization

Scanning Electron Microscopy

Particle morphology of the spray-dried powders was assessed using field emission scanning electron microscopy (Zeiss Sigma FE-SEM; Carl Zeiss, Oberkochen, Germany). Two samples were prepared for each experiment, one to assess the exterior morphology, and one to assess the interior structure. The

former was prepared by placing the sample on a carbon tape-covered aluminum stub. The latter was prepared by scraping the sample across an aluminum stub in an effort to break the particles open. These samples were subsequently sputter-coated with either gold (Denton Vacuum Desk II Sputter Coater; Denton, Moorestown, NJ, USA) to a thickness of approximately 16 nm or a mixture of 80% gold and 20% palladium (Leica ACE600 Carbon/Metal Coater; Concord, ON, Canada) to a thickness of 10-15 nm to remove any charging effects during imaging.

Moisture Content

Water content of the powders over the course of the stability study was assessed using Karl Fisher Calorimetry (Karl Fisher Coulometric Titrator Model C30; Mettler Toledo; Mississauga, ON, Canada). Results were reported as the percentage of water by mass relative to the total mass of the sample. Experiments were completed in duplicate.

Raman Spectroscopy

Solid phase of the powders was monitored over the course of the stability study by Raman spectroscopy on a custom instrument used in previous work [15]. Samples were measured at ambient temperature and an RH of ≤6% to minimize moisture uptake during the spectra acquisition. Raman spectra measurement was also completed on crystalline trehalose, crystalline trileucine, Tris, and squalene oil for their respective references. Reference spectra for amorphous trehalose and trileucine were also obtained from spray-dried trehalose and spray-dried trileucine respectively.

***In vitro* Aerosol Performance**

Aerosol performance of the dry powders was assessed using a method described elsewhere [16]. Briefly, the dry powders were loaded into a commercial dry powder inhaler (DPI) (Seebri Breezhaler, Novartis International AG; Basel, Switzerland) and actuated into an Alberta Idealized Throat connected to a Next Generation Impactor to obtain an accurate assessment of extrathoracic powder deposition [30]. Inhalation was simulated at 100 L/min for 2.4 s to obtain 4 L of inhaled air, as per the USP standard [31]. The interior of the mouth-throat model and the impactor plates was coated with silicone spray (Molykote 316

Silicone Release Spray, Dow Corning Corporation; Midland, MI, USA) to mitigate particle bounce [32]. Aerosol performance was assessed in terms of emitted dose and lung dose. Emitted dose was defined as the percentage of the loaded dose exiting the DPI, while lung dose was defined as the percentage of the loaded dose penetrating the mouth-throat model. Each DPI capsule was filled with approximately 40 mg of powder, and each run consisted of actuating three capsules to obtain enough powder for gravimetric analysis. Experiments were completed in triplicate.

The mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) of the powder that deposited on the impactor plates were calculated by fitting the data to a cumulative lognormal distribution function [33]. Correlation of mass distribution to particle size was based on stage cutoff diameters at 100 L/min flow rate, as determined elsewhere [34]. Data fitting was done using the MATLAB (MathWorks Inc., Natick, MA, USA) “Curve Fitting” application.

Reconstituted Powder Characterization

Although the powder is intended as a dried product, its reconstitution was necessary for characterization of the GLA-SE and ID93 components. This reconstitution also allowed for assessment of processing losses. For squalene, GLA, and ID93 content assays, the powders were first reconstituted to 3× concentration of the feedstock to maintain a high level of accuracy for the assays. The remaining liquid was then diluted to 1× feedstock concentration for dynamic light scattering measurements. Sample osmolality was measured at 1× feedstock concentration at each time point to ensure accurate dilution (data not shown). Measurements of the reconstituted powder were completed in triplicate unless otherwise stated.

Dynamic light scattering

Mean hydrodynamic diameter and polydispersity of the nanoemulsion droplets after reconstitution were measured using dynamic light scattering with a measurement angle of 173° (NanoZS; Malvern, Worcestershire, UK). The mean hydrodynamic diameter and polydispersity index were calculated by the instrument software from a cumulates analysis of the intensity autocorrelation function.

Reversed Phase HPLC

Squalene and GLA content were quantified by reversed-phase HPLC using an Agilent 1200 HPLC (1200 HPLC; Agilent Technologies; Santa Clara, CA, USA) equipped with a silica-based, C18 reversed-phase column (Atlantis T3 Column; Waters; Elstree, UK). Column temperature was held constant at 30 °C and analyte detection was accomplished using a charged aerosol detector (Corona CAD; ESA Biosciences; Chelmsford, MA, USA). Mobile phase A contained 75:15:10 (v/v/v) methanol:chloroform:water, 1% (v/v) acetic acid and 20 mM ammonium acetate, and mobile phase B contained 50:50 (v/v) methanol:chloroform, 1% (v/v) acetic acid, and 20 mM ammonium acetate. Samples were diluted in mobile phase B and injected with a gradient over 30 minutes for squalene content analysis or 18 minutes for GLA content analysis. Squalene content was quantified by peak area, while GLA content was quantified by peak height. Concentration measurements were made by interpolation of a curve generated from standards fitted with a second order polynomial.

SDS-PAGE

ID93 concentration was quantified using densitometry analysis of reducing SDS-PAGE based on a standard curve. Gel samples were prepared by mixing a 20% (w/v) sodium dodecyl sulfate solution (Thermo Fisher Scientific, Waltham MA, USA), 4X LDS Buffer (Thermo Fisher Scientific, Waltham, MA, USA) spiked with 5% (v/v) β-Mercaptoethanol, and formulated sample in a 2:1:1 ratio. Prepared samples were heated for 15 minutes in an 85 °C water bath and cooled in a room-temperature water bath. A precast, 4-20% Tris-Glycine SDS-PAGE gel (Thermo Fisher Scientific, Waltham, MA, USA) was prepared according to manufacturer's instructions. Cooled gel samples were centrifuged at 2000 RPM in a benchtop centrifuge (Thermo Fisher Scientific, Waltham, MA, USA) for 2 minutes to collect the sample, and then briefly vortexed prior to loading onto the prepared gel to ensure uniformity. The gel was run at 180V for 65 minutes and then stained overnight using SYPRO Ruby according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). Stained gels were imaged using the SYPRO Ruby protocol on the gel imaging system (ChemiDoc; Bio-Rad, Mississauga, ON, Canada). Only one

measurement was taken for the feedstock analysis and the twelve-month timepoint of the Trehalose-trileucine stability study. ID93+GLA-SE standards at 10 ng, 50 ng, and 100 ng protein load were prepared in the same manner and included on each gel. Densitometry analysis was performed using Image Lab 6.0 software (Bio-Rad Laboratories, Hercules, CA, USA). The three standards were used to generate a standard curve based on band intensity, and the quantity of ID93 in each sample was determined based on interpolation from the standard curve. An admix ID93+GLA-SE control was freshly prepared in the same manner as above and run on each gel to ensure accurate interpolation from the standard curve. Samples that were below the limit of quantitation were not included in the average and standard deviation; timepoints where these exclusions occurred were noted in the figures.

