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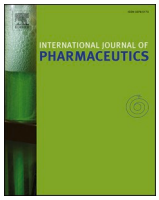

The interplay between trehalose and dextran as spray drying precursors for cationic liposomes

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ABSTRACT

Successful oral delivery of liposomes requires formulations designed to withstand harsh gastrointestinal conditions, e.g., by converting to solid-state followed by loading into gastro-resistant delivery devices. The hypothesis was that the use of dextran-trehalose mixtures for spray drying would improve the rehydration kinetics of dried liposomes. The objectives were to determine the protective capacity of trehalose-dextran dehydration precursors and to increase the concentration of liposomes in the dry formulation volume. The study successfully demonstrated that 8.5% dextran combined with 76.5% trehalose protected CAF®04 liposomes during drying, with the liposome content maintained at 15% of the dry powder. Accordingly, the rehydration kinetics were slightly improved in formulations containing up to 8.5% dextran in the dry powder volume. Additionally, a 2.4-fold increase in lipid concentration (3 mM vs 7.245 mM) was achieved for spray dried CAF®04 liposomes. Ultimately, this study demonstrates the significance of trehalose as a primary carrier during spray drying of CAF®04 liposomes and highlights the advantage of incorporating small amounts of dextran to tune rehydration kinetics of spray-dried liposomes.

1. Introduction

Oral administration of drugs and vaccines is currently a key focus with the intention to solve issues such as patient compliance as well as accessibility in low-income regions (Baryakova et al., 2023; Pollard and Bijker, 2021). Administration of drugs and vaccines orally can also be used to reduce the burden on the healthcare resources as this form of delivery does not require trained personnel for efficient administration (Taddio et al., 2012). Moreover, for vaccines, oral administration can be a good strategy for targeting mucosal immunity in the gut (Carlsen et al., 2023).

Liposomes have been established as potential carriers for delivery of active pharmaceutical ingredients (APIs) (Giordani et al., 2023; Liu et al., 2022). Liposomes are small spherical vesicles formed when lipids rearrange into bilayer membranes in aqueous medium. Liposomes are versatile delivery carriers as hydrophilic compounds can be in the aqueous core of the vesicles, whereas hydrophobic compounds can be embedded in the lipid membrane (Liu et al., 2022; Marasini et al., 2017). The versatility liposomes offer is broadly used in the vaccine delivery field. Here, liposomes have been established as excellent adjuvants, as they offer a platform for incorporating immunopotentiators, as well as a delivery system for antigens (Tretiakova and Vodovozova, 2022). The cationic adjuvant formulation (CAF®) platform is an example of such a liposome-based adjuvant system, which was developed to meet the need for novel and safer adjuvants that could elicit strong Th1 immune responses (Aroffu et al., n.d.; Tretiakova and Vodovozova, 2022). The main component in the CAF® platform is the quaternary cationic ammonium surfactant, N,N-dimethyl-N,N-dioctadecylammonium bromide (DDA), formulated into liposomes or emulsions, which are often unstable in aqueous medium (Davidsen et al., 2005). Consequently, compounds such as glycolipids were studied to create more stable DDA-based formulations, such as CAF04 (Fig. 1), which contains the glycerolipid monomycoloyl glycerol (MMG) as a stabilizing lipid (Pedersen et al., 2018).

Despite being a versatile and attractive delivery system, adaptation of liposomes for oral delivery still faces a lot of challenges such as *in vivo*

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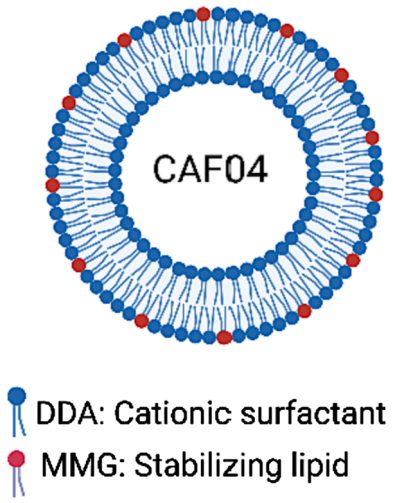

