Microparticle Encapsulation of Subunit Tuberculosis Vaccine Candidate containing Nanoemulsion Adjuvants via Spray Drying

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Spray drying is a technique that can stabilize biopharmaceuticals, such as vaccines, and allow for dry particles to be engineered for specific properties and applications. Compared to liquid pharmaceutical products, dry powder has the potential to reduce costs associated with refrigerated storage and transportation. In this study, spray drying was investigated for processing an adjuvanted tuberculosis subunit vaccine, formulated as an oil-in-water nanoemulsion, into a dry powder composed of microparticles. Applying *in-silico* approaches to the development of formulation and processing conditions, successful encapsulation of the adjuvanted vaccine within amorphous microparticles was achieved in only one iteration, with high retention (>90%) and integrity preservation of both the antigen and adjuvant system. Moisture controlled stability studies on the powder were conducted over one year at four different temperatures up to 40°C. Results showed that the powder was physically stable for all temperatures after one year. Physicochemical analysis after reconstitution showed nanoemulsion droplet size and component concentrations were maintained for all samples. The antigen remained present at all temperatures. Degradation of a TLR4 agonist component of the adjuvant at increased temperature storage was similar to that of a lyophilized comparator formulation. These results demonstrate the feasibility of a novel method of adjuvanted vaccine microencapsulation, with the possibility for stability improvement through further formulation development.

Key-words: nano-encapsulation, physical stability, gel microparticles, vaccine spray drying, adjuvant emulsion

Introduction

An important aspect of disease control is intervention by inducing widespread protective immunity through vaccination. Liquid pharmaceutical formulations, such as vaccines, usually must be kept refrigerated during transportation and storage in order to maintain their efficacy. Widespread global immunization is limited because vaccines often require refrigeration to remain effective [1]. Exposure to higher temperatures can lead to loss of efficacy of the active pharmaceutical ingredient and the possible formation of unsafe byproducts. Eliminating the refrigeration requirement would facilitate greater global distribution through cost reduction of storage and transportation. This would be especially beneficial towards interventions for diseases that are prevalent in population-dense locations that lack the required infrastructure to maintain refrigeration. Thermostability can be improved by converting a liquid product into a dry form that can be rehydrated for administration as needed. Desiccation processing methods, such as spray drying, have been widely used in the food processing, chemical, and pharmaceutical industries to preserve temperature-sensitive components [2]. Spray drying produces a dry powder made up of microparticles through the drying of atomized droplets via a drying gas. Once the solvent has evaporated, the dried particles are separated from the drying gas, e.g. via a cyclone.

Thermostability of vaccines can be improved by stabilization via spray drying. Experimental dry powder vaccines have been developed for the intervention of measles [3], influenza [4, 5, 6, 7], anthrax [8], herpes [9], whooping cough [10], and tuberculosis [11, 12]. The immunogenicity of spray dried vaccines can be maintained provided an appropriate stabilizing system and drying conditions are used. Successfully stabilized dry powder vaccines have been reported to elicit similar immunogenicity profiles as their liquid counterparts after reconstitution when administered in animal models [3, 4, 7, 10]. Ideally, a simple formulation is used to improve stability, however, multiple stabilizers may be required for biologics which are difficult to stabilize [13].

A disease whose eradication would be greatly advanced with the existence of a thermostable vaccine is tuberculosis (TB). The World Health Organization has noted that TB is the leading cause of death from a single infectious agent, having caused an estimated 1.5 million deaths in 2018 [14]. Approximately 1 in 4 people globally is infected with latent TB, wherein the immune response has not eliminated the pathogen and instead has contained it within granulomas [15]. Ten percent of those with latent TB are expected to develop active TB within their lifetime [14, 16]. There is currently no vaccine that is fully effective in preventing either TB incidence or latent TB becoming active in adults [14]. Due to these significant drawbacks to the current intervention strategy, an alternative TB vaccine must be developed. The WHO has explicitly noted that new TB vaccines must improve upon the current BCG vaccine with demonstrated efficacy in adolescent and adult subjects with and without latent tuberculosis, and must be safe for all subjects, including those with suppressed immune systems [16]. Subunit vaccines typically have better safety profiles than live attenuated vaccines; however, they often do not elicit strong immune responses on their own. Thus, if administered via parenteral injection, an adjuvant may be required to stimulate a robust immune response [17].

A vaccine candidate under development by the Infectious Disease Research Institute, ID93+GLA-SE, is a subunit TB vaccine based on a recombinant protein, ID93, and a nanoemulsion adjuvant system, GLA-SE. ID93 is composed of four *Mycobacterium tuberculosis* genes, Rv3619, Rv1813, Rv3620, and Rv2608, all associated with virulence or latency. These antigens were chosen on the basis of a comprehensive study identifying which antigens stimulate a strong cell mediated immune response [18]. The adjuvant system is formulated as a nanoemulsion and is comprised of glucopyranosyl lipid A (GLA), a synthetic Toll-like receptor 4 agonist, and a squalene oil-in-water stable emulsion (SE). Development and characterization of the GLA-SE system is discussed elsewhere [19]. The ID93+GLA-SE vaccine candidate has shown

promising preclinical and clinical results. Preclinical trials in mice, guinea pigs and nonhuman primates showed that administration of ID93+GLA-SE induced antigen-specific T-cell immune responses and significantly reduced viable bacteria in the lung after challenge with both TB and a multi-drug resistant strain of TB [20], and clinical testing has confirmed the benefit of GLA-SE in enhancing antigen-specific immune responses [21]. The current adjuvanted ID93 vaccine candidate has been administered in clinical studies in a two-vial system consisting of rehydrated lyophilized ID93 and the liquid adjuvant, which are mixed prior to administration [22]. The two-vial presentation is designed for short-term stability to support point-of-care mixing and immunization. However, a single vial presentation with long-term thermostability would require a different approach since a liquid ID93+GLA-SE sample experienced greater than 50% decrease in GLA concentration when stored at 25 °C for three months [23].

To improve thermal stability, a liquid product may be converted into a dry version through processing methods such as lyophilization or spray drying. Lyophilization, also known as freeze-drying, removes water by freezing the product and then removing the ice through sublimation, leaving behind a dry 'cake'. A previous study investigated lyophilization of the ID93+GLA-SE vaccine in a single vial as a method of improving thermal stability [23]. Reconstitution of the lyophilized vaccine showed that physicochemical characteristics, such as ID93 and GLA integrity, were maintained. Further formulation development led to several lyophilized vaccine candidates [22], which were stable after three months storage at 37 °C and still elicited immune response in mice after storage at 50 °C for three months. The best-performing candidate utilized a trehalose-Tris excipient system to stabilize the vaccine [22] and is currently undergoing a Phase I clinical trial [24]. Success in lyophilization of the ID93+GLA-SE vaccine led to consideration of spray drying as an alternative technique to produce a dry vaccine product. Spray drying is more cost-effective than lyophilization as it is a faster, more scalable process with reduced operation and installation costs [25, 26]. Nevertheless, there are technical difficulties associated with spray drying the ID93+GLA-SE formulation. One challenge is due to the structure of the ID93+GLA-SE vaccine as a nanoemulsion with a phospholipid emulsifier. Its emulsion droplets must be encapsulated with high efficiency within particles during the spray drying. Another challenge is to stabilize both the ID93 and GLA-SE active components of the vaccine within the dry powder for long-term room temperature stability. Both challenges must be overcome to produce a successful spray dried vaccine powder.

Stabilization requires an appropriate stabilizer and optimal spray drying conditions to ensure that desiccation does not lead to losses or changes in vaccine component structure. There has been successful stabilization via spray drying of vaccines formulated as dispersions. Enveloped viral vector vaccines [6], an outer membrane vesicle vaccine [10], and a subunit vaccine consisting of an antigen and liposomal adjuvant system [12] have all been stabilized through spray drying with the disaccharide trehalose as an excipient. Trehalose acted as a glass stabilizer to stabilize both the protein and lipid components of the vaccines in order to create a successful dry powder product. Trehalose is a commonly used glass stabilizer due to its high glass transition temperature, high solubility [27], low toxicity [28], and ability to act as both a protein [29] and liposome [30] stabilizer during spray drying. The glass transition temperature (T_g) can be defined as the temperature at which the brittle properties of an amorphous material undergo a reversible change to more rubber-like ones. Glass stabilizers are hypothesized to physically stabilize proteins during drying and storage through two main mechanisms: water replacement and vitrification [2, 29, 31, 32]. Water replacement theory states that biologic structure is maintained in water through hydrogen bonding. During drying, the hydrogen bonds between the water and the biologic are replaced by hydrogen bonds with the glass stabilizer. To inhibit protein denaturing, the glass stabilizer must be capable of forming many hydrogen bonds and maintain an amorphous state in order to match the biologic structure [2, 29]. Vitrification theory states that the biologic is rendered immobile within a glassy matrix [2, 29]. Protein stability is largely dependent on the molecular mobility of the stabilizer at temperatures near the glass

transition temperature [2, 29]. It has been reported that glass stabilizers also stabilize the phospholipid layer of liposomes through both the water replacement and vitrification mechanisms [30]. The hydrogen bond replacement occurs between the stabilizer's hydroxyl groups and the phosphate groups of the liposomes' lipid head groups [30]. As with the stabilization of proteins, it is critical that the stabilizer be amorphous such that the hydroxyl groups are able to orient themselves into the required bond with the lipid head group without changing the liposome structure. Trehalose is reported to be an effective stabilizer for phospholipid membranes as it forms stronger hydrogen bonds with phosphate groups than other stabilizers, such as mannitol [6]. Vitrification stabilizes the liposomes by rendering them immobile and by creating a barrier between adjacent liposomes, thus inhibiting liposome fusion [30].

In this paper, we stabilize the tuberculosis subunit vaccine ID93+GLA-SE via spray drying using trehalose as a stabilizer. A successful spray dried ID93+GLA-SE product must stabilize the ID93 protein and GLA long-term as well as encapsulate the GLA-SE nanoemulsion droplets with high efficiency. An *in-silico* engineering approach was used to calculate spray drying process parameters based on material properties to minimize vaccine component losses. The resulting powder was placed on stability study at various temperatures for a year and analyzed to assess both the physical stability of the powder and the chemical stability of the vaccine components.

Materials and Methods

Materials

Trehalose dihydrate (CAS 6138-23-4; Fisher Scientific Ottawa, ON, Canada) with a purity of 98% was used as the excipient for spray drying. 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) were used as a buffer system. Formulations were made with HPLC grade water (CAS 7732-18-5; Fisher Scientific Ottawa, ON, Canada).

The ID93 antigen and GLA-SE adjuvant components of the vaccine were produced separately. The construction, expression, and purification of the ID93 recombinant fusion protein has been described previously [20]. Briefly, ID93 was expressed in *E. coli*, purified under denaturing conditions by chromatography, and analyzed by SDS-PAGE. ID93 protein was stored in aliquots of 1.2 mg/mL at -80 °C prior to use. GLA-SE was formulated with squalene droplets and dimyristoyl-sn-glycero-3-phosphocholine (DMPC) as an emulsifier. Manufacture of GLA-SE generally followed the same procedure as described in Orr et al. [23], except that the oil phase in the present work included the addition of α-tocopherol (0.05% w/v), and that glycerol and buffer were omitted from the aqueous phase. GLA-SE nanoemulsions with a squalene concentration of 10% v/v and GLA concentration of 50 µg/mL were stored in a refrigerator prior to use. The initial emulsion droplet size was determined to be 90.6 nm in diameter, with a polydispersity index of 0.06.

Two formulations were prepared for spray drying; an adjuvant system-only formulation and a formulation containing both the antigen and adjuvant system. The former will hereafter be referred to as spray dried trehalose+GLA-SE (SD-TG) and the latter as spray dried trehalose+GLA-SE+ID93 (SD-TGI). The adjuvant system-only formulation was investigated for applications where different antigens can be added to the dose after reconstitution of the adjuvant system. The feedstock was prepared by first dissolving trehalose to 200 mg/mL and Tris to 40 mM in HPLC grade water. This solution was then pH adjusted to a pH of 7.5±0.1 using hydrochloric acid. A separate solution of GLA-SE or GLA-SE+ID93 was diluted with water to twice the working concentration (4% [v/v] squalene, 20 µg/mL GLA; 8 µg/mL ID93). The trehalose-Tris solution and the nanoemulsion were then mixed in a 1:1 ratio such that the final concentrations, summarized in Table 1, were achieved. A lyophilized vaccine candidate of ID93+GLA-SE

was established in prior stabilization work and used in this study for comparison [22]. It utilized an excipient combination of 10% trehalose (w/v) and 20 mM Tris buffered to a pH of 7.5.