Statistical Analysis

Results are reported as mean ± standard deviation. Statistically insignificant differences were indicated for $p>0.05$, as determined by a two-tailed student's t-test.

Results and Discussion

Processing losses

Table 2 compares the ID93+GLA-SE vaccine properties before and after spray drying. Emulsion size targets are from previous studies [15, 16], and component concentration targets were ±20% of the values listed in Table 1. Properties of the adjuvant system were within the target values for both formulations, with the exception of the higher squalene content measured in the reconstituted powder. Higher values post-spray drying may be due to assay variability. There was no significant change in nanoemulsion droplet diameter, polydispersity index and GLA content after the trehalose-trileucine formulation was spray dried. Overall, it is apparent that the adjuvant system was well stabilized during spray drying. Following these results, the characterization for the stability study was conducted by reconstituting the powder to 3× the concentration of the feedstock to improve accuracy.

Measured ID93 concentration in the feedstock was higher than expected, approximately 2× the target concentration. The ID93 concentration was reduced by 68% and 66% from the feedstock concentration for the control and trehalose-trileucine formulations upon reconstitution, respectively. Further work has suggested that ID93 loss was highly variable, with several cases showing no processing loss of ID93 under the same conditions (data not shown). Further refinement is needed to obtain consistently low processing losses in ID93.

Table 2. Comparison of key properties of the ID93+GLA-SE system before and after spray drying the control and trehalose-trileucine formulations. Spray-dried powder was reconstituted to 1× the feedstock concentration to allow for direct comparison. Values marked with a club (♣) indicate statistically insignificant differences (p>0.05) as compared to the liquid feedstock. ID93 content in the feedstock was measured only once.

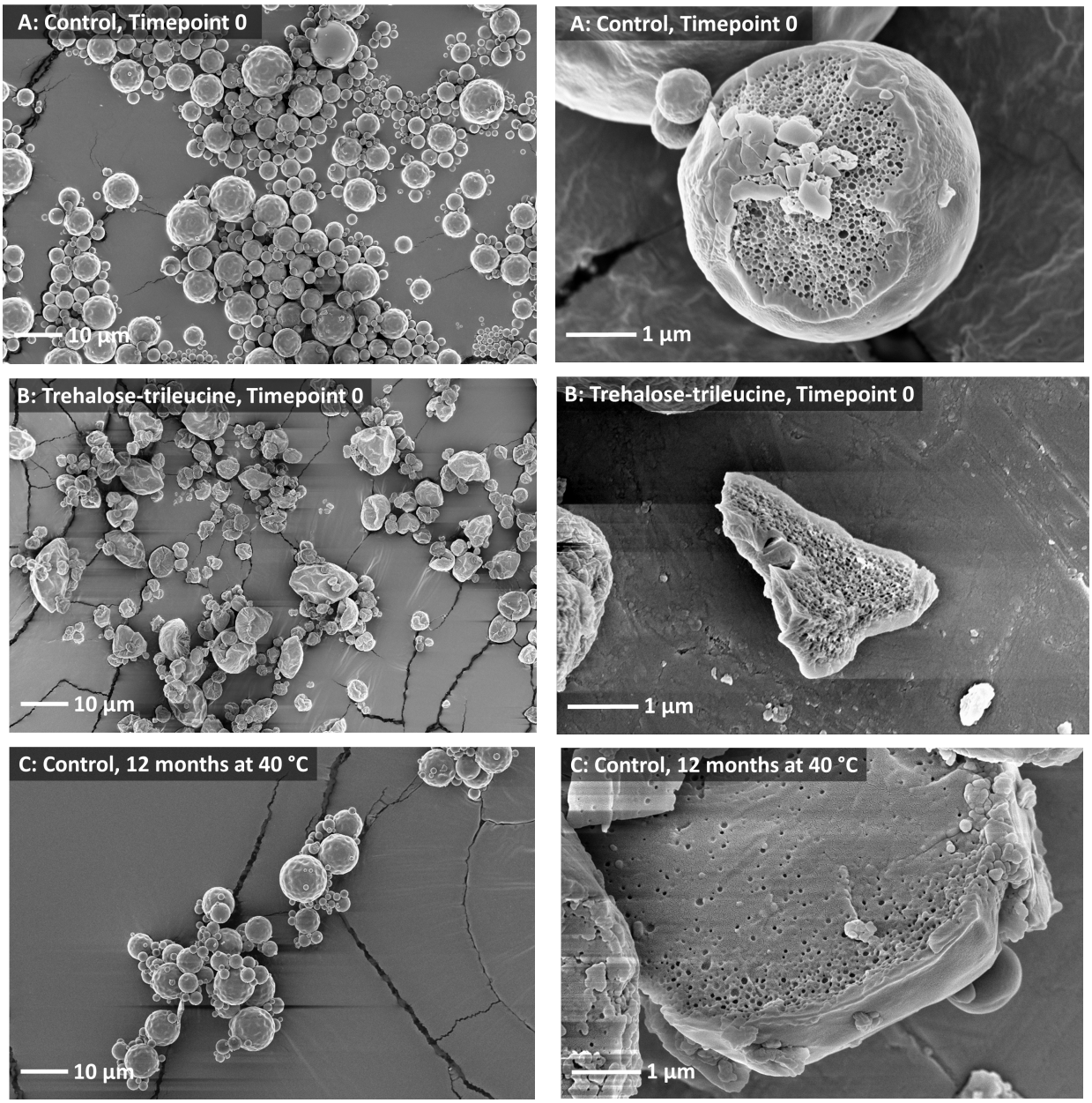
ID		Adjuvant System				Antigen
		Nanoemulsion Droplet Diameter (nm)	Polydispersity Index	Squalene Content (mg/mL)	GLA Content (µg/mL)	ID93 Content (µg/mL)
Target		80-160	<0.20	4.58-6.88	2.67-4.00	1.07-1.60
Control	Feedstock Liquid	115.9±0.8	0.04±0.01	6.21±0.09	3.27±0.14	2.86
	Reconstituted Powder	108.2±1.2♣	0.06±0.01♣	6.83±0.04	3.64±0.05	0.92±0.05
Trehalose-trileucine	Feedstock Liquid	111.0±2.5	0.07±0.00	6.32±0.02	3.38±0.05	2.90
	Reconstituted Powder	112.0±1.8♣	0.06±0.01♣	6.94±0.09	3.48±0.06♣	0.98±0.01

Powder Stability

Particle Morphology

The particle morphology of the spray-dried control and trehalose-trileucine formulations at timepoint 0 and after twelve months of storage at 40 °C are given in Figure 1. Similar images were taken throughout the stability study. The exterior morphology of the control formulation indicates that the polydisperse powder consists of spherical particles with relatively smooth or lightly dimpled surfaces. The addition of trileucine in the trehalose-trileucine formulation led to a rugose and folded particle morphology due to the accumulation of trileucine on the surface. Cracked particles show that the nanoemulsion droplets were

encapsulated within the particle wall in both formulations, as represented by the voids within the particle shell. These results are consistent with the morphology exhibited in a previous study [16].