Fig. 1. Theoretical schematic of CAF04 liposome structure.

instability and formulation challenges which result in batch-to-batch variations (Carlsen et al., 2023). One way of addressing such challenges is by converting the liposomes to a solid-state form. Converting liposomes to a dry powder has been extensively studied (Ingvarsson et al., 2013a; Thakur et al., 2022; Yu et al., 2021), and found to enhance their storage stability, as the aqueous environment is removed, and the generated powders can be rehydrated back to liposomes (Barbălată et al., 2023; Chowdhary et al., 2023; Mohammadi et al., 2021). However, adjacent bilayers could fuse during drying, posing a risk of large aggregates forming (Ingvarsson et al., 2011). Therefore, it is important to maintain the structural integrity of the membrane and avoid fusion, which could result in leaking of liposomes (Mohammadi et al., 2021).

Several drying techniques have been extensively studied and reviewed, namely: freeze drying (Franzé et al., 2018; Guimarães et al., 2019; Nowak and Jakubczyk, 2020) spray drying- including supercritical CO_2-assisted spray drying (Costa et al., 2021; Ingvarsson et al., 2011; Ingvarsson et al., 2013b; Moura et al., 2019; Sosnik and Seremeta, 2015), and spray-freeze drying (Adali et al., 2020; Yu et al., 2021). Spray drying is widely used as it is cheap, fast and easily scalable (Sosnik and Seremeta, 2015). Additionally, during spray drying, the powder particle can be engineered to attain different particle properties such as size and porosity (Ingvarsson et al., 2013b). Previously, spray drying of CAF01 has been studied using design of experiments, where trehalose was established to be the optimal spray drying excipient for protecting liposomes (Ingvarsson et al., 2013a; Ingvarsson et al., 2013b). Disaccharides such as trehalose, are widely used in protection of biologicals during drying (Dieplinger et al., 2023). There are many theories supporting the protective mechanism of such sugars. A good stabilizer has a low number of reducing groups, as the reducing sugars tend to interact with groups such as amine groups present on DDA lipids, which can destabilize the formulations (Alhajj et al., 2021). Trehalose having few reducing groups, protects the liposomes from collapsing by replacing the water molecules in the core of the vesicles. Upon rehydration, water is drawn back into the vesicles (Giuffrida et al., 2017; Jójárt-Laczkovich et al., 2016). This is known as the water replacement phenomenon. Consequently, to spray dry CAF-based liposomes at higher concentrations, the ratio between the reducing sugars present in trehalose and amine groups should be adjusted accordingly. This often results in bulky powders with higher carrier content and a higher amount of effective dose would therefore be required. Bulky powders further present a potential issue e.g., limited loading capacity of devices for oral delivery (Carlsen et al., 2023). Therefore, there is a need to increase the ratio of the active drug, i.e., the liposomes without increasing the bulk volume of the formulations.

Additionally, a good stabilizer has a low tendency for crystallization as crystal growth would lead to aggregation of the liposomes during storage (Jójárt-Laczkovich et al., 2016). Trehalose dihydrate exists in a crystal form, and upon spray drying, is thermodynamically modified into an amorphous form (Jójárt-Laczkovich et al., 2016). Crystallization is generally accepted to be a challenge when the difference between the glass transition temperature (T_g) and the storage temperature is less than 50 °C (Newman and Zografi, 2020). Therefore, sugars with high T_g are preferred as stabilizing agents during drying. Additionally, in the presence of moisture, there is a tendency for recrystallization, which could affect the storage stability of spray dried products (Jójárt-Laczkovich et al., 2016).

Another theory for protection during drying is the vitrification theory. Vitrification is the process where amorphous compounds form a rigid, glassy matrix, reducing molecular mobility and thus, maintain stability of the liposomes (Susa et al., 2021; Trenkenschuh and Friess, 2021). However, the kinetic immobilization and stabilizing properties of sugar glasses are lost above the T_g of the sugar (Ubbink, 2016). Consequently, oligosaccharides and polysaccharides such as dextran, having higher T_g's, are stable glass formers (Mensink et al., 2017), but also exhibit polymeric properties (Haeuser et al., 2020; Moreno Raja et al., 2019). Dextran has a high T_g (103 °C at 23% RH) and is, thus, expected to act as a suitable glass former (Moreno Raja et al., 2019; Song et al., 2012), stabilizing molecules for longer duration of time (Imamura et al., 2003; Li et al., 2022), and show less crystallization tendencies during storage.