Component	Feedstock Concentration	
	SD-TG	SD-TGI
Trehalose	100 mg/mL	100 mg/mL
Tris (buffer)	2.42 mg/mL (20 mM)	2.42 mg/mL (20 mM)
Squalene	17.2 mg/mL	17.2 mg/mL
GLA	10 µg/mL	10 µg/mL
ID93	-	4 µg/mL

Table 1 Final concentration of the main components in the adjuvant-only SD-TG and antigen containing SD-TGI feedstock formulations

Theory

Particle Design

Use of a glass stabilizer is required to stabilize both the ID93 protein and the GLA-SE nanoemulsion droplets. Trehalose was chosen as the glass stabilizing excipient for this study due to its relatively high T_g and demonstrated ability to stabilize spray dried proteins and lipid based dispersions. A relatively high solute concentration (100 mg/mL) of the excipient was chosen. Increasing the solids content increases the generated particle size. Larger particles have a lower surface-to-volume ratio, which increases the probability for nanoemulsion droplets to be embedded within the particle. This is because a lower surface area will decrease availability for the droplets to accumulate on the surface. Increased encapsulating agent concentration supports an earlier shell formation [33], thus trapping the nanoemulsion droplets within the interior of the forming particle. Additionally, Carrigy et al. [13] previously demonstrated that spray drying 100 mg/mL trehalose concentration provided better stabilization to phages than 20 mg/mL trehalose.

An amorphous solid phase is desired in order to promote stabilization of the antigen, agonist, and phospholipid membrane of the adjuvant system. Studies on the lyophilization of the GLA-SE vaccine have shown that crystalline lyophilisate increased GLA-SE emulsion droplet size after reconstitution [22]. Amorphous trehalose microparticles can be designed to be stable for storage at a specific temperature by ensuring low molecular mobility. With a low molecular mobility, the amorphous structure will maintain its shape as a brittle glass. The molecular mobility of these materials is a function of the storage temperature [34]. Storage at or below the Kauzmann temperature—that is, the temperature at which the molecular mobility is insignificant—maximizes physical stability. The Kauzmann temperature is approximately 50 K below the glass transition temperature, T_g, for pharmaceutical powders [35]. The success criteria for the lyophilized formulation was long-term stability at 37 °C. Hence, the T_g of the spray dried powder must be $\geq$ 88 °C. As described by the Gordon-Taylor equation [36], T_g for sugar-water mixtures can be determined based on their mass fractions and an empirical parameter *k*. Chen et al. [37] modelled the trehalose-water glass transition curve as a function of powder moisture content by fitting literature data to the Gordon-Taylor equation. For low water content systems, a *k* of 5.9 was determined based on a trehalose T_g of 387.1 K [37, 38] and a water T_g of ~138 K [37]. For a spray dried powder with a T_g greater than or equal to 88 °C, the moisture content of the spray dried trehalose powder must not exceed 2-3%. The moisture sorption isotherm of trehalose indicates that subjecting trehalose powder to 10% relative humidity (RH) leads to an approximate 2-3% moisture content [39, 40]. Therefore, in order to obtain a T_g greater than or equal to 88 °C, the relative humidity at the collection point of the spray dryer must be less than 10%. Additionally, some level of moisture content may be necessary for protein stability as over-drying may lead to increased degradation [41].

Another critical processing parameter is the spray dryer outlet temperature. This must be much lower than the T_g to prevent powder adhesion to the dryer surfaces and crystallization of the resulting powder. Furthermore, a relatively low inlet drying gas temperature of 65 °C was chosen in order to prevent possible evaporation of the nanoemulsion droplets during the spray drying process and the deactivation of GLA and ID93. Experiments involving twin fluid atomizers to spray dry oil-in-water emulsions reported that a high air-liquid-ratio will decrease the emulsion size distribution [42]. An air-liquid ratio of 8 was selected in order to minimize any changes to the emulsion droplet structure during the atomization step of spray drying.

Particle Formation and Structure

The encapsulation of very small particles or droplets within a protective material via spray drying has been summarized elsewhere [43]. This process, known as microencapsulation or nanoencapsulation via spray drying, has been studied for the main uses of protecting the encapsulated product from the surrounding environment, encapsulating a toxic product, or controlling the rate of release of the encapsulated product. Spray drying has been shown to successfully encapsulate food ingredients [44, 45, 46] as well as lipid-based drug delivery systems [47] as dry emulsions. Briefly, oil-in-water emulsions can be spray dried to produce a powder containing the encapsulated droplets. The properties of these particles are dependent on the processing parameters, as well as on the properties of both the encapsulated ingredient and the encapsulating agent. The resulting particles can be spherical or irregular, and the droplets can be encapsulated as singular droplets or as multiple droplets embedded in a continuous matrix of the encapsulating agent [43].

Wang et al. [33] investigated the particle surface formation mechanics of algae oil containing emulsion droplets drying on a glass filament. The study summarized the drying process of emulsions into three stages. The first stage consisted of constant-rate drying before initial particle shell formation where the emulsion droplets are evenly distributed through the aqueous phase. The second phase consisted of the initial shell formation where a soft outer layer forms due to saturation of the components on the droplet surface. The final stage was defined as the thickening of the fully formed shell. These stages summarized Wang et al.'s [33] findings on the general formation mechanics of spray dried emulsions; however, the distribution of components and the particle morphology are also dependent on the material properties and processing conditions of the spray dryer. Appropriate processing conditions, such as drying temperature, is contingent on the properties of the given formulation. A sufficiently high temperature must be used to form a thin shell around the drying droplets early in the drying process in order to entrap the emulsion droplets and obtain a high encapsulation efficiency. However, the temperature must also be low enough to prevent possible evaporation of emulsion droplets or inactivation of temperature-sensitive components [48]. Encapsulation efficiency, defined here as the mass of the dispersed phase retained within the powder after spray drying, has been reported to increase with the concentration of the encapsulating agent [49, 50]. An encapsulated ingredient to encapsulating agent ratio of 1:4 is often used in the spray drying of emulsions in order to obtain a high encapsulation efficiency [51, 52, 53]. These findings can be applied to the encapsulation of vaccines formulated as dispersions via spray drying.

Based on Wang et al.'s findings, a high encapsulation efficiency is obtained when the first stage is short enough for the initial shell formation to occur quickly. An early shell formation will prevent the emulsion droplets from reaching the surface of the drying droplet and thus be lost during the evaporative process. Droplet drying time, τ_D, can be approximated using Eq. 1, where d_0 is the initial droplet diameter and κ is the evaporation rate [54].

$$\tau_D = \frac{d_0^2}{\kappa} \quad \mathbf{1}$$

In order to predict the shell formation process, a particle formation model that describes the radial distribution of components within the atomized droplets during the drying process was used, in which a dimensionless Peclet number, *Pe*, was defined as Eq. 2, where D_i refers to the diffusion coefficient of component i and κ stands for the evaporation rate of the solvent [55]. Therefore, *Pe* is a parameter affected by both material properties of the components and processing parameters and can be used to predict final particle morphology [55].

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

For the nanoemulsion system studied in this case, the nanoemulsion droplets can be treated as nanoparticles, assuming they remain stable in an aqueous dispersion. The diffusion coefficient of nanoparticles can be approximated through the Stokes-Einstein equation, given in Eq. 3, where D is the diffusion coefficient for the emulsion droplet, k_B is Boltzmann's constant, T is temperature, μ is viscosity, and d_h is hydrodynamic diameter. From this equation it is apparent that larger nanoparticles and nanodroplets will have a low diffusion coefficient, typically much lower than that of dissolved molecules, leading to a much larger *Pe*.

$$D = \frac{k_B T}{3\pi\mu d_h} \quad \textbf{3}$$

In general, for a component with low *Pe*, material migration of the given component inside of an atomized droplet is quick relative to the speed of the receding droplet surface [55]. Hence, solutes with a low *Pe* distribute evenly in the atomized droplet during evaporation, typically forming spherical, solid particles if they do not crystallize. For components with very large *Pe*, such as nanoemulsion droplets, the diffusion is slow compared to the evaporation rate, rendering the component effectively immobile, and thus the component will accumulate near the surface as the atomized droplet dries. Using on the particle formation model, it is possible to manipulate the distribution of the main components trehalose and the nanoemulsion droplets within the spray dried powder.

The *Pe* for trehalose was designed to be approximately 1 in order to achieve a successfully spray dried powder. The trehalose must be evenly distributed in order to increase coverage of the nanoemulsion droplets to facilitate stabilization during the drying process. The diffusion coefficient for trehalose in water at 100 mg/ml is 5×10^{-10} m^2/s [55]. For a *Pe*=1 for trehalose, based on Eq. 2, the evaporation rate must be 4×10^{-9} m^2/s. The GLA-SE droplet size has been measured as approximately 92 nm [22]. The diffusion coefficient of GLA-SE nanoemulsion droplets with a diameter of 92 nm has been measured via dynamic light scattering as 5.3×10^{-12} m^2/s (data not shown). For a theoretical evaporation rate of 4×10^{-9} m^2/s, the *Pe* of the nanoemulsion droplets is ~ 95. The nanoemulsion droplets are expected to accumulate near the surface of the atomized droplet. At a very high *Pe*, the nanoemulsion droplets are expected to accumulate and possibly coalesce near the surface due to the force from the receding particle surface and convective flux of water towards the surface. Based on similar studies, trehalose is expected to form solid, spherical amorphous particles when spray dried [55, 56].

Methods

Spray Drying

Spray drying was conducted using a custom research spray dryer [57] with a twin fluid atomizer operated at an air-liquid ratio of 8, which corresponds to an atomized droplet diameter of approximately 9 µm [35]. The outlet humidity target was less than 10% RH to achieve a T_g greater than 88 °C. A spray dryer process mass and energy balance model [58] was used to determine processing conditions to achieve an outlet temperature of 36 °C and outlet RH of 7%. The feedstock was supplied to the atomizer at a rate of 0.6 mL/min using a peristaltic pump (Model 77200-60; Cole-Parmer, Montreal, QC, Canada). The atomized droplets dried in an air flow rate of 200 SLPM, where the drying gas temperature at the inlet was 65 °C. Dry particles were separated from the air by a cyclone, and powder was collected in glass jars. These jars were sealed and stored in an environmental chamber set to 25 °C and 7% RH until powder packaging.

Stability Study

The design targets for the spray dried formulation required that the vaccine concentration and integrity be retained post-spray drying and that the powder maintains stability for at least 3 months at 40 °C. Stability of the spray-dried powder was assessed using the following acceptance criteria: no particle fusing or crystallization, emulsion droplet size change less than or equal to 50%, polydispersity index less than 0.2, squalene content loss less than or equal to 20%, GLA content loss less than or equal to 20%, and ID93 is present. Similar criteria were used previously for the assessment of lyophilized candidates [22].

The powders were stored at -20 °C, 2-8 °C, 25 °C, and 40 °C. The stability arm was chosen for 40 °C rather than 37 °C as recommended by the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines for pharmaceutical stability studies [59]. Similarly, time points were chosen based on the ICH guidelines. Characterization tests for chemical stability of the reconstituted powder were conducted at the beginning of the study, and then at multiple time points after storage at two weeks, one month, two months, three months, six months, nine months, and 13.5 months. Similarly, characterization tests for physical stability of the dry powder were conducted at the beginning of the study, and after one month, three months, and twelve months of storage. The final chemical characterization timepoint was originally planned for the same date; however, due to equipment repairs the chemical and colloidal characterization for the 12-month time point was delayed and characterization was completed 13.5 months after the initial timepoint.

Dry Powder Packaging for Stability Study

Spray dried powder will equilibrate to the new water activity when placed in an environment with a different RH than its initial value [60]. Desiccants, such as silica gel, are hygroscopic agents that can be used to modify or buffer the RH within a closed package. Chemical degradation due to a change in RH has been shown to be mitigated by the inclusion of desiccant within the package [60]. Desiccants can be equilibrated to the desired moisture content when placed in an environmental chamber at the desired RH value [2]. The time required to equilibrate is influenced by several factors, such as the difference in RH value and temperature and the permeability of the packaging seals.