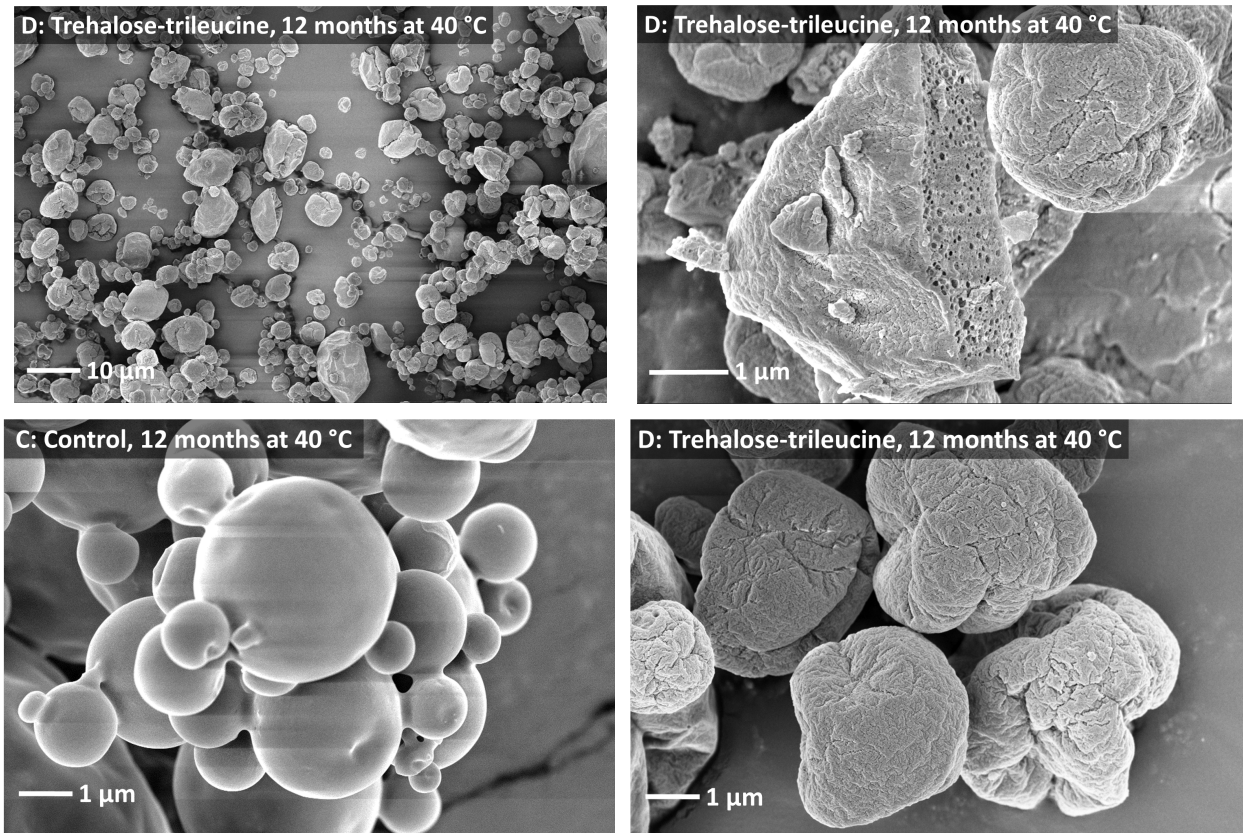


Figure 1. SEM images of the control and trehalose-trileucine formulations at timepoint 0 and after twelve months of storage at 40 °C. Images depict the exterior and interior morphology of both formulations after storage. Scale bars are given on the respective images. A: Control, Timepoint 0; B: Trehalose-trileucine, Timepoint 0; C: Control, 12 months at 40 °C; D: Trehalose-trileucine, 12 months at 40 °C

Low magnification images of the control and trehalose-trileucine particles indicate that the overall exterior particle morphology was maintained after twelve months of storage at 40 °C. Similarly, interior particle morphology was maintained for all samples, as shown by the presence of distinct voids within the particles' walls. Similar preservation of particle morphology was observed in the trehalose-trileucine sample held at 50 °C for seven months (not shown). However, high magnification images show bridging between the smaller particles in the control formulation, whereas the trehalose-trileucine particles remain distinct. This result reveals that the addition of trileucine prevented high-temperature particle fusing over the studied timeframe.

Powder Moisture Content

Initial moisture content for the control formulation was 2.4±0.1%. Initial moisture content for the two trehalose-trileucine batches was 2.1%±0.0% and 1.8±0.1%. Moisture content for both the formulations did not change significantly after twelve months at any of the storage temperatures. The protective packaging method mitigated exposure to humidity, thereby preventing powder moisture sorption. This packaging system was effectively used in a previous study [15] to prevent moisture uptake. Prevention of moisture exposure is critical for inhalable pharmaceutical powders. Increase in water content of sugars is known to depress the glass transition temperature, [17] and protein stabilization is less effective at storage temperatures close to the transition temperature [35].

Solid Phase Analysis

Raman spectra for the control and trehalose-trileucine formulations at timepoint 0 and after twelve months of storage are shown in Figure 2. The deconvoluted spectra, plotted under each of the respective sample spectra, were obtained after subtracting reference amorphous trehalose, squalene, and amorphous trileucine spectra. Reference spectra for amorphous trehalose, squalene, crystalline tris, amorphous trileucine, crystalline trehalose, and crystalline trileucine are given in Figure 3. Similar analysis was completed at different timepoints throughout the stability study.

Analysis of both the control and trehalose-trileucine spectra taken at timepoint 0 confirms that the formulations were composed of fully amorphous trehalose and fully amorphous trileucine. Additionally, the residual spectra remain flat for all samples obtained after twelve months of storage. Similar results were obtained for the trehalose-trileucine formulation stored at 50 °C for seven months (not shown). The lack of crystalline peaks in the residual spectra suggests that both spray-dried powders remained amorphous for all storage temperatures and had undergone no detectable phase change after twelve months of storage as compared to timepoint 0, demonstrating a good solid-state stability of the spray-dried respirable vaccine formulation.