Crowe *et al.* established that a desirable spray drying carrier is one which has excellent kosmotropic as well as vitrification abilities (Crowe et al., 1997), but it is challenging to find an excipient with both of these properties. One way to achieve this, is by combining a good glass forming carbohydrate, for example dextran 6000 (T_g: 103 °C), with one that easily forms hydrogen bonds with the lipid head groups, e.g., trehalose. Combining the large polysaccharide, dextran, with the small disaccharide, trehalose, has previously been explored in the lyophilization of actin (Allison et al., 2000). Thus, it was hypothesized for this study that combining trehalose for its kosmotropic effect and dextran for its glass forming ability could further improve the protection of liposomes upon spray drying. Additionally, dextran could further protect the liposomes during storage by acting as a spacer as a result of steric hinderance due to its branching and high molecular weight (Penzol et al., 1998).

The purpose of this study was therefore to investigate the interplay between trehalose and dextran as precursors for spray drying CAF04 liposomes. Additionally, we aimed to increase the active ingredient concentration by increasing the liposome concentration in the formulation.

2. Materials and methods

2.1. Materials

N,N-dimethyl-N,N-dioctadecylammonium bromide (DDA) (purity 99.9%) and glycerolipid monomycoloyl glycerol (MMG) (purity 99%) were obtained from Niels Clauson-Kaas A/S (Farum, Denmark). Trehalose dihydrate (purity 99.9%) and Dextran 6000 (purity 99%) were from Sigma-Aldrich (St. Louis, Missouri, USA). All reagents used were of analytical grade.

2.2. Methods

2.2.1. Preparation and spray drying of liposomes

The liposome dispersions were prepared using the thin film hydration method as previously described (Zhang, 2017). DDA and MMG lipid powders were weighed in a 5:1 wt/wt ratio and dissolved in 99% ethanol. Ethanol was evaporated overnight, followed by flushing with nitrogen to remove residuals, resulting in a lipid film. Following that, the lipid film was rehydrated in milliQ water containing trehalose or a blend

of trehalose and dextran (Table 1). The liposomes were prepared by high shear mixing using an Omni GLH 850 (Omni International, Georgia, USA) at 21,000 rpm at 60 °C for 15 min.

The liposome dispersions were spray dried using air as the drying gas on a Büchi B-290 mini spray dryer (Büchi Labortechnik, Flawil, Switzerland). The spray dryer was operated in open-loop configuration with a B-296 dehumidifier (Büchi Labortechnik) and a pressure nozzle with a diameter of 0.7 mm. Centrifugal forces were used to separate the dry particles from the airstream using a high-performance cyclone (Büchi Labortechnik). The liposome concentration was maintained at 15% w/w or 20% w/w of the total formulation (4.5 mg/mL or 6 mg/mL), and the carbohydrate content was fixed at 80 or 85% wt. of the total formulation (20 mg/mL or 25.5 mg/mL). Process control parameters applied during spray drying of CAF04 liposomes were selected based on previous studies on spray drying of CAF01 (Ingvarsson et al., 2013a) (Supplementary Table 1).

The dry powder yield was determined as the difference in weight of the collecting vessel before and after spray drying. The weight difference was compared to the initial total dry mass included in the feed, and the yield (w/w) in % was calculated. The resulting powders were stored in airtight glass vials at −20 °C until further use.

2.2.2. Residual moisture content

Moisture content in the samples was analyzed by thermogravimetric analysis (TGA) as previously described (Ingvarsson et al., 2013a). Briefly, 10 mg of the dried powder samples were placed on platinum pans, and nitrogen purging was applied at 50 mL/min. The samples were heated from 25 °C to 110 °C at a constant rate of 10 °C/min in a Discovery TGA (TA Instruments, New Castle, USA). The % weight loss caused by water evaporation was determined using TRIOS software 5.1.1 (TA Instruments, New Castle, USA) and defined as the residual moisture content.