A packaging process involving the use of desiccants was utilized to prevent moisture uptake in the powder during temperature-controlled storage of the stability study. Packaging preparation involved placing silica gel pouches into an environmental chamber set to 25 °C and 7% RH for 3-4 days in order to equilibrate the desiccant to the outlet RH of the spray dryer. An equal number of silica gel pouches were equilibrated to 0% RH in a regulated glove box over the same time period. The packaging process took place within a custom glovebox set to 0% RH. The powder was aliquoted into low bind snap cap tubes (Product Z768820; Sigma Aldrich, Oakville, ON, Canada). The powder-containing tubes were then placed into an aluminum

bag, along with a 7% RH desiccant pouch to prevent powder moisture changes. However, moisture transfer rates through the seals increase with a larger difference between the environments. To counteract this, the aluminum bag was then double heat-sealed and placed into another aluminum bag, along with a 0% RH desiccant pouch to minimize the moisture difference between the innermost package and the external environment. This external bag was also double heat-sealed and labelled. A simplified schematic summarizing the configuration is shown in Figure 1.

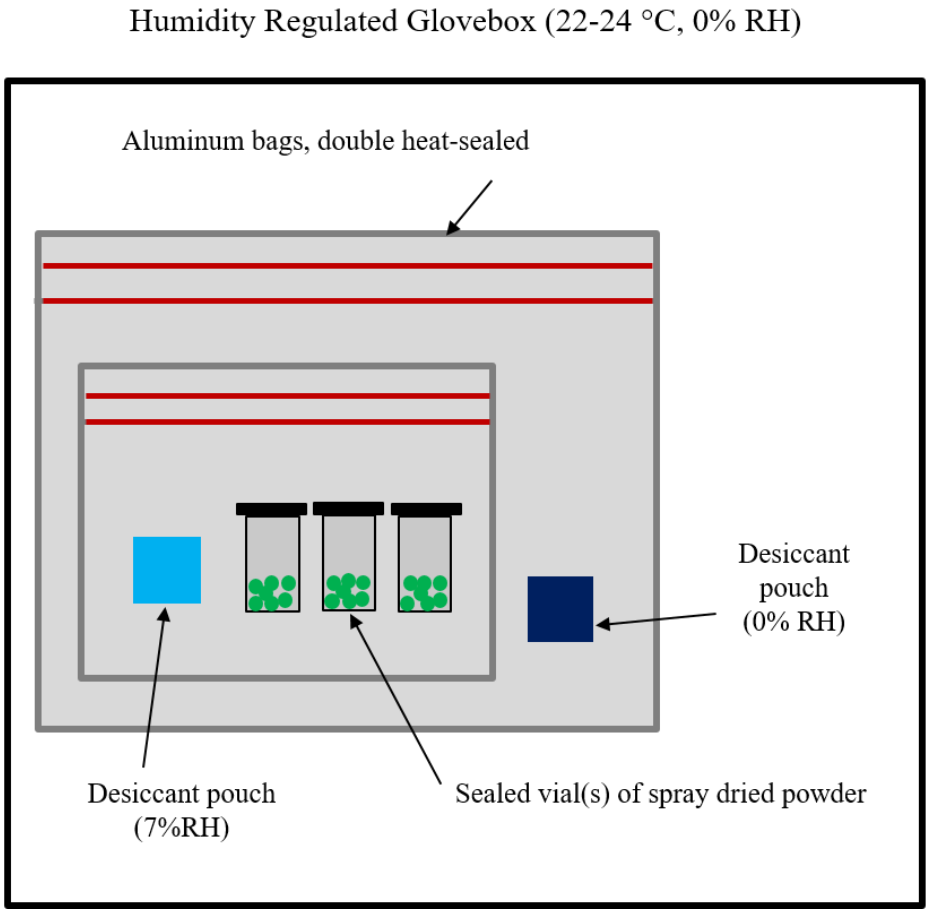

Figure 1 Simplified schematic for packaging protocol to ensure powder moisture content control during long-term powder storage

Dry Powder Characterization

Field Emission Scanning Electron Microscopy (Zeiss Sigma FE-SEM; Carl Zeiss, Oberkochen, Germany) was used to determine whether powder morphology changed over time. Powder samples were dispersed directly onto aluminum SEM stubs (Product 16111; Ted Pella, Inc.; Redding, CA, USA) and pressed against the surface of the stub to intentionally produce cracked particles for imaging. To prevent damage to the electron microscope, these samples were placed in a desiccator connected to an in-house vacuum system for 2-4 days to remove the exposed nanoemulsion droplets. Subsequently, the samples were sputtered with a coating of 80% gold and 20% palladium (Leica ACE600 Carbon/Metal Coater; Concord, ON, Canada) to a thickness of 10-15 nm. Images ranging from magnifications of 500 to 20000× were taken at a working distance of 5.3-6.3 mm using an accelerating voltage of 3-4 kV.

The solid phase of the powder was monitored by Raman spectroscopy to assess whether or not the spray dried powder remained amorphous during storage. A custom dispersive Raman spectroscopy system was utilized for this purpose. The system included a 671 nm diode-pumped solid-state laser (Ventus Solo MPC6000; Laser Quantum, Stockport, UK). A detailed description of a similar apparatus has been published elsewhere. [61]. Samples were placed in a closed sample chamber under nitrogen to prevent moisture exposure. All spectra were measured at a temperature of 22.0-23.0 °C and at less than 5% RH. In addition to measured SD-TG and SD-TGI powder samples, Raman spectra analysis was also conducted on neat amorphous and crystalline trehalose powder samples as references. Similarly, reference spectra were also obtained for liquid samples of squalene and crystalline Tris powder samples. A deconvolution process was used to assess the contributions and solid phase of each component. The deconvolution process is described elsewhere [62].

Karl Fisher Calorimetry (Karl Fisher Coulometric Titrator Model C30; Mettler Toledo; Mississauga, ON, Canada) was used to measure the water content of powder samples by mass. Results were displayed as a percentage based on the measured sample mass. Oven temperature for the method was 110 °C. Moisture content of the powder was determined by measuring and averaging results of two vials of the same powder. Analysis of experiments to determine moisture content of a HYDRANAL water standard (Honeywell; Mexico City, Mexico) showed that the machine variance was 0.3%.

Reconstituted Powder Characterization

At each stability time point, approximately 108 mg of spray-dried powder was reconstituted with the appropriate volume of freshly dispensed MilliQ water to yield sample concentrations expected in the liquid drug product. The exact reconstitution volume was based off the exact mass of powder in each vial and an empirically determined expansion factor. Each test was performed in triplicate on separate vials of reconstituted powder for the first three months. Remaining timepoints were performed on single vials. Replicates of measurements of the feedstock were made from the same vial. The stability study involved storage of the spray-dried powders at several storage temperatures: -20, 2-8, 25 and 40 °C. For each temperature, at a given time point the powders were reconstituted and assessed for nanoemulsion droplet diameter, polydispersity index, squalene contentration, GLA concentration, ID93 presence, and pH. Unlike other chemical characterization tests, osmolality was only measured initially, after three months, and after 13.5 months for all storage temperatures.

A dynamic light scattering technique (Zetasizer APS; Malvern, UK) was used to measure the mean hydrodynamic diameter and polydispersity of the nanoemulsion droplets in the liquid formulations reconstituted from spray-dried powders. Details of the measurement process have been described elsewhere [63]. Liquid feedstock measurements were from one sample analyzed three times.

GLA content in the powder was quantified using a reversed-phase HPLC method. Separation of GLA from the sample was performed on an Agilent 1200 series HPLC (Agilent Technologies; Santa Clara, CA, USA) equipped with a silica-based, C18 reversed-phase column (Atlantis T3 Column; Waters; Elstree, UK) with a gradient from 50% mobile phase B to 90% mobile phase B over 18 minutes and a flowrate of 1.0 mL/min. Mobile phase A contained 75:15:10 (v/v/v) methanol:chloroform:water, 1% (v/v) acetic acid, and 20 mM ammonium acetate. Mobile phase B contained 50:50 (v/v) methanol:chloroform, 1% (v/v) acetic acid, and 20 mM ammonium acetate. Samples were diluted 10-fold in mobile phase B, and the injection volume was 100 µL. Column temperature was held constant at 30 °C. A charged aerosol detector (Corona CAD; ESA Biosciences; Chelmsford, MA, USA) was used for analyte detection. A standard curve was prepared from GLA in mobile phase B. The peak heights from the standards were fit with a second order polynomial per

the charged aerosol detector manufacturer's directions. Sample analyte concentrations were calculated by interpolation. Liquid feedstock measurements were done in two replicates, each analyzed once.

Squalene content in the powder was quantified using a reversed-phase HPLC method. Separation of squalene from the sample was done with the same equipment and the same mobile phases used for GLA as described above. Samples were diluted 100-fold in mobile phase B, and 10 µL of volume was injected with a gradient from 50% mobile phase B to 90% mobile phase B over 30 minutes and a flowrate of 1.0 mL/min. Column temperature was held constant at 30 °C. The peak area from the standards were fit with a second order polynomial per the charged aerosol detector manufacturer's directions. Sample analyte concentrations were calculated by interpolation. Liquid feedstock measurements were completed in two replicates, each analyzed once.

SDS-PAGE was used to detect the presence of the ID93 protein in the samples. Samples were reduced in LDS buffer (NP0007; Thermo Fisher Scientific, Waltham, MA, USA) spiked with β-Mercaptoethanol to a final concentration of 1.25% (v/v). Samples were heated at 90 °C for 15 minutes and then cooled to room temperature. Once cooled and centrifuged at 2000 rpm for 30 seconds, samples and molecular weight marker (LC5925; Thermo Fisher Scientific, Waltham, MA, USA) were run in 4-20% Tris-Glycine gels (XP04205BOX; Thermo Fisher Scientific, Waltham, MA, USA). Gels were silver stained (PROTSIL1-1KT; Sigma Aldrich, St. Louis, MO, USA) and imaged (AlphaImager EC; Protein Simple, San Jose, CA, USA). At each time point, two SDS-PAGE samples were prepared and analyzed per vial to confirm the presence of the ID93 protein.

Reconstituted powder pH was measured using a pH meter (Orion ROSS Ultra Semi-micro pH Electrode, Thermo Fisher Scientific, Waltham, MA, USA). Liquid feedstock measurements consisted of one sample. Measurements of reconstituted samples were performed in triplicate for the first three months; measurements were performed once for the remaining timepoints.

Osmolality of the reconstituted powder was measured using an osmometer (Model 2020; Advanced instruments, Norwood, MA, USA) to determine if the reconstituted powder was isotonic and suitable for injection. Liquid feedstock measurements consisted of one sample. Measurements of reconstituted samples were performed in triplicate for the first three months; measurements were performed once for the remaining timepoints.

Statistical Analysis

Mean results are reported, and the indicated error is the standard deviation of replicate measurements. Number of replicates for each method are indicated above. A two-tailed student's t-test was used for analysis, where statistically significant differences were reported for $p<0.05$.

Results

Powder Manufacturing

Process calculations predicted the nominal solids throughput in the spray dryer to be 75 mg/min. During powder manufacturing for the stability study, the actual rate to produce SD-TG and SD-TGI powders was 49 mg/min and 45 mg/min, corresponding to spray drying yields of 65% and 60%, respectively. Considering the relatively small spray drying batch size of 16.4 g and 17.3 g, produced over 5.5 hours and 6.5 hours, respectively, the yield was typical and well within the acceptable range for early development.

The measured properties of the formulations before and after spray drying (post-reconstitution) are shown in Table 2. The target values given are the same as for the lyophilized vaccine product. All measured vaccine properties were well within the target values. Average nanoemulsion droplet size was nearly the same as

the liquid vaccine product. Hydrodynamic diameter increased only slightly by 2-3% after spray drying. The polydispersity index of the nanoemulsion droplets did not change significantly (p>0.05) over the course of spray drying for either formulation.

Squalene content levels before and after spray drying are very similar, indicating a high encapsulation efficiency. The results show high retention (>90%) of the squalene component for both formulations. The high retention indicates that the evaporation losses during low temperature spray drying for this system are less than 10%. The GLA content did not change significantly (p>0.05) over the course of spray drying for either formulation, indicating that the GLA component, a synthetic lipid, was successfully stabilized by the trehalose. The retention of GLA content indicates that this component does not degrade over the course of spray drying, which was a concern, as GLA is sensitive to thermal stress [22]. Similarly, ID93 protein was shown to be present in both the liquid and reconstituted formulations. Likewise, the pH did not change significantly (p>0.05) over the course of spray drying for either formulation.