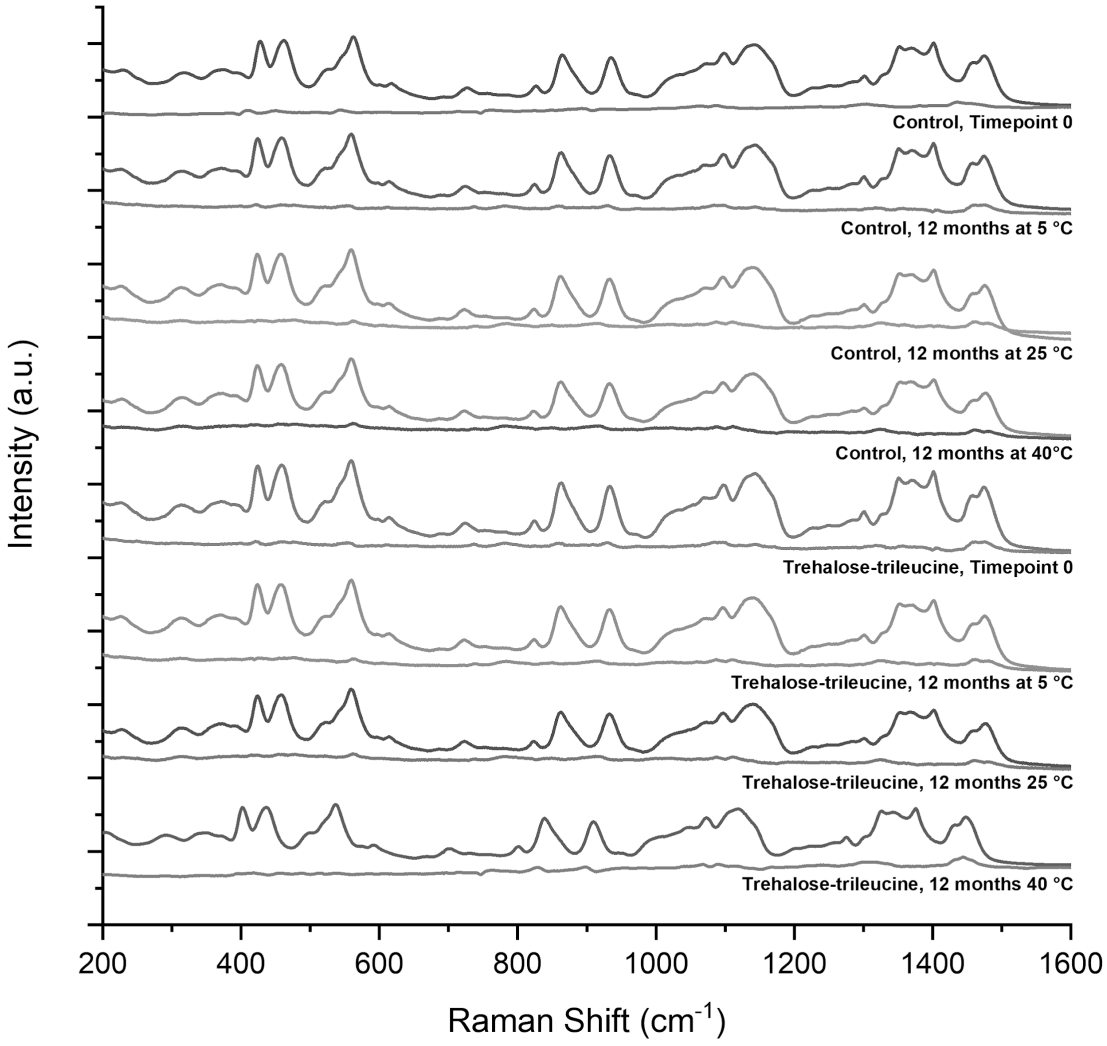


Figure 2. Sample spectra of the control and trehalose-trileucine formulations at timepoint 0 and at twelve months of storage at 5, 25, and 40 °C. The residual spectra obtained after deconvolution are given under the respective sample spectra. The low intensity of the residual spectra indicates that the powders remained amorphous after seven months at all storage temperatures.

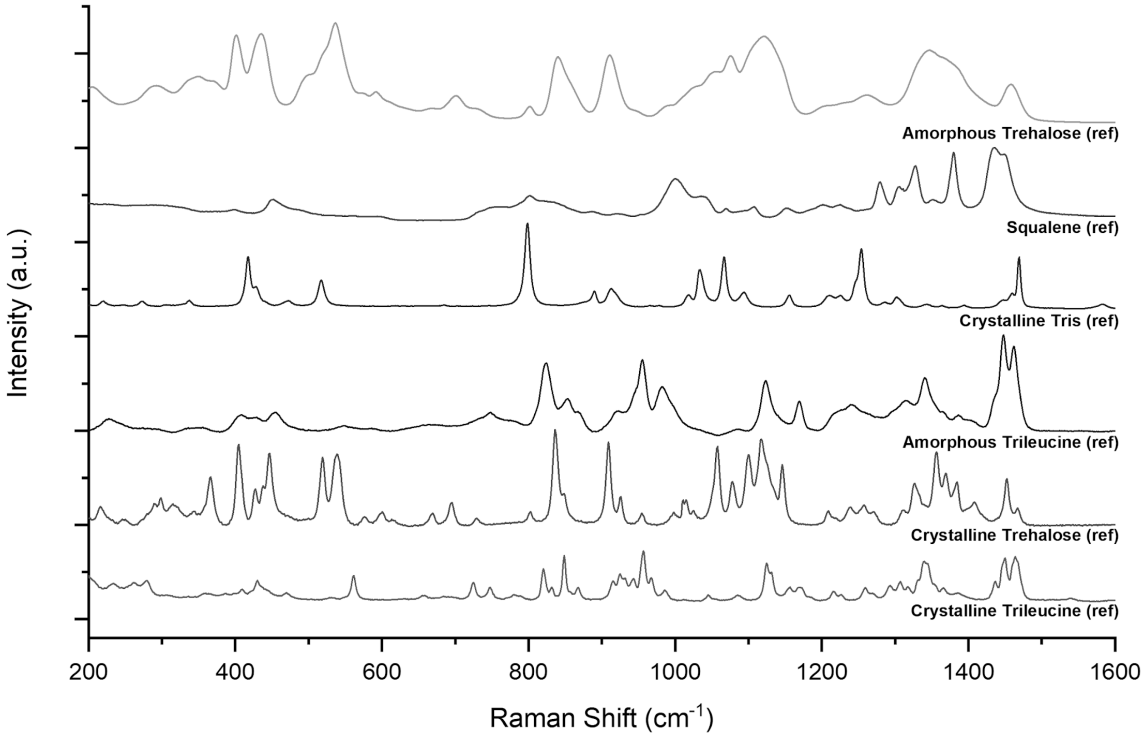


Figure 3. Reference spectra for the main components of the spray-dried control and the spray-dried trehalose-trileucine formulations.

Aerosol Performance

The emitted dose and lung dose for the control and trehalose-trileucine formulations over the twelve-month stability study are plotted in Figure 4. The emitted dose for both spray-dried formulations was high and did not change significantly for any of the storage temperatures after twelve months of storage. The emitted dose of these experimental formulations is better than or comparable to the emitted dose of commercial DPIs [36, 37, 38]. These powders exhibit very reliable administration with a DPI even after long-term storage at high temperatures.