2.2.3. Morphology characterization of dried powder particles and of liposomes after rehydration

The powder particle morphology was examined by scanning electron microscopy (SEM) using Hitachi Tabletop SEM TM3030 (Hitachi High Technologies, Tokyo, Japan). The dried powder was transferred onto a flat standard stub covered with double-adhesive carbon tape and examination was performed at an acceleration voltage of 15 kV and a working distance of 6.3 mm.

Morphology and size of redispersed liposomes were analyzed using Cryo-Transmission electron microscope (cryoTEM). 3 μL of the redispersed liposomes were placed onto glow discharged lacey carbon coated 300 mesh copper TEM grids (Ted Pella Inc., Redding, California, United States), blotted for 5 s, and plunge frozen in liquid ethane using a Leica EM GP2 plunge freezer (Wetzlar, Germany). Samples were transferred to the TEM at −175 °C using a gatan single tilt cryo holder (Gatan Pleasanton, California, United States) and imaged using a FEI Tecnai T20 G2 transmission electron microscope (Hillsboro, Oregon, United States) operated at 200 KeV in low dose mode with a TVIPS XF416 CCD camera (TVIPS, Gauting, Germany). Images were analyzed using ImageJ, software version 1.53 t (National Institute of Health, Bethesda, Maryland, USA).

Table 1
Composition of spray drying precursors.

Spray drying carrier	% w/w in solid feedstock (15% CAF04)	% w/w in solid feedstock (20%CAF04)
Trehalose	85% w/w trehalose	80% w/w trehalose
90% trehalose	76.5% w/w trehalose-8.5% w/w dextran	72% w/w trehalose-8% w/w dextran
50% trehalose	42.5% w/w trehalose-42.5% w/w dextran	40% w/w trehalose-40% w/w dextran
10%trehalose	8.5% w/w trehalose-76.5% w/w dextran	8% w/w trehalose-72% w/w dextran

2.2.4. Size, polydispersity and surface charge

The mean particle diameter (Z-average), polydispersity index (PDI) and Zeta Potential (mV) of the liposomes were determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) equipped with a 633 nm laser and 173° detection optics. Malvern DTS v.6.20 software (Malvern Instruments) was used for data acquisition and analysis. The samples were rehydrated in milliQ water corresponding to the initial total solid weight i.e. 3% wt. (4.5 or 6 mg/mL CAF04), and then diluted 10-fold in milliQ water, thus the osmotic pressure was maintained. Samples for surface charge measurements were rehydrated in the same manner and diluted 100-fold. The measurements were repeated 3 times for each sample. The values of pure water were used for viscosity and refractive index. A Nanosphere™ Size Standard (220 ± 6 nm, Duke Scientific Palo Alto, CA, USA) was used to verify the performance of the instrument.

2.2.5. Thermotropic phase behavior and solid solubility of trehalose and dextran

The thermotropic phase behavior of the liposomal dispersions was determined by differential scanning calorimetry (DSC) using a MicroCal PEAQ-DSC (Malvern Panalytical Ltd., Cambridge, UK). After rehydration to a total solid concentration of 3% wt. (corresponding to the initial total solid weight), 250 μL of the formulations were heated from 20 °C to 60 °C at a scanning rate of 0.5 °C/min. The exothermic scans of each sample were used for data analysis.