ID		Nanoemulsion Droplet Diameter (nm)	Polydispersity Index	Squalene Content (mg/mL)	GLA Content (µg/mL)	ID93	pH	Osmolality (mOsmol/kg)
Target		120 ± 40	<0.2	13.6 – 20.5	10 ± 2.5	Present	7.5 – 8.5	315 – 415
SD-TG	Feedstock Liquid	95.3 ± 0.9	0.05 ± 0.04	19.1 ± 0.4	9.2 ± 0.5	N/A	7.62	383
	Reconstituted Powder	96.9 ± 0.2	0.07 ± 0.02	18.8 ± 0.3	9.5 ± 0.4	N/A	7.57 ± 0.04	376 ± 8
SD-TGI	Feedstock Liquid	94.9 ± 0.8	0.06 ± 0.01	18.8 ± 0.3	9.3 ± 0.4	Present	7.56	389
	Reconstituted Powder	97.6 ± 1.1	0.09 ± 0.01	17.3 ± 0.6	9.0 ± 0.4	Present	7.55 ± 0.01	351 ± 8

Table 2 Chemical and colloidal properties before and after spray drying for adjuvant-only (SD-TG) and antigen containing (SD-TGI) formulations.

Physicochemical Stability

Particle Morphology

The SEM images for the spray-dried SD-TG and SD-TGI formulations are shown in Figure 2. Analysis of the SEM images indicates that spray drying the formulations produced a polydisperse powder sample with microparticles ranging in geometric diameter from ~1-20 µm. These images show that the spray dried microparticles were spherical with surfaces ranging from smooth to slightly dimpled. The spray dried powders containing the emulsion droplets were relatively flowable and easy to handle. The similarity of the microparticles from the SD-TG and SD-TGI formulations indicates that the presence of the protein ID93 does not affect the particle morphology. Microparticles of neat trehalose spray dried from a 100 mg/mL aqueous solution under the same processing conditions are also shown in Figure 2 for comparison. The trehalose particles have an outer particle morphology very similar to those of the SD-TG and SD-TGI formulation, that is, spherical particles with smooth or slightly dimpled surfaces and a similar geometric diameter. Spray dried bacteriophage encapsulated in trehalose [13, 64] and outer membrane vesicle pertussis vaccine spray dried with trehalose [10] produced particles with a similar exterior morphology. These particles, composed mainly of trehalose, were also spherical, had a similar geometric diameter range and exhibited a smooth or dimpled surface.

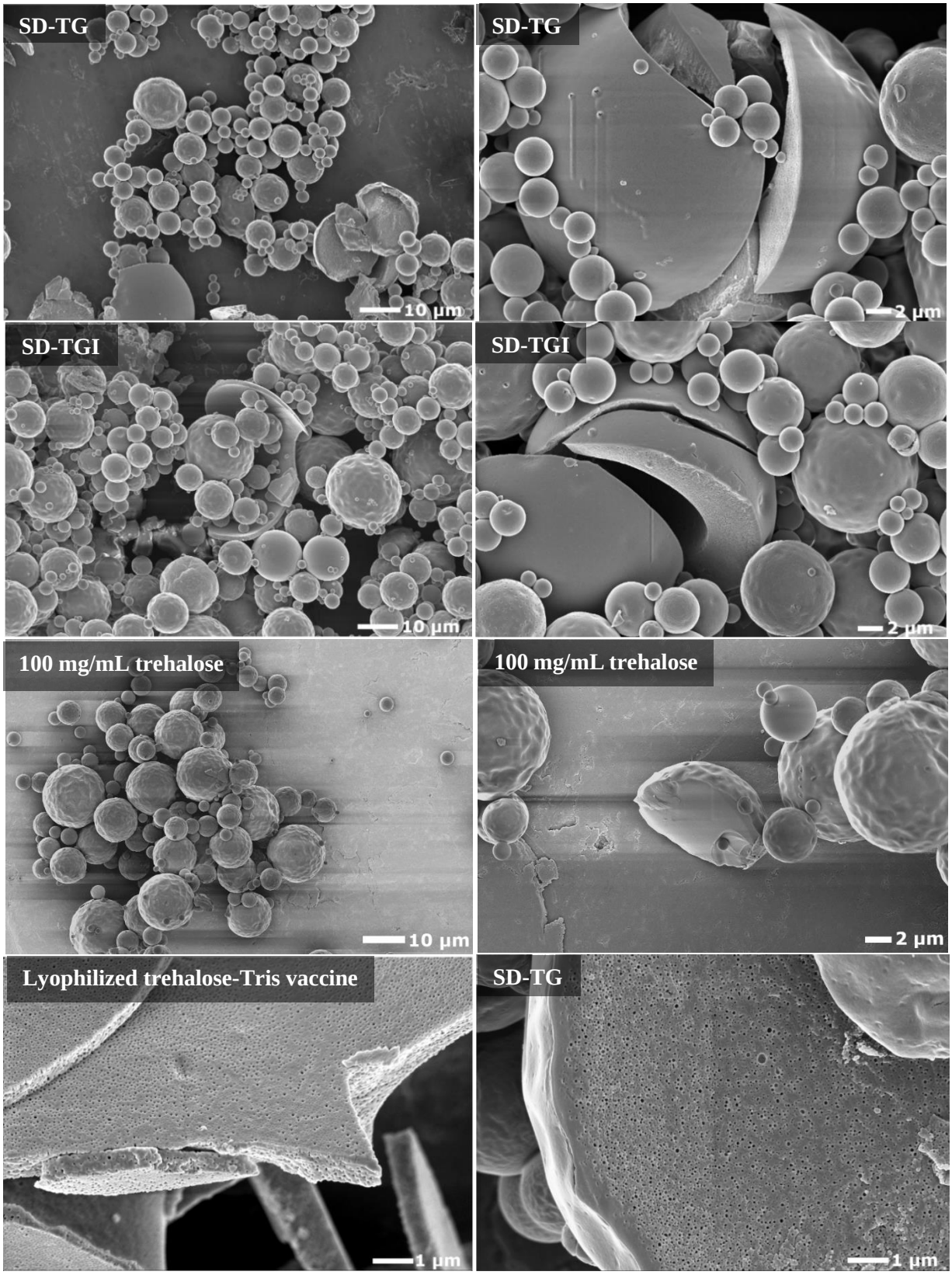

Figure 2 Comparison of morphology for the adjuvant-only SD-TG formulation, antigen-containing SD-TGI formulation, spray dried trehalose and the lyophlized vaccine. External morphology of all spray dried powders shows similar spherical,

polydisperse particles that are not fused. Interior structure of the lyophilized vaccine and the spray dried particles shows similar voids left by the encapsulated emulsion droplets. Scales are provided on the respective images.

SEM images of the cracked microparticles of SD-TG and SD-TGI all show that these particles can be hollow or solid, with a range of shell thicknesses within a given sample. The images show that the interior structure of this shell differs from shells formed with spray dried trehalose. Trehalose particles show a solid interior whereas the interior of the SD-TG and SD-TGI particles appears to be foam-like with numerous voids within the trehalose matrix. These voids observed in the SEM images of SD-TG and SD-TGI also appear in images of the lyophilized trehalose-Tris formulation (Figure 2). The geometric diameter of these voids appears to be approximately the same as that of the nanoemulsion droplet hydrodynamic diameter. It can be concluded that these voids are left behind by nanoemulsion droplets that evaporated during the imaging preparation process. Higher magnification images of the surface (not shown) show few of these voids on the surface of the particle.

Similar interior morphologies have been shown in SEM images particles generated by emulsion encapsulation via spray drying of isoeugenol in a glucose syrup matrix [65], spray drying of D-Limonene emulsion with different combinations of the excipients gum arabic, maltodextrin, and trehalose [66], and spray drying of fish oil in a whey protein isolate matrix [67]. The particles in the latter paper are especially like the particles shown in this study. Both studies generated intact thin-shelled particles with the emulsion droplets embedded within the particle wall. A central void was also found in the particles generated in a study on surface formation of drying emulsions [33].

There appears to be a slight gradient in nanoemulsion droplet distribution within the particle, with a higher concentration of droplets closer to the surface of the particle than to the center, as demonstrated by the cracked SD-TG microparticle in Figure 2. As per the theoretical particle formation theory calculations previously discussed, the *Pe* number of the nanoemulsion droplets is >>1 and the *Pe* of trehalose is ≈1. Theory suggests that the nanoemulsion droplets accumulated near the surface of the drying droplet because the nanoemulsion droplets were unable to diffuse to the centre faster than the particle was forming. The lack of visual nanoemulsion droplet aggregation and relatively even distribution in the SD-TG and SD-TGI particles suggests that an appropriate drying temperature was chosen.

Similar SEM images of the powder samples were taken over the course of the stability study. Figure 3 shows the SD-TGI formulation after three months' and 12 months' storage at 40 °C. A lower magnification view, shown in Figure 3a, shows that the sample stored at 40 °C has maintained overall exterior particle structure even after 12 months of storage. Smaller particles may experience some bridging, as shown by the smaller particles attaching to the larger particles. As shown in Figure 3b, the interior structure of the particle is also maintained after storage at 40 °C, as the voids left by the nanoemulsion droplets are clearly distinct. Cavities within the central void are too large to be nanoemulsion droplets; they may be caused by ruptured nanoemulsion droplets, which led to the pockmarked inner surface. The pockmarked inner surface was not consistently exhibited in cracked particles. The morphology for the samples stored at lower temperatures also displayed a maintenance of interior and exterior particle structure over 12 months of storage. No particle bridging was apparent in the samples stored at lower temperatures.

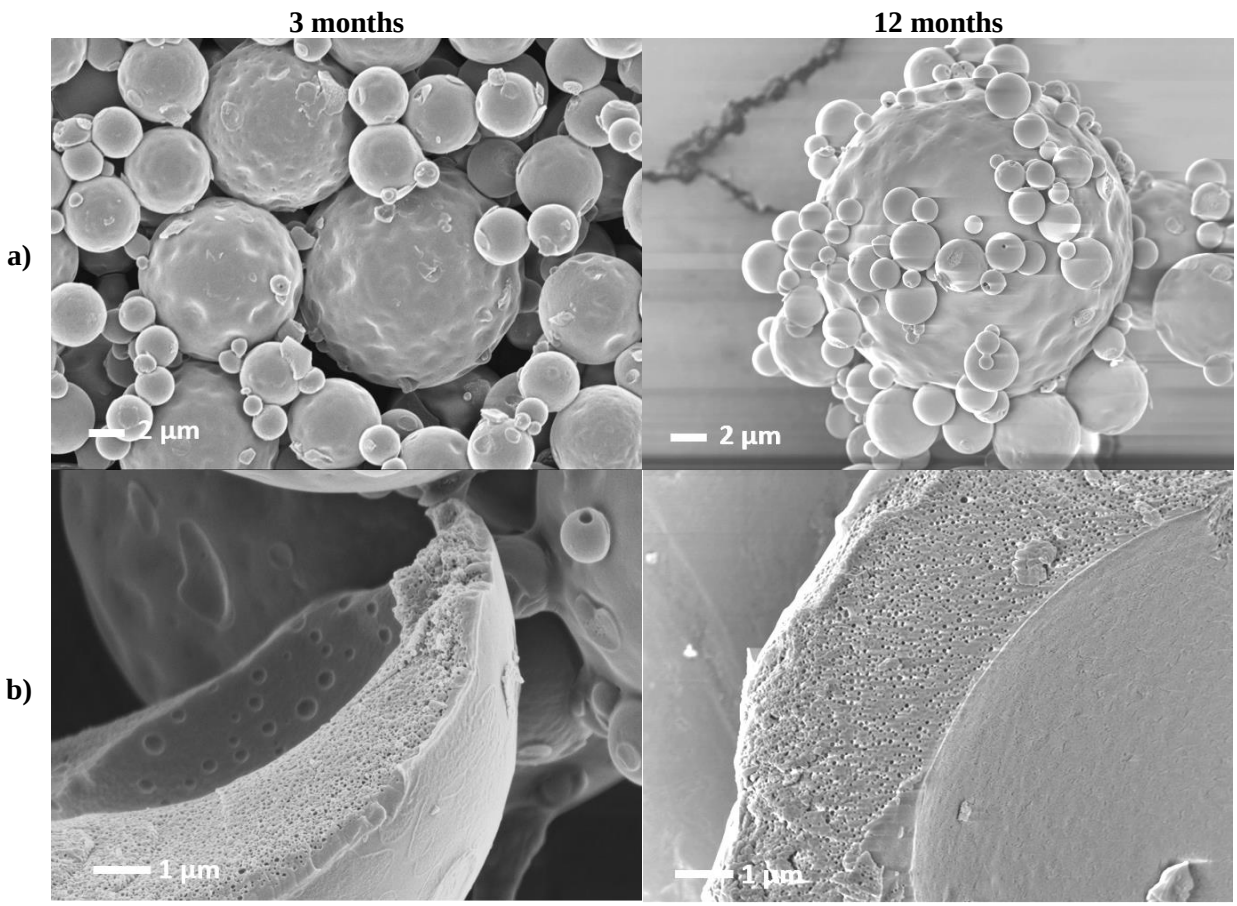

Figure 3 SEM images of antigen containing SD-TGI powder after 3 months and 12 months of storage at 40 °C indicating a) sample maintains external morphology after accelerated storage, b) interior particle structure is maintained. Scales are provided on the respective images.