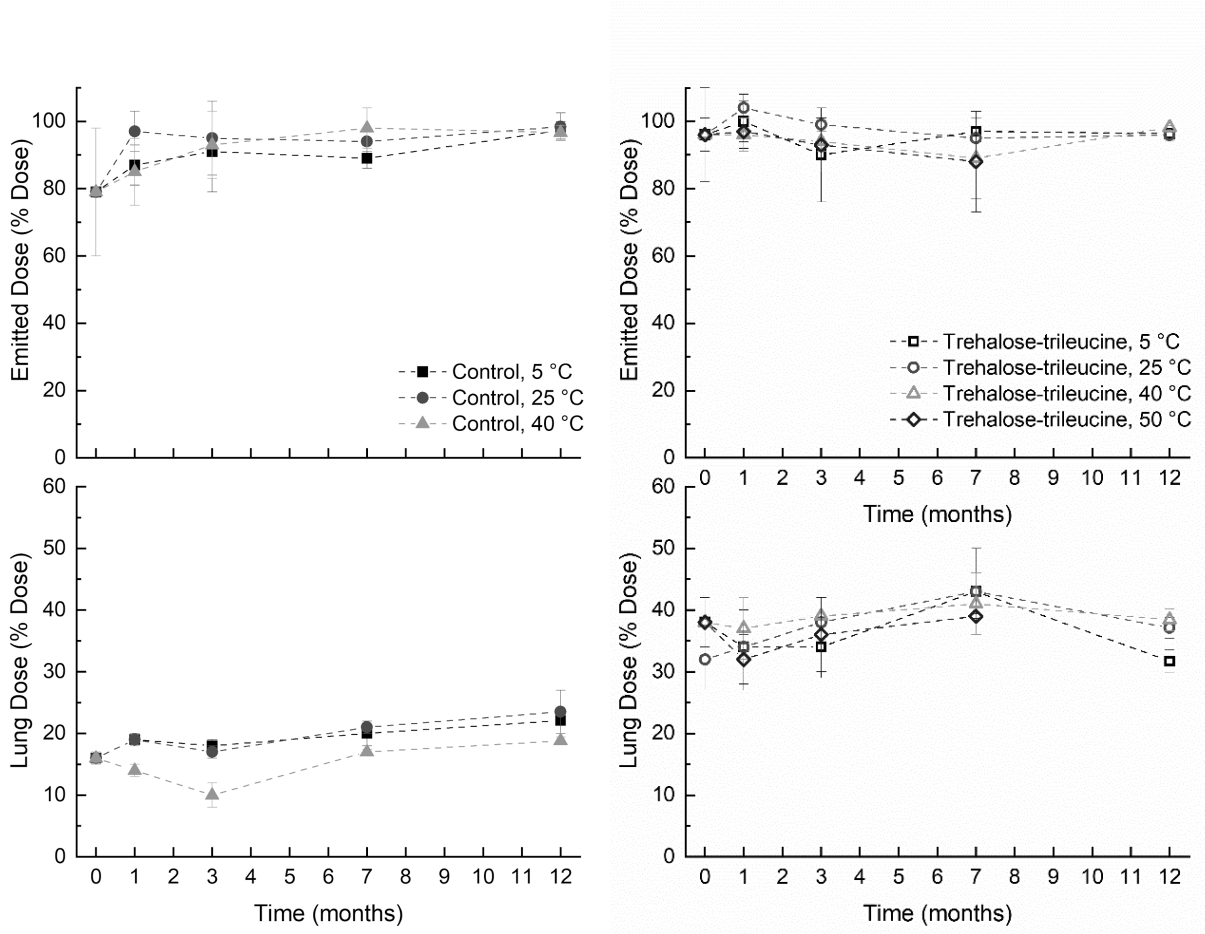


Figure 4. Aerosol performance, in terms of emitted dose and total lung dose of the spray dried formulationsover twelve months of storage at various temperatures. Marker color and shape indicate the temperature arm (black squares – 5 °C, red circles – 25 °C, green triangles – 40 °C, blue diamonds – 50 °C). Closed symbols represent the control formulation (left) and open symbols represent the trehalose-trileucine formulation (right). Aerosol performance was calculated as a percentage of the loaded powder dose. Results shown are the average of triplicate measurements; error bars represent the standard deviation of triplicate measurements.

Comparison of the aerosol performance at timepoint 0 indicates that the inclusion of trileucine in the trehalose-trileucine formulation significantly improved lung dose (p<0.05) as compared to the control formulation. The rugose morphology due to the accumulation of trileucine at the surface of the trehalose-trileucine particles reduced contact area and thus improved dispersibility as compared to the smooth-surfaced control particles. The lung dose did not decrease significantly after twelve months of storage for either formulation at all tested storage temperatures. The measured lung dose of the trehalose-trileucine formulation over the stability study (32-43%) was much better than the reported lung

dose of many commercial products [39, 40]. Lung dose may be further improved through the use of high-efficiency DPIs [23, 36]; however, DPI optimization was outside the scope of this study.

Clearly, the intensive packaging procedure used in the stability study effectively protected the powders from moisture uptake and potential consequent moisture-induced crystallization. Increase in moisture content may decrease powder dispersibility due to capillary forces between particles [41], and crystallization of amorphous solids may potentially impact the powder aerosolization. Inconsistent dosing due to moisture-induced crystallization has been reported by Shetty et al. [42], where crystallization of amorphous spray-dried ciprofloxacin powders stored under different humidities led to variable measured emitted dose and fine particle fraction after actuation from a DPI.

The MMAD and GSD of the control and trehalose-trileucine powder that deposited within the impactor are shown in Figure 5. This size distribution measurement of the lung dose was obtained to predict peripheral lung deposition, in which smaller MMAD suggests deeper lung penetration. The MMAD of the control formulation stored at 40 °C was significantly higher ($p<0.05$) than that of the control powders stored at 5 and 25 °C for a given timepoint. The higher MMAD for the control samples stored at 40 °C is likely due to fusing among small particles, as demonstrated by the SEM images. The fusing of the smaller particles in a polydisperse powder would lead to these aggregates behaving aerodynamically as a large particle, thus increasing the measured MMAD. By comparison, the trehalose-trileucine powder MMAD was not significantly different ($p>0.05$) at any of the different storage temperatures for a given timepoint, and particle morphology analysis did not show any particle fusing. GSD of the deposited powder was high throughout the stability study for both formulations, suggesting a wide distribution of powder throughout the lungs.