The melting point depression method, previously described to determine the miscibility of crystalline drugs and amorphous polymers (Marsac et al., 2006), was used to determine solubility of the precursors used for spray drying. Physical mixtures of trehalose and dextran were prepared by mortar and pestle in various w/w ratios (Supplementary Table 2). 2–4 mg of the physical mixtures were then weighed into DSC pans and hermetically sealed. To obtain the melting temperature of the samples, a DSC method from 25 °C to 140 °C was applied with the scanning rate of 1 °C/min. The heating curves were analyzed using TRIOS software 5.1.1 (TA Instruments, New Castle) to determine the melting point (T_m onset) and the melting enthalpies (ΔH_m). To investigate the solubility of trehalose and dextran, the experimental melting enthalpies were plotted into the Flory Huggins equation (Eq.1) and χ was used to extrapolate the theoretical miscibility point.

$$\frac{\Delta Hm}{R}\cdot\left(\frac{1}{T_m}-\frac{1}{Ta}\right)=\ln(v_{\mathrm{Tre}})+\left(1-\frac{1}{\lambda}\right)\cdot(1-v_{\mathrm{Tre}})+\mathrm{X}\cdot(1-v_{\mathrm{Tre}})^2 \quad (1)$$

Where ΔH_m is the melting enthalpy of trehalose, T_m the melting point of trehalose, R the gas constant, λ the molar volume ratio of dextran 6000 to trehalose, χ is the Flory- Huggins interaction parameter, T_a the annealing temperature and V_{Tre} the volume fraction of trehalose in the physical mixtures.

2.2.6. Statistics

Where relevant, results are expressed as mean ± standard deviation. Origin® software 9.9.0.225 (OriginLab, Northampton, MA, USA) was used to analyze data from the DSC, using the first and third scans. GraphPad Prism 10.0.2 (232) (GraphPad Software, Boston, MA, USA) was used to analyze particle size, PDI and particle surface charge data, as well as statistical analysis. Two-way ANOVA with Tukey's multiple comparison was applied in statistical analysis of particle size, PDI and zeta potential before and after spray drying. One-way ANOVA with Dunnett's multiple comparison test was applied for analysis of process yield and residual moisture content of formulations.

3. Results and discussion

3.1. Combining trehalose and dextran as spray drying precursors

The combination of trehalose and dextran for lyophilization of

proteins has previously been studied, where it was established that dextran increases the T_g of the formulations and consequently, extends the storage stability of freeze-dried actin (Allison et al., 2000). Though freeze-drying and spray drying rely on different mechanisms of drying, when dehydrating liposomes, the two techniques require the use of carriers such as disaccharides to preserve the structural integrity of liposomes (Yu et al., 2021). Trehalose is applied in freeze-drying for its kosmotropic properties, while dextran is used to reduce the freeze-drying time by lowering the primary drying time (Christensen et al., 2007; Larsen et al., 2019). Spray drying also relies on the water-replacement theory of protection as well as vitrification. It is yet to be established which protective mechanism is more significant in each of the drying techniques. Therefore, understanding the interplay of trehalose and dextran in protecting proteins or liposomes is critical in protection upon spray drying.

3.1.1. Determining solubility of trehalose and dextran

To investigate the interaction between the two components, solubility studies using the melting point depression method and the Flory-Huggins model were performed. The model has been used to effectively predict drug-polymer solubility using onset melting temperatures of physical mixtures between the two components and extrapolating their solubility to ambient temperature (Marsac et al., 2006; Rask et al., 2018; Romhányi et al., 2018). Applying this approach, the interaction parameter describes the solubility of trehalose-dextran (Fig. 2). Therefore, according to the Flory-Huggins model, trehalose and dextran are not soluble as 1.1% of dextran is the highest amount of dextran that would allow the system to maintain entropy.

The Flory-Huggins formula predicts the interaction parameter or solubility of two components using molecular weights. Therefore, phase separation is plausible due to the differences in the molecular weight of trehalose (378.33 g/mol) and dextran (6000 D). This is consistent with an earlier study which describes the phase separation of trehalose-dextran solid solutions (Aksan and Toner, 2004). Although, a seemingly homogeneous system can be obtained upon preparation of trehalose-dextran mixtures, phase separation may occur due to moisture-induced plasticity upon storage (Aksan and Toner, 2004; Vasanthavada et al., 2004). Furthermore, solubility describes a system that is thermodynamically stable, while miscibility describes a monophasic system, which is not thermodynamically stable but physically stable. Therefore, a solubility value close to 0 indicates that the two components will eventually phase separate or crystalize, but despite that, could be physically stable for years.