Powder Moisture Content

Maintenance of moisture content is critical to avoid lowering of the T_g, which would result in powder crystallization. Crystallization of amorphous spray dried powder will inactivate the biological components [2]. The moisture content of the powder by wet basis after manufacture was measured to be 2.6 ± 0.1% in this study. Moisture content of the SD-TGI powder over 12 months of storage is shown in Figure 4. The moisture content after 12 months of storage at 2-8, 25, and 40 ℃ was 2.5%, 2.1%, and 1.8%, respectively. This lack of moisture uptake signifies that the implemented packaging system is very robust.

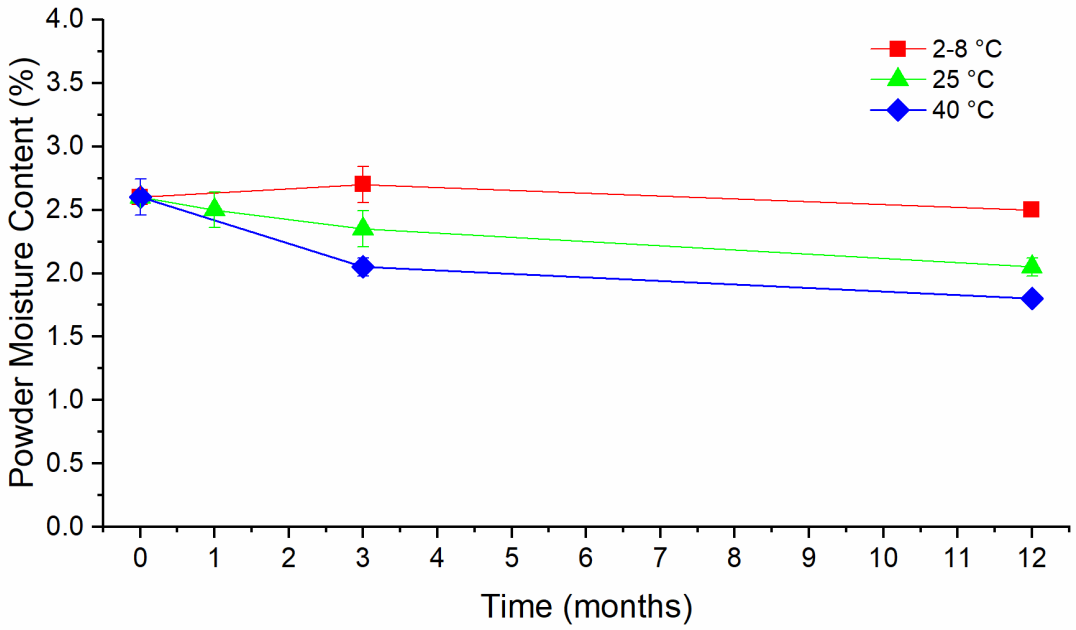

Figure 4 Graph indicating the powder moisture content of the antigen containing SD-TGI powder sample after 12 months of storage at 2-8, 25, and 40 °C. The minimal change at each temperature arm of the stability study indicates that the moisture content of the powder is preserved and the robustness of the implemented packaging system.

Solid State Analysis

Raman spectroscopy analysis was completed on SD-TG and SD-TGI powders at various time points during the stability study in order to confirm the presence of initially formulated components within the sample and to determine any changes in solid phase. Sample spectra for SD-TG powder obtained at timepoint 0, as well as reference spectra for amorphous trehalose, squalene, crystalline Tris, and crystalline trehalose, are shown in Figure 5. The ID93 protein and GLA contributions were not considered due to their low mass fraction.

Inspection of the sample spectra showed the trehalose component to be completely amorphous. This is evidenced by the presence of amorphous trehalose characteristic peaks at 425, 460, 560, 865, 935, and 1145 cm^{-1} in the sample and further supported by the lack of crystalline trehalose characteristic peaks at approximately 395, 455, 695, 980, and 1235 cm^{-1}, to cite a few examples. The squalene in the sample was identified by its characteristic peak appearing at 1400 cm^{-1}. The solid phase of trehalose is further confirmed by the low normalized intensity of the residual spectrum, shown in Figure 5. The residual spectrum was obtained by subtracting the reference spectra for amorphous trehalose (mass fraction 81%), squalene (mass fraction 14%) and Tris (mass fraction 2%) from the sample spectrum. Similar analysis was completed on the SD-TGI powder (not shown), also confirming the amorphous structure of the trehalose component and the presence of squalene and Tris buffer in the sample. It is expected that SD-TG and SD-TGI have similar results, as the only difference in formulation between the samples is the presence of ID93 in the latter.

Sample spectra for SD-TGI at time point 0 and after 12 months of storage at 2-8, 25, and 40 °C are shown in Figure 6. The residual spectrum after subtracting the SD-TGI spectrum collected after 12 months at 40 °C from the timepoint 0 spectrum is also shown in Figure 6. The subtracted spectrum of the most extreme storage condition from the initial spectra was extremely low. The spectra for all storage temperatures were very similar, indicating that the solid phase of the powder had not measurably changed over the course of the stability study. Indeed, deconvolution of the SD-TG and SD-TGI spectra collected at different points of the stability study confirmed that all samples showed presence of squalene and Tris, and that trehalose

remained amorphous. This ties into the moisture content data, in that if moisture content did not rise, it is unlikely that the trehalose crystallized as the T_g was theoretically >88 °C.

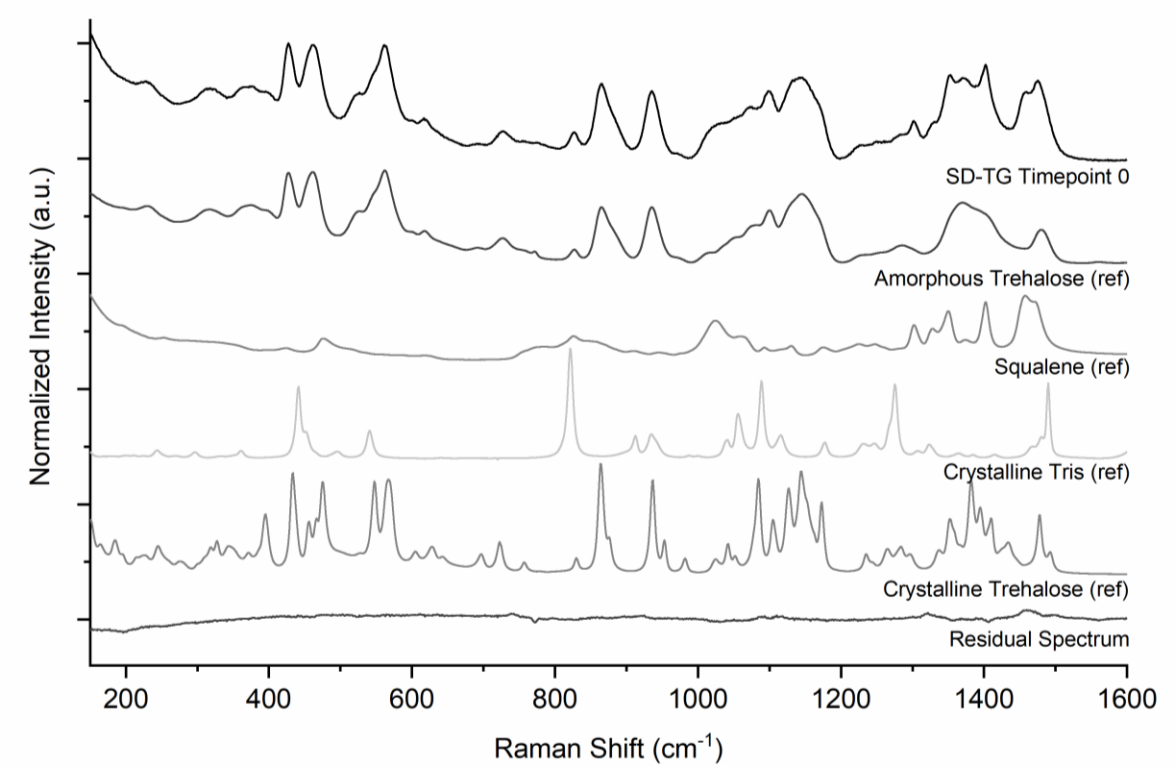

Figure 5 Normalized Raman spectrum of the adjuvant-only SD-TG powder sample, and reference spectra of amorphous trehalose, squalene, Tris, and crystalline trehalose. Also shown is the normalized residual spectrum, obtained by subtracting individual component contributions from the raw measured SD-TG spectrum.

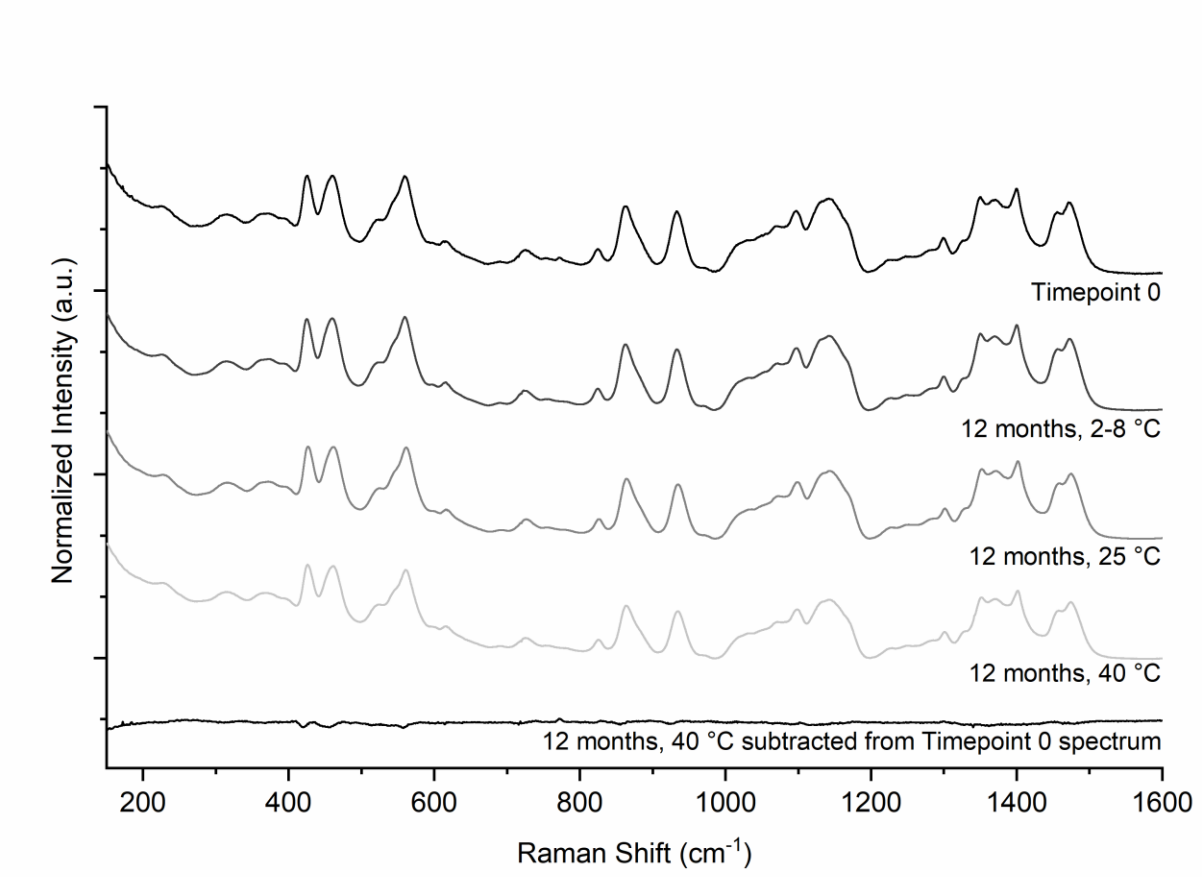

Figure 6 Normalized Raman spectra of the antigen containing SD-TGI powder formulation, shown after 12 months of storage at 2-8, 25, and 40 °C, and at time point 0. All samples shown were normalized according to the peak at 460 cm^{-1}. Also shown is residual spectrum obtained from subtracting the 12 month, 40 °C spectrum from the spectrum obtained at time point 0. The residual spectrum indicates that the SD-TGI powder stored for 12 months at 40 °C does not undergo any solid phase changes.