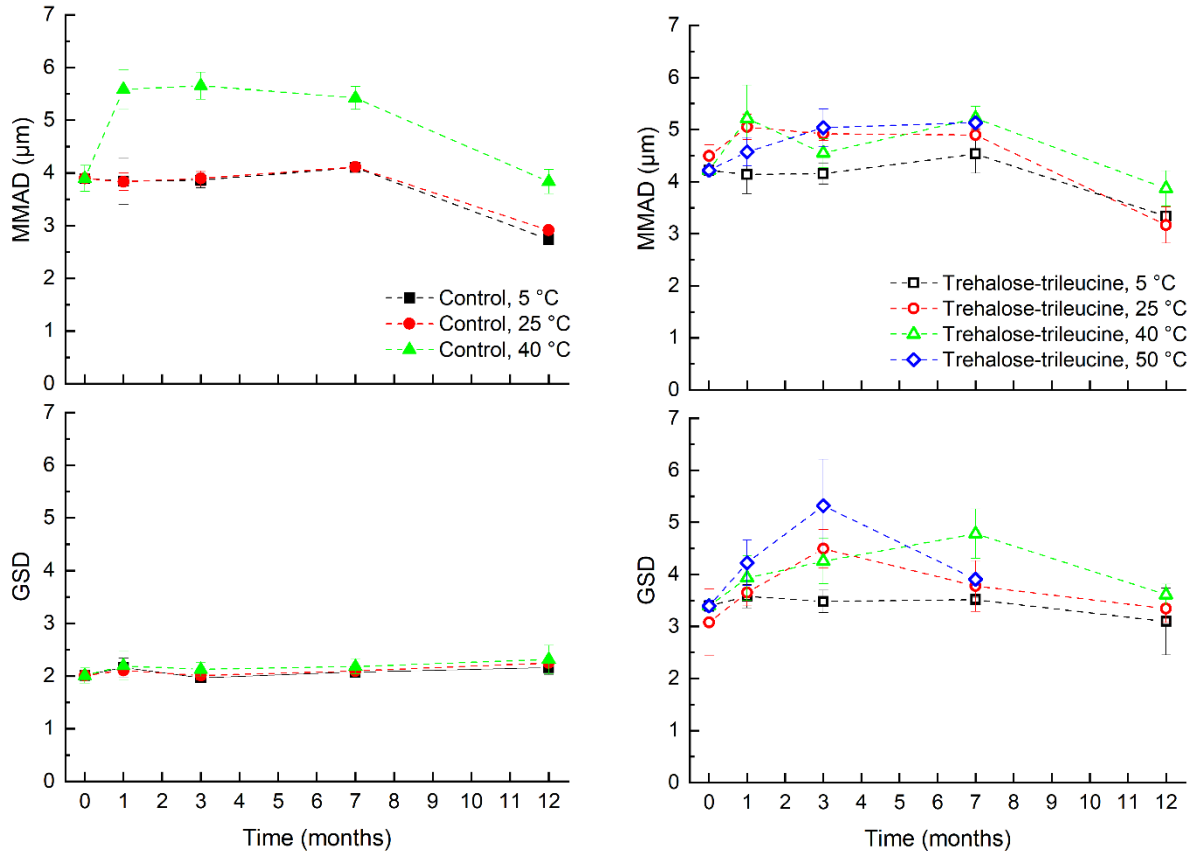


Figure 5. The mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) of the spray dried powder deposited on the impactor stages over twelve months of storage at various temperatures. Marker color and shape indicate the temperature arm (black squares – 5 °C, red circles – 25 °C, green triangles – 40 °C, blue diamonds – 50 °C). Closed symbols represent the control formulation (left) and open symbols represent the trehalose-trileucine formulation (right). Results shown are the average of triplicate measurements; error bars represent the standard deviation of triplicate measurements.

The preserved excellent aerosol performance of the trehalose-trileucine as compared to the control formulation was likely due to the presence of trileucine at the surface of the former. Accumulation of trileucine on the surface of particles has previously been reported to decrease surface energy, and rugose particles have reduced contact area between adjacent particles, thus lowering cohesive forces.

Adjuvant Stability

Nanoemulsion Size Distribution

The hydrodynamic diameter (Z-Ave) of the GLA-SE nanoemulsion droplets over twelve months of the stability study is shown in Figure 6. Trehalose is a well-known stabilizer of lipid membranes in its amorphous glass form through the vitrification or hydrogen-bonding stabilizing mechanisms [43]. The

control samples underwent an 11% increase in droplet diameter as compared to a larger increase of 33% in the trehalose-trileucine samples after twelve months at 40 °C, suggesting that the addition of trileucine reduced the stability of the nanoemulsion membrane. Other studies have shown that trileucine is able to penetrate and thereby disrupt lipid-based membranes due to its hydrophobic sidechains [44]. However, both the control and trehalose-trileucine formulations demonstrated less than 50% change in emulsion diameter, and the measured polydispersity index was less than 0.20 at twelve months of storage at 40 °C. The preservation of droplet size distribution for both formulations exceeds the minimum preset requirement of three months of stability at 40 °C storage, indicating that on the whole the nanoemulsion droplets were well stabilized within the trehalose matrix.

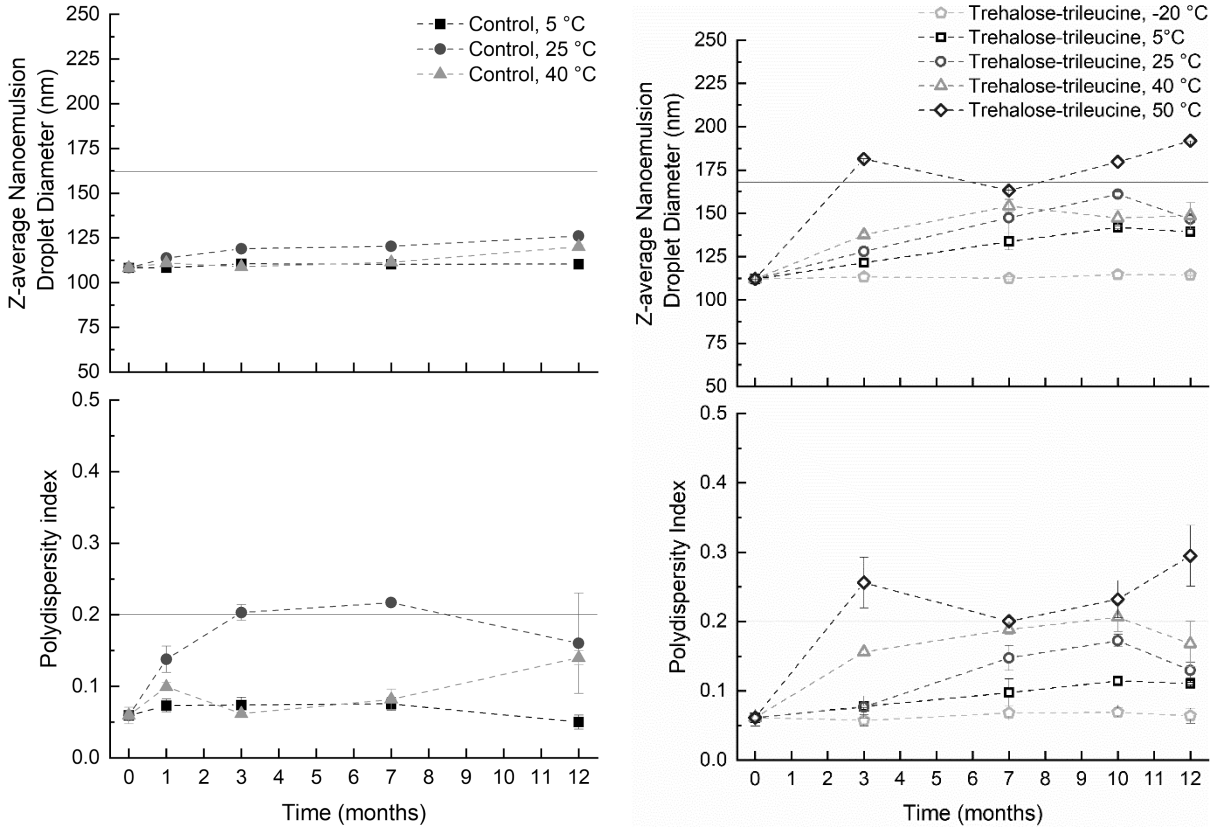


Figure 6. Nanoemulsion size distribution, in terms of average nanoemulsion droplet diameter and polydispersity index, for the spray dried formulations over twelve months of storage at various temperatures. Marker color and shape indicate the temperature arm (light blue pentagons - -20 °C, black squares – 5 °C, red circles – 25 °C, green triangles – 40 °C, blue diamonds – 50 °C). Closed symbols represent the control formulation (left) and open symbols represent the trehalose-trileucine formulation (right). Preset stability criteria were ≤50% change in droplet diameter and polydispersity index ≤0.20 after at least three months of storage at 40 °C, as represented by the solid line. Results shown are the average of triplicate measurements; error bars represent the standard deviation of triplicate measurements.