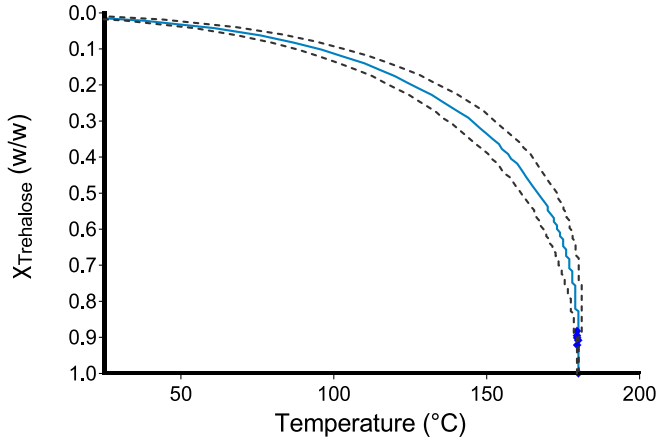

Fig. 2. A temperature-dependent equilibrium solubility curve for trehalose in dextran. All data points are illustrated as averages ($n = 3$). The solubility data has been fitted using the Flory-Huggins model (blue curve), along with the inclusion of a 95% prediction interval (dotted curves). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Although the melting point depression method is a useful tool to determine polymer miscibility, the method could overestimate or underestimate solubility (Knopp et al., 2015). T_gs of mixtures can also be used to characterize miscibility, as mixtures having multiple T_gs are deemed immiscible and those with one T_g indicate miscibility (Ajitha and Thomas, 2020). Despite chemical similarities and the prediction of immiscibility according to the Flory-Huggins parameter, DSC thermograms of the physical mixtures indicated uniform T_gs (Supplementary Fig. S1). Moreover, both trehalose and dextran are readily soluble in water and did not phase separate in our application during preparation of liposomes. Ultimately, monophasic trehalose-dextran solutions were used to rehydrate the lipid films, followed by spray drying.

3.1.2. The influence of spray drying carrier composition on liposome dehydration- rehydration critical quality attributes

Ingvarsson et al. highlighted the significance of a fast-drying rate during spray drying, i.e., slower drying rates often result to phase separation and lipid accumulation on the surface of the particles (Ingvarsson et al., 2013a). Therefore, critical quality attributes such as particle size, polydispersity, thermotropic behaviour and powder particle morphology were used to characterize the protective capacity of trehalose-dextran solutions on liposome colloidal stability.

Formulations prepared with 90 and 100% trehalose in the carrier matrix maintained the particle size before and after spray drying (p-value = 0.593 and 0.90 respectively) (Table 2, Supplementary Fig.S3). Additionally, the PDI value of ≤ 0.3 indicated that the particles were monodisperse after rehydration of the powders (Danaei et al., 2018). Conversely, the sizes of formulations prepared with excess dextran (10 and 50% trehalose) in the carrier matrix showed a 3-fold increase in the sizes after spray drying, and the PDIs indicated polydisperse particles after rehydration (Table 2). CAF04 liposomes are characteristically small liposomes (<250 nm) with a low PDI (<0.3). Therefore, formulations with particle sizes greater than 250 nm and PDI greater than 0.3 were considered unstable.

The results indicated that liposomes prepared with 90 and 100% trehalose were protected sufficiently during spray drying and rehydrated back to the initial sizes. Contrary, the DLS results from formulations with excess dextran (10 and 50% trehalose) indicated insufficient protection due to particle aggregation during rehydration. Aggregation often occurs as a result of merging of the bilayer, as in the case for formulations spray dried with excess dextran.

The zeta potential of all formulations remained positive and above 20 mV showing that the liposomes retained their cationic state (Table 2). Changes to the zeta potential of the particles would be expected in case of surface interactions between DDA and MMG upon rehydration, a phenomenon previously reported (Davidsen et al., 2005). As a result, the lipid ratio between MMG and DDA is more critical to the surface charge than carbohydrate composition.

SEM was used to further characterize the formulations after spray drying, and the images showed powder particles with different characteristics (Fig. 3). Formulations prepared with 90 and100% trehalose

Table 2
Characterization of formulations with varying trehalose-dextran solutions before (BSD) and after (ASD) spray drying. (Mean ± SD, $n = 3$).