Emulsion Droplet Size and Distribution

Properties of the emulsion droplet size of the GLA-SE vehicle over 13.5 months of the stability study are shown in Figure 7. The changes are all within the pre-determined target stability criteria mentioned above; that is, <50% change in droplet size; and polydispersity index <0.2 after three months storage' at 40 °C. Both the initial change in emulsion diameter and the subsequent size change over storage are important in evaluating a spray dried emulsion. After three months' storage, droplet size increased only 1-3% for the SD-TG formulation. Similarly, droplet size increased only 2-4% for the SD-TGI formulation. The polydispersity index did not change significantly over the course of three months' at any given storage temperature (p>0.05). The nearly constant droplet diameter and polydispersity index indicates that the nanoemulsion droplets did not coalesce or shrink during the three-month storage or on reconstitution, even at the highest temperature of 40°C, proving that the nanoemulsion droplets were well protected by the trehalose stabilizer as designed.

Nanoemulsion droplet diameter for all samples after 13.5 months was within -4 to 2% of the initial measurement. These results show that even after long term storage the powder is well within the <50% change in diameter stability criteria. Similarly, the polydispersity index is below 0.2 after 13.5 months of

storage. Low measured polydispersity index indicates that the nanoemulsion droplet diameter distribution remains relatively monodisperse over the course of the stability study.

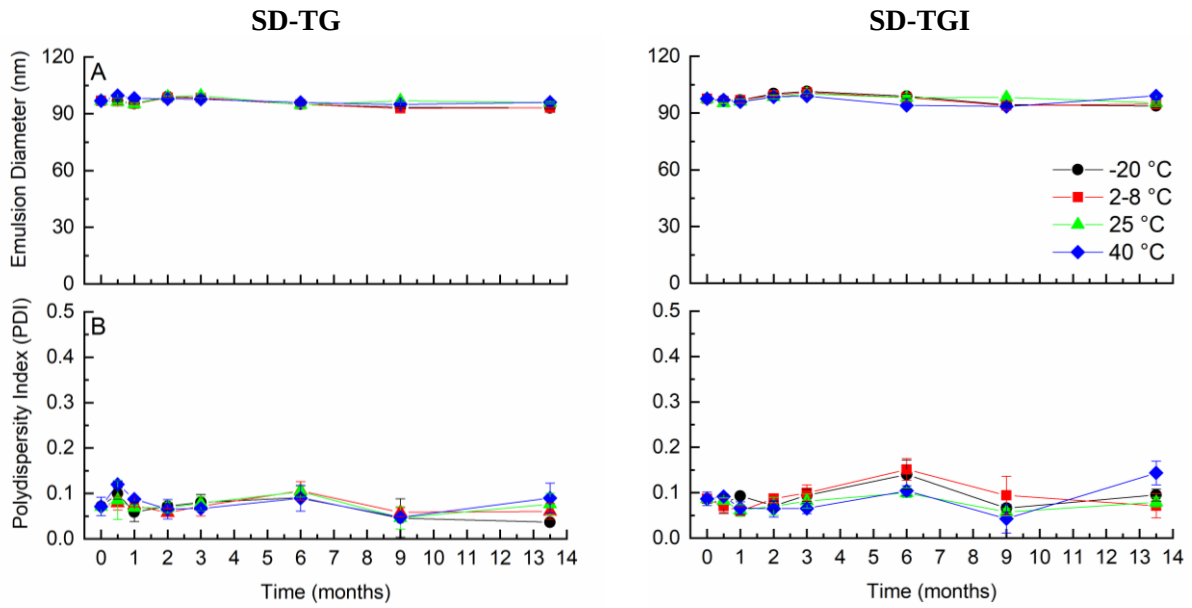

Figure 7 Plots showing A) nanoemulsion droplet diameter size and B) polydispersity index of droplets for the reconstituted adjuvant-only SD-TG powder (left) and reconstituted antigen containing SD-TGI powder (right) stored over 13.5 months at the indicated temperatures. Legend indicates the storage temperatures for the powder. Parameters are within targets over time at all temperatures. Error bars indicate the standard deviation of three measurements.

Squalene Content

Squalene retention of the SD-TG and SD-TGI powders over 13.5 months of the stability study are shown in Figure 8. The changes are all within the pre-determined target <20% loss for squalene content defined previously. Squalene content was preserved after three months at high temperature storage, with 84% retention for the SD-TG formulation and 91% retention for the SD-TGI formulation, when compared to the initial measurement. After 13.5 months of storage, the squalene content for the SD-TG formulation was 83% to 89% retained. Similarly, after 13.5 months of storage, the squalene content for the SD-TGI formulation was 91 to 94% retained. Squalene content analysis for both formulations shows a similar pattern where the squalene loss trend among all storage temperatures are similar, but appears to increase with increasing temperature. Squalene loss appears to occur over the first three months of storage and then remains relatively constant afterwards for a given storage temperature. Reported increases in squalene content may be due to assay variability.

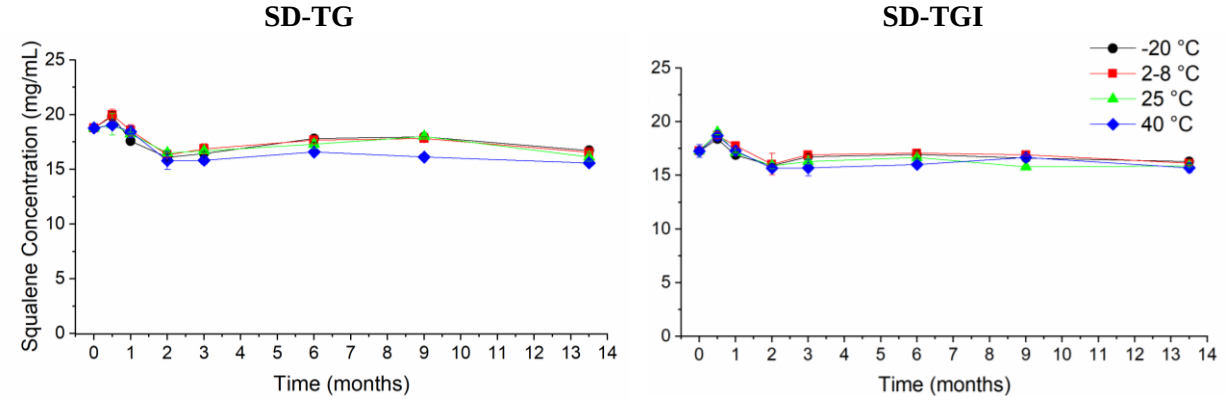

Figure 8 Plots showing squalene content of the reconstituted adjuvant-only SD-TG powder (left) and reconstituted antigen containing SD-TGI powder (right) stored over 13.5 months at the indicated temperatures. Legend indicates the storage temperatures for the powder. Parameters are within targets over time at all temperatures, indicating that the squalene component of the vaccine is stabilized within the dry powder. Error bars indicate the standard deviation of three measurements.

GLA Content

The pre-determined target stability criteria for the agonist GLA was <20% loss of GLA after three months storage at 40 °C. GLA retention of the SD-TG and SD-TGI powders over 13.5 months of the stability study are shown in Figure 9. GLA retention is high for storage at -20 and 2-8 °C after three months for both powders (87-93% retained). A decrease of GLA concentration of 40% for the SD-TG formulation and 43% for SD-TGI formulation stored at 40 °C for three months was observed.

After 13.5 months of storage, the GLA content for the SD-TG and SD-TGI formulation remained stable when stored at -20 and 2-8 °C (92-98% retained). Retention of GLA for the SD-TG and SD-TGI formulations stored at 25 °C was 83% and 75%, respectively. Both the formulations underwent 56-58% loss in GLA after storage at 40 °C for 13.5 months.

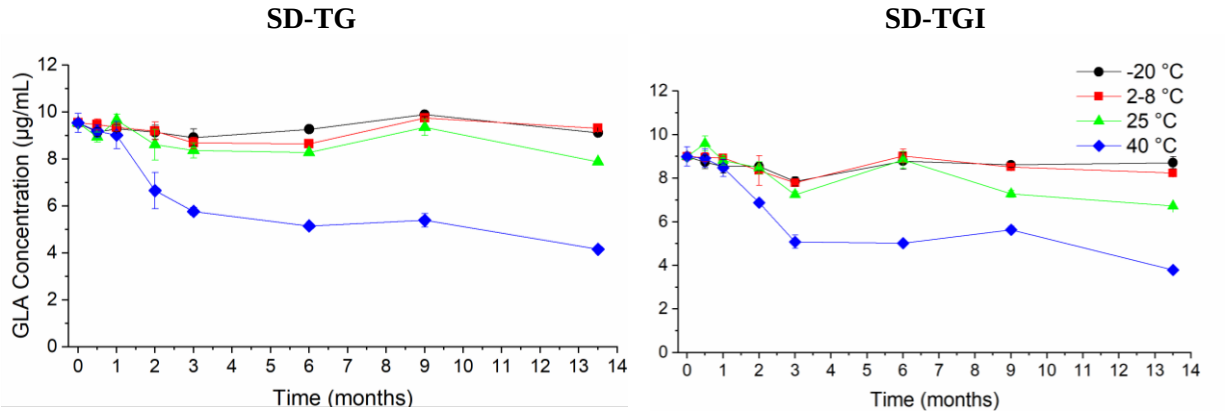

Figure 9 Plots showing GLA content of the reconstituted adjuvant-only SD-TG powder (left) and reconstituted antigen containing SD-TGI powder (right) stored over 13.5 months at the indicated temperatures. Legend indicates the storage temperatures for the powder. Error bars indicate the standard deviation of three measurements.

ID93 Content

SDS-PAGE results of the samples after 3 months and 13.5 months of storage were compared to an ID93 control and a two-vial ID93+GLA-SE control. Oil smear shown at the top of the gel was due to GLA-SE [68]. The presence of the ID93 band in all samples, shown in Figure 10 and Figure 11, confirms that ID93 is present at all storage temperatures after 3 months and 13.5 months, respectively. The results indicate that the ID93 protein survives in the spray dried powder over 13.5 months of storage even at temperatures up to 40 °C. All samples showed similar band intensity to the control at timepoint 0, after 2 weeks, 1 month and 2 months of storage (data not shown). The reduced intensity of the band in the 25 and 40 °C samples as compared to the control may suggest that there is some loss in ID93 protein after 3 months' storage.