Squalene and GLA Content

The squalene and GLA retention of the control and trehalose-trileucine powders over the stability study are shown in Figure 7. The squalene loss was statistically insignificant for all samples stored for twelve months at 25 °C and below. After twelve months of storage at 40 °C, both the control and trehalose-trileucine formulations underwent less than 20% loss in squalene (18% and 19%, respectively), well within the preset stability criteria for squalene retention. The control formulation showed less than 7% loss in GLA after storage for twelve months at 25 °C and below, and the GLA losses in the trehalose-trileucine formulation were statistically insignificant under the same conditions. Reported increase in GLA concentration at the twelve-month timepoint was not statistically significant ($p>0.05$) relative to the ten-month timepoint. GLA loss at 40 and 50 °C storage appeared to follow an overall downward trend over time toward complete degradation, with an increasing rate of loss shown to correspond to an increase in temperature. For comparison, a stability study on the single-vial liquid ID93+GLA-SE product showed greater than 50% GLA loss after only three months of storage at 25 °C [29]. Additionally, GLA loss after three months of storage at 40 °C was 31% and 34% for the control and trehalose-trileucine formulations, respectively, whereas GLA was completely degraded in the liquid product after three months of storage at 37 °C [29]. Spray drying the ID93+GLA-SE vaccine with either the control or trehalose-trileucine formulation clearly improved short-term high-temperature stability of GLA and greatly improved long-term room-temperature stability as compared to the liquid product.

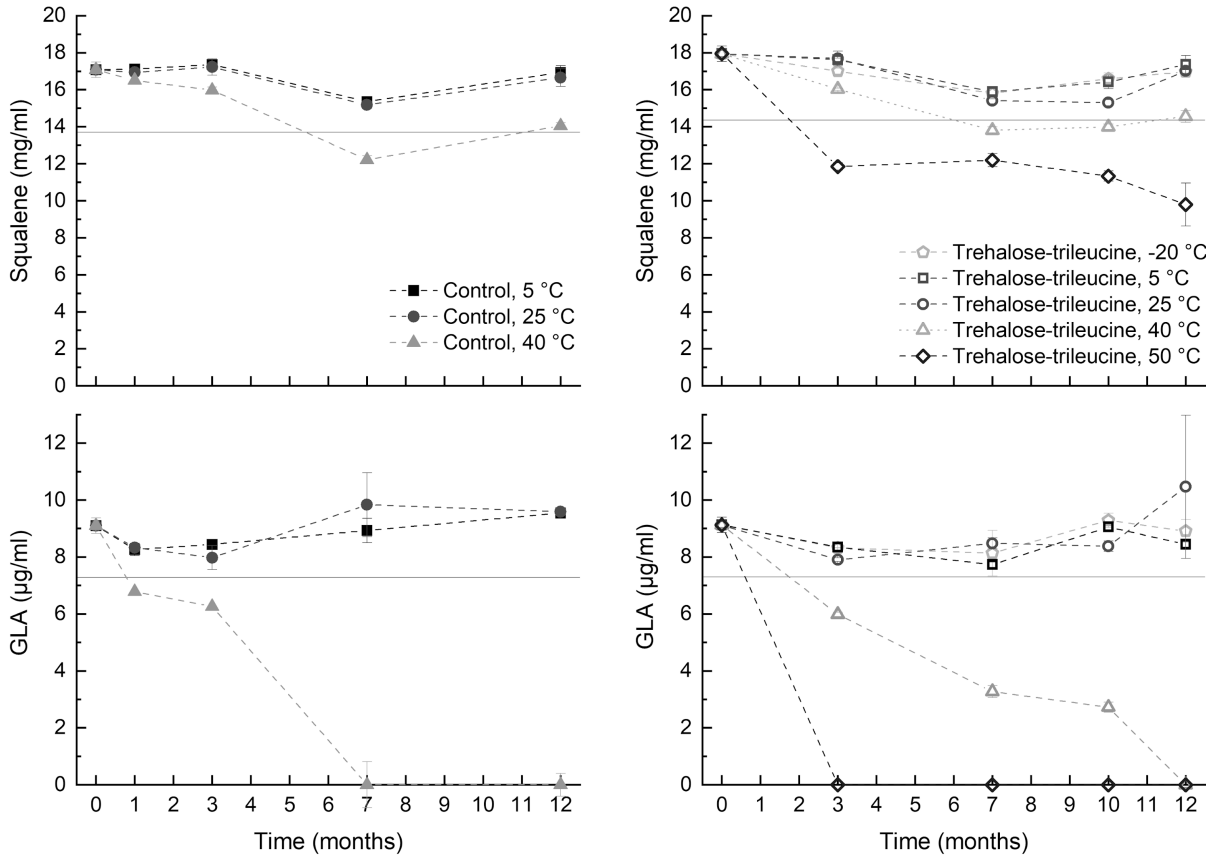


Figure 7. Squalene content and GLA content for the spray dried formulations over twelve months of storage at various temperatures. Marker color and shape indicate the temperature arm (light blue pentagons - -20 °C, black squares – 5 °C, red circles – 25 °C, green triangles – 40 °C, blue diamonds – 50 °C). Closed symbols represent the control formulation (left) and open symbols represent the trehalose-trileucine formulation (right). Preset stability criteria were ≤20% loss in squalene and GLA content after at least three months of storage at 40 °C, as represented by the solid line. Results shown are the average of triplicate measurements; error bars represent the standard deviation of triplicate measurements.

Antigen Stability

Retention of the ID93 antigen in the control and trehalose-trileucine powders over the duration of the stability study is shown in Figure 8. Compared to the content at timepoint 0, only 45% of the antigen was still present in the control formulation stored at 5 °C for twelve months, and ID93 had completely degraded in the control samples stored at 25 and 40 °C. The trehalose-trileucine samples stored at -20 °C did not undergo significant loss in ID93 content and the samples held at 5 °C showed 88% ID93 retention. Characterization of the samples held at 25, 40, and 50 °C demonstrated an average of 55% loss. These

trehalose-trileucine samples show complex degradation behavior wherein samples held at temperatures above refrigeration appear to undergo an initial period of loss before stabilizing. The ID93 content of the samples stored at 40 and 50 °C do not change significantly from the three-month timepoint onward, and the ID93 concentration of trehalose-trileucine samples stored at 25 °C does not change significantly from the seven-month timepoint onward.