Formulation		10% trehalose	50% trehalose	90% trehalose	100% trehalose
Size (d.nm)	BSD	249 ± 116	221 ± 70	180 ± 28	212 ± 56
	ASD	699 ± 114	582 ± 56	211 ± 35	204 ± 20
PDI	BSD	0.3	0.2	0.3	0.3
	ASD	0.7	0.5	0.2	0.2
Zeta potential (mV)	BSD	47 ± 3	48 ± 0	51 ± 2	49 ± 2
	ASD	38 ± 3	37 ± 4	44 ± 2	49 ± 3
% Process yield		44 ± 14	39 ± 13	45 ± 11	41 ± 13
% Moisture content		5.0 ± 1.4	3.8 ± 1.5	3.6 ± 1.4	3.2 ± 1.2

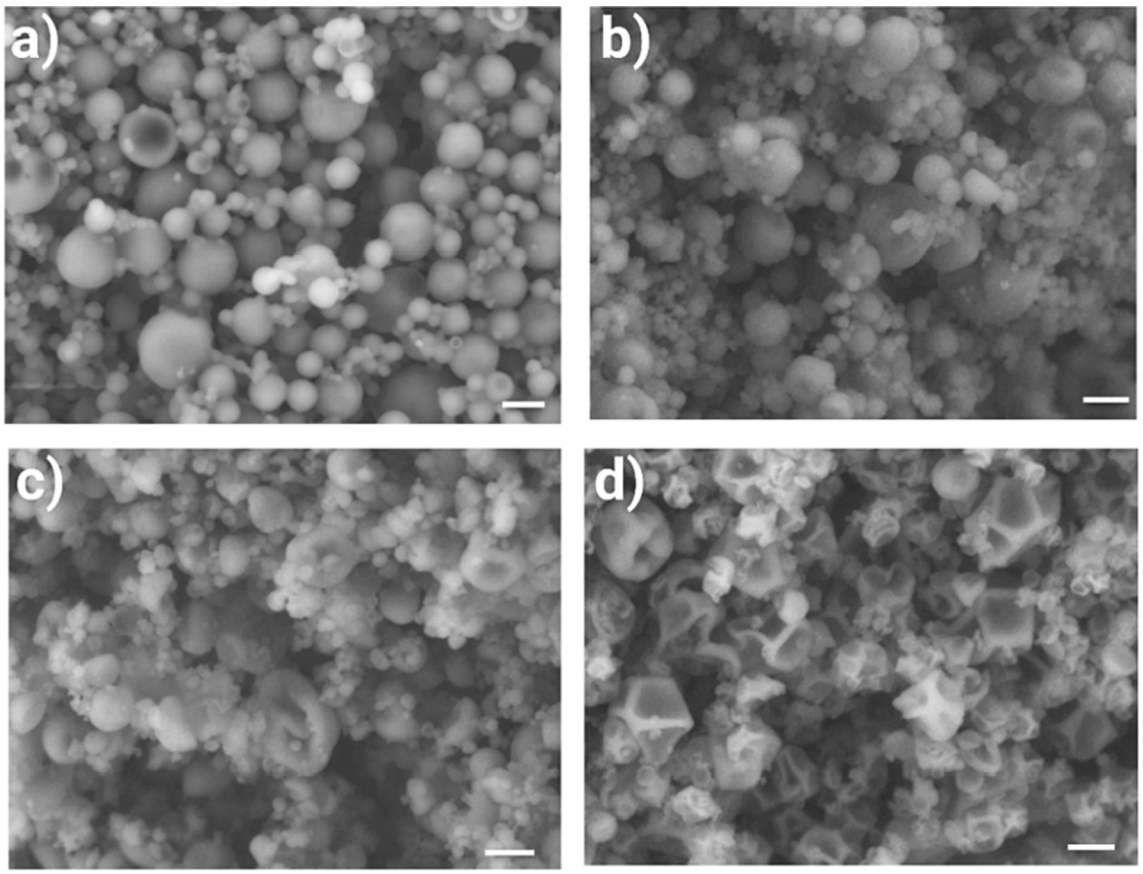

Fig. 3. SEM micrographs of CAF04 (15% w/w) dried powder particles with varying ratios of trehalose-and dextran as spray drying precursors. a)-100% trehalose, b)-90% trehalose, c)-50% trehalose, d)-10% trehalose. Scale bar – 3 μm.