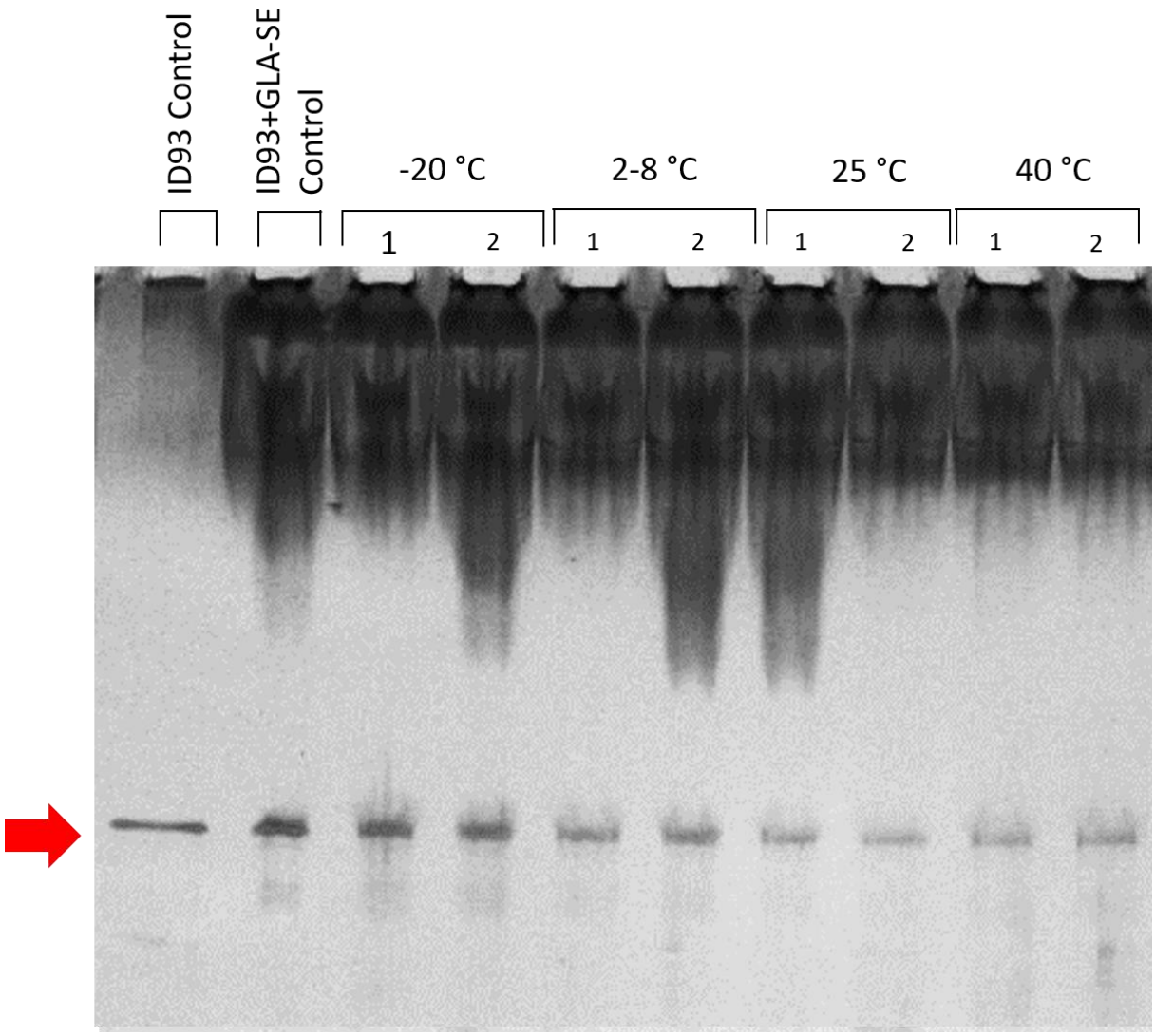

Figure 10 SDS-PAGE results of SD-TGI powder after 3 months storage at temperatures -20, 2-8, 25, and 40 °C. ID93 and ID93+GLA-SE control samples are shown for comparison. Results shown are from a single run. The red arrow points to the ID93 protein band.

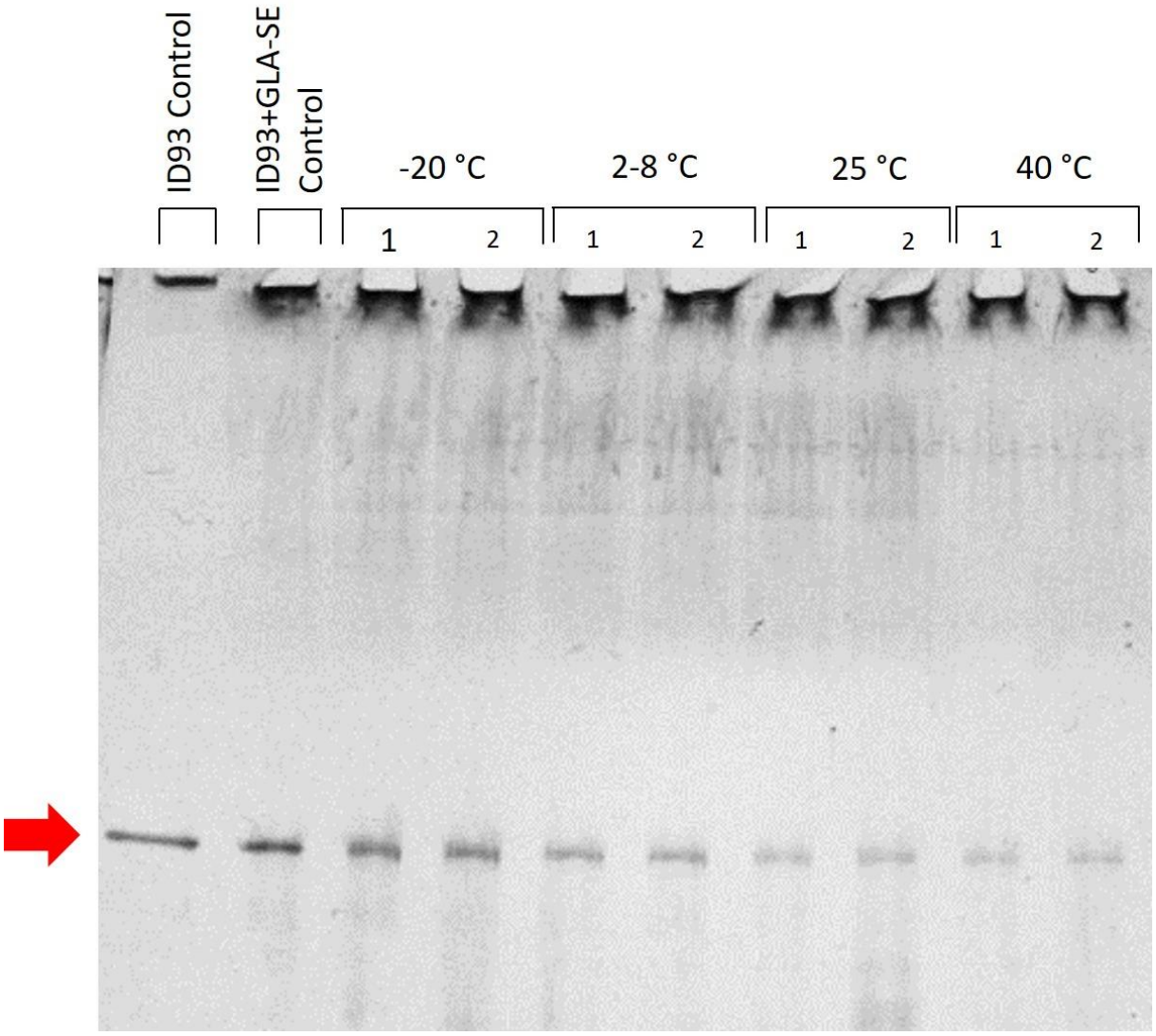

Figure 11 SDS-PAGE results of SD-TGI powder after 13.5 months storage at temperatures -20, 2-8, 25, and 40 °C. ID93 and ID93+GLA-SE control samples are shown for comparison. Results shown are from a single run. The red arrow points to the ID93 protein band.

pH and Osmolality

A previous study found that increased aggregation of ID93 occurs at and below neutral pH [22]. Formulating the vaccine at pH 7.5 or 8 minimizes aggregation and maximizes the temperature at which unfolding occurs [22]. Overall, pH of the reconstituted liquid trended slightly downward over time for all temperatures. After three months of storage, the greatest pH change was 0.07- 0.16 pH units; comparatively, the lead lyophilized vaccine candidates stored at 37 ℃ for three months showed similar pH decreases of less than 0.12 pH units [22]. The pH of the liquid, two-vial and lyophilized proof-of-concept samples exhibited pH decreases of ≥0.2 pH units. Thus, the spray dried formulations appear to offer greater protection against pH change than the liquid vaccine presentations and similar protection as the lead lyophilized candidates. After storage for 13.5 months at -20, 2-8, and 25 ℃, the greatest pH change in the spray dried powders was 0.1 pH units. Somewhat greater pH decrease (0.3 pH units) was evident in reconstituted SD-TG and SD-TGI powders after storage at 40 ℃ for 13.5 months.

Osmolality was monitored through the stability study to ensure that the reconstituted formulation is isotonic and suitable for injection. Osmolality changed only slightly after 13.5 months for all samples. For the SD-TG sample, osmolality increased 0.4% for the samples stored at 40 °C and decreased by 1-3% for the samples stored at -20, 2-8, and 25 °C. Osmolality of the SD-TGI powder increased 1-5%. The osmolality of all samples remained within the 300±100 mOsmol/kg target.

Discussion

This study reports the development of a thermostable presentation of an adjuvanted subunit TB vaccine candidate via spray drying. Spray drying a vaccine into a dry powder form can greatly improve long term stability over its liquid counterpart; however, loss of efficacy can occur due to suboptimal formulation and processing parameters, causing loss or structural change of the vaccine components [69]. Our results showed that spray drying of the ID93+GLA-SE vaccine candidate with trehalose as a stabilizer successfully preserved all vaccine components. The nanoemulsion droplets maintained their size on reconstitution after spray drying. The lack of membrane fusing suggests that the chosen processing parameters effectively facilitated the stabilization at the phospholipid monolayer membrane of the GLA-SE nanoemulsion droplets within trehalose matrix. The overall preservation of nanoemulsion droplet diameter is critical as it is has been shown that smaller nanoemulsion droplets are more stable over time than large ones [70]. Additionally, the ID93+GLA-SE vaccine candidate targets uptake by dendritic cells to induce immunity and particle size is known to influence dendritic cell uptake.

The maintenance of droplet size shows that the shearing forces on the formulation due to the twin fluid atomizer were not sufficient to cause droplet size change. Similar preservation of membrane integrity was achieved by Kanoija et al.'s [10] spray drying of outer membrane vesicle pertussis vaccine. Their study also used trehalose as a stabilizing excipient and found that vesicle size before and after spray drying was approximately 130 nm. Conversely, spray drying of H56/CAF01, which was formulated as a liposomal dispersion, resulted in size change of the CAF01 adjuvant [12]. H56/CAF01 was also spray dried with trehalose, further indicating that stabilization must also consider the processing parameters rather than just the stabilizing excipient. Similarly, the method of manufacture plays a role in preserving phospholipid membrane integrity. Spray drying the vaccine showed little change in GLA-SE droplet diameter; however, after lyophilization, GLA-SE diameter increased 31% and 18% for trehalose-containing and sucrose-containing lead candidates, respectively [22]. The discrepancy in diameter changes for the formulations with similar excipients but differing manufacturing methods indicates that there are additional stresses in the lyophilization process that may cause the nanoemulsion droplets to coalesce.

In additional to nanoemulsion droplet size preservation, results show that a high encapsulation of squalene within the spray dried particles was achieved, suggesting that trehalose was an appropriate choice of wall material for this vaccine candidate. High retention of GLA further signifies that trehalose provided suitable protection during desiccation. Preservation of these components over spray drying was a concern as both need to be stabilized during the desiccation process. The chosen drying temperature was clearly not too high as the integrity of these components was preserved. For the chosen drying gas temperature, the droplet temperature was predicted to be near the wet bulb temperature, i.e. approximately 28 °C. Protection of these components against desiccation stress is likely due to glass stabilization by trehalose. Similar stabilizing performance was shown in the lyophilization study, where GLA concentration did not significantly decrease below the target after lyophilization for any of the samples [22].

High encapsulation efficiencies via spray drying have been previously achieved for nanoemulsions at the 100 nm scale. Nanoemulsions containing the drug tioconazole were formulated with a mean size of 182 nm and spray dried using lactose as an excipient [71]. The authors reported that nanoemulsion size was

preserved and that there was no loss of drug over the spray drying process. Successful encapsulation of H56/CAF0, a TB liposomal vaccine candidate at the nano-scale within trehalose microparticles has been demonstrated by Thakur et al. [12]. The size of the tested liposomal dispersions ranged from approximately 370-770 nm. Encapsulation efficiency was not directly measured; however, similar immunogenicity profiles were elicited from the reconstituted vaccine as compared to the liquid formulation. The similarly generated immune responses suggest that a high amount of the active ingredient were encapsulated within the particles.