These powders do not meet the preset stability criteria of less than 20% loss of ID93 after at least three months of storage at 40 °C or one year of storage at 25 °C. However, the control and trehalose-trileucine spray-dried formulations still demonstrate improved thermostability of ID93 as compared to the liquid formulation. A stability study on the liquid ID93+GLA-SE presentation found that ID93 could not be detected after only one month of storage at 37 °C [15]. By contrast, 38% and 50% of ID93 were retained in the control and trehalose-trileucine samples held at 40 °C for three months, respectively, and approximately 45% of the ID93 antigen was still present in the trehalose-trileucine powder after twelve months of storage even at the highest temperature of 50 °C, indicating excellent stability of the protein fraction, which did not degrade in the initial period of loss. This apparent difference in long term stability of ID93 at high temperatures as compared to the GLA component suggests that the antigen and adjuvant systems experience different destabilization mechanisms. The mechanism of ID93 degradation in response to thermal stress is possibly due to conformational changes [45] in a fraction of the protein present. Improved long-term retention of the ID93 antigen in the trehalose-trileucine formulation as compared to the control formulation suggests that addition of trileucine provided a protective effect. Formation of an outer particle shell and the subsequent encapsulation of the antigen away from the particle surface may lead to further improved long-term stability, as surface mobility is much higher than bulk mobility [46] and reduction of mobility during storage is necessary to preserve protein conformation [47]. However, reduction of the antigen in relation to the agonist at room temperature storage may not lead to significant reduction in induced immune response. Penn-Nicholson et al. [48] assessed three different doses of ID93/GLA in a Phase I clinical trial – 10 ug ID93/2 ug GLA; 2 ug ID93/2 ug GLA; and

10 ug ID93/5 ug GLA. Their results found that increasing the dose did not lead to increased CD4+ T-cell or antibody responses at the tested timepoints.

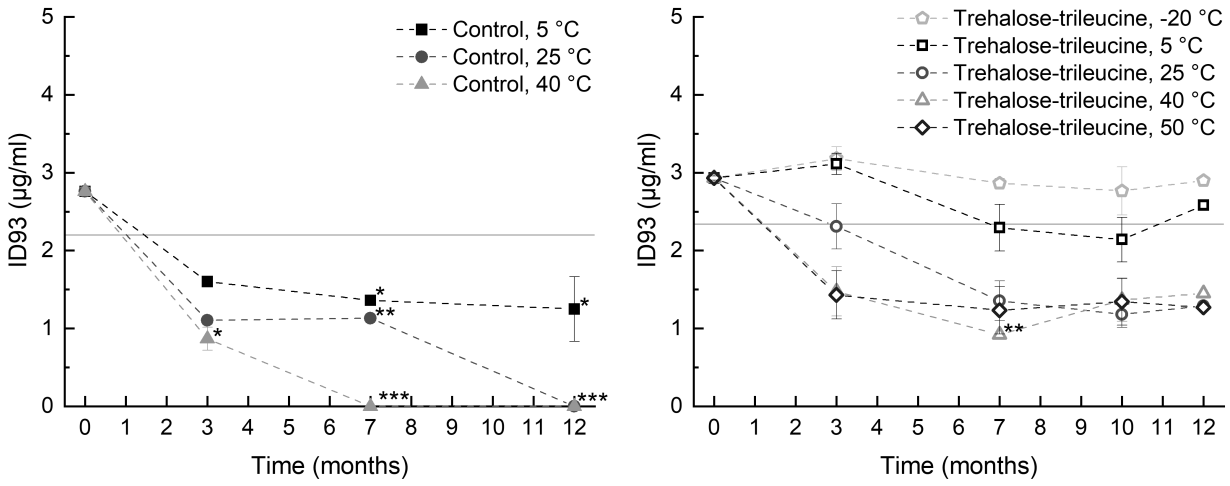


Figure 8 ID93 content for the spray dried formulations over twelve months of storage at various temperatures. Marker color and shape indicate the temperature arm (light blue pentagons - -20 °C, black squares – 5 °C, red circles – 25 °C, green triangles – 40 °C, blue diamonds – 50 °C). Closed symbols represent the control formulation (left) and open symbols represent the trehalose-trileucine formulation (right). Preset stability criteria were ≤20% loss in ID93 content after at least three months of storage at 40 °C, as represented by the solid line. The initial measurement was multiplied by a factor of three to allow for direct comparison of the remaining points, where characterization was conducted by reconstituting the powder to 3× the concentration of the feedstock. Results shown are the average of triplicate measurements; error bars represent the standard deviation of triplicate measurements, with the exception of the trehalose-trileucine twelve-month timepoint; only one measurement could be taken for these samples. Timepoints marked with asterisks indicate that some samples for the given timepoint were below the limit of quantitation. Number of asterisks signifies the number of samples below the limit of quantitation for the given timepoint.

Conclusion

Global vaccine distribution is greatly facilitated when storage and transportation are not limited to a specific temperature range. One method of developing thermostable vaccine presentations is through desiccation into a dry powder product, which can also be suitable for respiratory delivery. The development of alternative routes of administration may further improve the utility of the vaccine. The present work investigated a spray-dried version of a TB vaccine whose dry dosage form was designed to be thermostable and suitable for pulmonary administration. To the best of our knowledge, these are the first published results on an inhalable vaccine presentation with improved thermostability that uses a

trileucine-trehalose excipient system, as well as the first inhalable presentation of the GLA-SE nanoemulsion adjuvant with demonstrated thermostability.

This system demonstrated excellent physical dry powder stability, with no significant decrease in *in vitro* lung dose after long term storage at temperatures as high as 50 °C. The use of trileucine in the formulation protected against temperature-dependent loss in aerosol performance. Additionally, the emulsion-based adjuvant system was maintained for one year at temperatures up to 25 °C. The addition of trileucine also improved antigen stability, with approximately 45% of the antigen retained after twelve months of storage at 50 °C, whereas the antigen could not be detected in the trileucine-free formulation after seven months of storage at 40 °C or in the liquid version after one month of storage at 37 °C.

These results suggest that the use of trileucine as a dispersibility enhancing agent in combination with trehalose as a stabilizing excipient is a promising system for developing inhalable and thermostable nanoemulsion protein-based pharmaceuticals in dry dosage form. Furthermore, the addition of the surface-active tripeptide trileucine has the potential to improve long-term stability as compared to a single-excipient system. Improved thermostability with preserved aerosol performance is expected to expand the ability to conduct global vaccination campaigns. Furthermore, maintenance of global vaccine stockpiles over long storage times may benefit from the use of such dry dosage forms. While these results are promising, *in vivo* studies are required to assess preservation of potency of the formulation after storage above refrigerated temperatures.

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