The ID93+GLA-SE vaccine candidate is formulated as a nanoemulsion with a phospholipid membrane. The successful stabilization of this candidate with trehalose is consistent with the stabilization of the phospholipid based vaccines via spray drying. Kanoija et al. [5] spray dried several formulations of an influenza vaccine with trehalose as an excipient and held the powder at 60 °C for three months. For all tested formulations, greater than 86% of the antigen was retained after the stability study. Conversely, the liquid influenza vaccine when stored under the same conditions lost all antigenicity after only 5 days. However, appropriate stabilizer is dependent on the structure of the vaccine. Toniolo et al. [6] spray dried enveloped and non-enveloped viral vaccines using combinations of trehalose, mannitol, dextran, and lactose as stabilizers and stored the powders at 37 °C. The thermostability of the spray dried vaccines was improved over the liquid vaccine, with up to 50% titer remaining for certain vaccine and excipient formulations after 30 days. Titer for the liquid vaccine was reduced to 0 after only 15 days when stored under the same conditions. The different types of vaccines had different levels of titer remaining for the same processing conditions and stabilizer used. The enveloped viral vaccines had different levels of stabilization success but showed a similar trend in titer loss as compared to the non-enveloped vaccine. Trehalose and trehalose mixtures stabilized the enveloped vaccines best, whereas the non-enveloped viral vaccine was best stabilized by the mannitol-dextran mixture. Given such differences, the optimal formulation depends on the vaccine type.

However, success of long-term vaccine stabilization is also dependent on the spray drying parameters. Analysis of HSV-2 vaccine spray dried with either trehalose or sucrose as the stabilizing excipient under various processing conditions after storage at 45 °C over 10 days showed differences in activity loss among samples [9]. Trehalose-containing HSV-2 powders exhibited suitable thermostability. Sucrose was not as effective as a stabilizer. The HSV-2 vaccine powders stabilized with sucrose had a large range of activity loss depending on the spray drying parameters used, indicating that the processing conditions will affect the long-term stabilization of the vaccine. To this end, this study also assessed the long-term stability of the spray dried SD-TG and SD-TGI powders to determine if processing conditions and trehalose were appropriate for conferring thermostability.

A protective packaging method was necessary to effectively evaluate the stability of dry powders. This study showed that moisture content of the spray dried vaccine powder remained low over throughout the stability study for all storage temperatures. Proper packaging method likely conferred protection of powders against moisture uptake. Proper packaging is required to preserve powder integrity, as shown for spray dried anthrax vaccine powders held at 25 and 40 °C for three months [8], where the moisture content of the anthrax vaccine powders was within 0.5% of initial measurement after three months of storage due to the use of an effective packaging method. Similarly, Price et al. [72] stored spray dried BCG vaccine powders at different temperatures up to one year. The vaccine powders were stored in either a scintillation vial or a protective packaging system that utilized desiccant, reactive oxygen species scavengers, and inert gas. Powders stored at 40 °C without humidity protection showed significant titer losses.

The long-term stability study results showed that the spray dried powders maintained amorphous structure for all storage temperatures over one year. The extremely low normalized residual spectrum of the 40 °C

SD-TGI sample after one year storage subtracted from the timepoint 0 spectra indicates that the two samples are remarkably similar and therefore the solid phase of the powder has been maintained over this period, even with the added thermal stress placed on the powder. As shown in these results, trehalose, when stored dry, does not crystallize. This was also shown for spray dried anthrax vaccine powders using a packaging system including desiccant [8]. These powders also used trehalose as the main stabilizing excipient. The moisture content of the powders remained close to the initial measurement, and the powders remained amorphous. Vandenheuval et al. [64] spray dried bacteriophages in trehalose powders and stored them at 4 and 25 °C at 0 and 54% RH. They found that phage was inactivated due to the crystallization of amorphous trehalose. This result is due to the storage of unprotected trehalose-based powders at high humidity. The spray dried SD-TG and SD-TGI powders did not crystallize in this study, indicating that the packing system effectively protected the powders from crystallization caused by exposure to high humidity.

Suboptimal encapsulation of the emulsion resulting from inappropriate processing parameters can lead to accumulation of emulsion droplets on the surface of the particle [73]. Particles with a high surface oil content were reported to have small protrusions on the outer surface [73] or exhibit a pockmarked surface [33, 73]. The studies suggested that the protrusions are caused by the covered oil droplets and the pockmarks are from the surface oil droplets rupturing, leaving behind small craters in the outer particle wall. As neither of these surface morphologies is seen in either of the SD-TG or the SD-TGI particles SEM, it appears that no nanoemulsion droplets accumulated directly on the outer surface. Additionally, images of the particles obtained over the course of the stability study showed that interior and exterior morphology was overall preserved. Some bridging was shown between the small particles of the spray dried vaccine powder stored at 40 °C. However, bridging is to be expected after long term storage at high temperatures. Particle fusing among smaller particles was also shown in spray dried HAC1 vaccine with trehalose after storage for three months at 50 °C [7]. Bridging between particles is due to transition to a more energy favorable state through surface area reduction. This bridging is facilitated by increased molecular mobility due to increased temperature. However, the overall lack of particle fusing, shape change, and maintenance of internal structure indicates physical stability was achieved. Physical stability at a higher temperature over 12 months is a strong indicator of long-term physical stability at room temperature.

Reported nanoemulsion droplet diameter change after 13.5 months of storage was within a few percent of the initial measurement for all storage temperatures. These results can be compared to a stability study completed on the liquid single-vial presentation, the two vial clinical presentation, the proof of concept lyophilized candidate, as well as the lead lyophilized candidates [22]. Compared to three-month stability studies at 37 °C conducted on lead lyophilized candidates and liquid single-vial presentation, the spray-dried powders have a comparable or better performance regarding droplet size change [22]. Additionally, the droplet diameter of the spray-dried candidates show less change than the 2-vial clinical presentation and the proof-of-concept lyophilized candidate [22]. The droplet size of the 2-vial clinical presentation increased to over 240 nm, and the droplet size of the lyophilized proof-of-concept candidate increased to over 200 nm after three months' storage at 37 °C. It is apparent that the spray dried powders have greatly improved the thermostability of the ID93+GLA-SE vaccine candidate. Emulsion diameter results combined with the results of particle morphology, moisture content, and solid-state analysis further indicate that the powder is well-stabilized physically by trehalose. Malila et al.'s [74] experiments of spray drying w/o/w methyltestoterone emulsion droplets initially showed similar preservation of droplet diameter immediately after spray drying. However, after one month of storage in a desiccator the nanoemulsion droplet sizes increased by a factor of 2-8. In this particular case, the stabilizer of stearic acid was unable to prevent droplets from aggregating. By contrast, our study shows that trehalose was an effective stabilizer of the vaccine's phospholipid membrane and offered significant thermal protection. Pereira and Hünenberger [75]

showed that the bilayer structure of DPPC liposomes in water was thermally disrupted at 475 K, but the structure was preserved when trehalose was added to the solution.

High levels of squalene were retained after 13.5 months of storage, even at high temperatures. Approximately 40% GLA losses were shown in the samples stored at 40 °C after 3 months of storage, failing the target stability criteria. However, this result is comparable to the reported GLA loss in the lyophilized proof-of-concept formulation. When stored at 37 °C for three months, the lyophilized proof-of-concept formulation exhibited a decrease of 48% in GLA concentration [22]. GLA loss for the lead lyophilized candidates and two-vial clinical presentation ranged from no losses up to 36% loss in content [22]. The spray dried formulations perform comparably to the proof-of-concept lyophilized formulation. The lead lyophilized candidates generally outperform the spray dried formulations in terms of GLA retention. However, these lead candidates were developed and tested after an extensive design-of-experiments approach. The spray dried candidates in this paper are a first iteration and clearly exhibit some improved protection against degradation as compared to the liquid vaccine. Approximately 40% of the GLA content was retained after storage at the most extreme condition, whereas all GLA was degraded when stored as a liquid product at similar temperatures [22]. Degradation of GLA was shown to increase with increasing temperature storage. Approximately 80% of GLA was retained for the spray dried samples stored at room temperature after 13.5 months. This is to be expected as GLA similarly showed temperature-influenced degradation in the lyophilized candidates [22]. The presence of antigen ID93 was confirmed after 13.5 months of storage at all temperatures. These results indicate that spray drying the ID93+GLA-SE vaccine candidate conferred some level of thermostability in terms of chemical preservation.

Squalene loss and GLA degradation did not exhibit a linear pattern over time. Squalene and GLA degradation appeared to have occurred primarily during the first three months of the stability study. Measured content for the remaining timepoints appear to be either relatively constant or decreasing slightly. Powder crystallization may lead to loss of stabilizing hydrogen bonds with the phosphate head groups of the GLA-SE membrane. However, characterization of the physical stability of the powder indicates that the powder remains amorphous and that therefore GLA is not degrading due to crystallization of the powder. Air-dried enveloped viral vaccines stabilized in trehalose-pullulan films exhibited a similar degradation pattern of initially rapid loss followed by a period of gradual loss [76]. It was suggested that this degradation pattern indicates the existence of multiple degradation mechanisms.

Conclusion

A subunit vaccine candidate containing a nanoemulsion adjuvant system with trehalose and Tris buffer as additional excipients was spray dried and the stability of the dry powder product was evaluated over one year. Two formulations were tested for stability: (1) the oil-in-water nanoemulsion adjuvant system, and (2) the oil-in-water adjuvant system with the protein antigen. *In-silico* formulation and process development were utilized, significantly reducing the required amount of empirical work. This approach is especially prudent for work that involves highly complicated formulations. To the authors' best knowledge, these are the first published results regarding the stabilization of a subunit vaccine formulated as a nanoemulsion at the 100-nm diameter scale using trehalose via spray drying and showing long-term stability potential.

These results are especially meaningful in light of global health applications as refrigeration infrastructure required for vaccine distribution is not always available. Because our dry power is stable at a wide temperature range, it alleviates the necessity for such refrigeration infrastructure. The dry powder product was physically stable after 12 months of storage at a high temperature up to 40 °C, suggesting that the product is physically stable for long-term storage under ambient temperature. Besides, formulating the vaccine as a dry powder and reconstituting as needed will significantly reduce the costs associated with

transport and storage. In comparison to the lyophilization treatment of these biological formulations, which has been proven to be a beneficial method of storage [22], spray drying presents a promising alternative with its potentially lower processing cost and improved scalability [25]. The increased production capability of spray drying can be utilized to stockpile larger amounts of thermostable vaccine for pandemic situations. Moreover, spray dried vaccines have the potential to be administered directly by inhalation without the need for reconstitution [3].

Comparison of the formulation feedstock and the reconstituted spray dried powder showed that the integrity of the emulsion was maintained for both size and squalene concentration. The agonist was shown to have a similar concentration and the antigen presence was confirmed post-spray drying. The preservation of physicochemical properties upon reconstitution strongly suggests that the efficacy of the vaccine is preserved over the course of spray drying. These spray-dried powders were then assessed for stability over one year, up to a temperature of 40 °C, through characterization of the reconstituted powder and the dry powder itself. The results showed that the powders maintained physical stability for all temperatures after one year. Powder stability at one year at a higher temperature suggests long-term stability at room temperature. Physicochemical characterization for both formulations, at all temperatures, showed that the average nanoemulsion droplet size and polydispersity did not change appreciably; they performed significantly better than a lyophilized proof-of-concept and on par with the lead lyophilized candidates [22]. Agonist preservation was on par with the proof-of-concept lyophilized version [22]. After being held for 3 months up to 50 °C, immune cell production for the lyophilized vaccine was maintained whereas the liquid vaccine lost most of its immunogenicity under the same conditions. Immunogenicity studies have not been carried out on the spray dried vaccine; however, the spray dried vaccine appears to be comparable to the lyophilized vaccine in terms of biophysical characterization.

This study showed that integrity of both the biologic and the adjuvant system was preserved over the spray drying process. Given the small scale, the efficiency of encapsulation and preservation of the nanoemulsion droplets is especially notable. The dry powder version of oil-in-water adjuvant systems can be combined with subunit vaccines for other diseases. Further formulation development must be conducted to improve long term chemical stability. Spray drying, unlike lyophilization, allows for the engineering of properties such as particle size and flowability. These properties open doors to other delivery routes, such as pulmonary and intranasal, rather than intramuscular injections. The demonstrated preservation of the physical state of the spray-dried powder suggests that it will maintain aerosol performance even after long-term storage at high temperatures.

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Disclosures

The authors declare that there are no conflicts of interest.

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