displayed spherical particles (Fig. 3a and b). In contrast, formulations prepared with 10 and 50% trehalose showed wrinkled particles (Fig. 3c and d). The wrinkled particles was hypothesized to be an indication of varying drying kinetics which is specific to the composition of the spray drying precursors. *Both et al.* linked the dried powder particle morphology to the rheological properties of the feedstock concentration and the drying rate of the particles. It was found that during drying, if particle shell formation occurred faster than phase separation, there would be regions rich in one of the components resulting in wrinkled particles due to slower drying rates (Both et al., 2019). *Ekdahl et al.* further determined that the Peclet number had an influence in the drying mechanics of particles. Briefly, atomized particles having a low Peclet number have a lower internal solvent partial pressure causing the particle to collapse (Ekdahl et al., 2019). The Peclet number is influenced by the viscosity of the solutions and how fast droplets form upon atomization. The morphology of the powder particles could affect the ease of powder dissolution, as the shape affects the surface area of contact between the particles and the solution.

We aimed to investigate the effect of particle shape on powder rehydration. It was expected that spherical particles having even and large surface area would have more contact with the medium and therefore achieve rapid lipid rehydration. Therefore, the thermotropic phase behavior of the liposomes was used to further determine the ease of redispersibility after spray drying. DSC uses the phase transition temperature (T_p) to determine the extent of lipid redispersion in the bilayer. T_p is a characteristic temperature at which change in the lipid physical state occurs from the ordered gel phase, where the hydrocarbon chains are fully extended and tightly packed, to the disordered liquid crystalline phase, where the hydrocarbon chains are randomly oriented and fluid (Kim, 2016).

The main phase transition temperature was found at 42 °C across the formulations before spray drying (Fig. 4), as reported elsewhere in literature (Davidsen et al., 2005). This peak remained dominant also after spray drying for formulations with 90 and100% trehalose. Additionally, formulations with 90 and100% trehalose showed one small post transition peak at 46 °C (Fig. 4). In contrast, formulations containing 10–50% trehalose showed multiple peaks after spray drying, one small peak at 42 °C, a large peak at 45 °C and another at 50 °C (Fig. 4).

A single peak in a DSC thermogram of liposomes indicates homogeneous lipid dispersion within the bilayer, or phase equilibrium with complete lipid dispersion. T_p may be applied as a vesicle fingerprint and used for identification of liposome quality (Blandamer et al., 1992; Fan et al., 2021; Feitosa et al., 2000). The main phase transition temperature of neat DDA is 47 °C (Feitosa et al., 2000). Stabilizing lipids and molecules reduce the T_p of CAF® liposomes such as CAF04 to 42.62 ± 0.02 °C (Davidsen et al., 2005). Therefore, the occurrence of the main peak at 42 °C in formulations with 90 and 100% trehalose confirms the presence of vesicles before spray drying, which were maintained during spray drying, i.e., successful redispersion of the lipids after spray drying. For formulations with 10 and 50% trehalose, it is possible that liposomes are not fully rehydrated after spray drying, possibly due to the powder particle morphology, which reiterates the importance of the particle shape on the rehydration of the particles. Furthermore, the main peak for formulations with 10 and 50% trehalose is closer to the main phase transition temperature of neat DDA vesicles (47 °C) (Christensen et al., 2007; Davidsen et al., 2005).Therefore, the presence of large multiple peaks post the T_p is indicative of highly ordered DDA-rich domains which are difficult to rehydrate, or incomplete redispersion of excess DDA into the bilayer.

Interestingly, the carbohydrate composition seemed affect the moisture content, since the formulations with a high ratio of dextran appeared to retain more moisture compared to samples with less

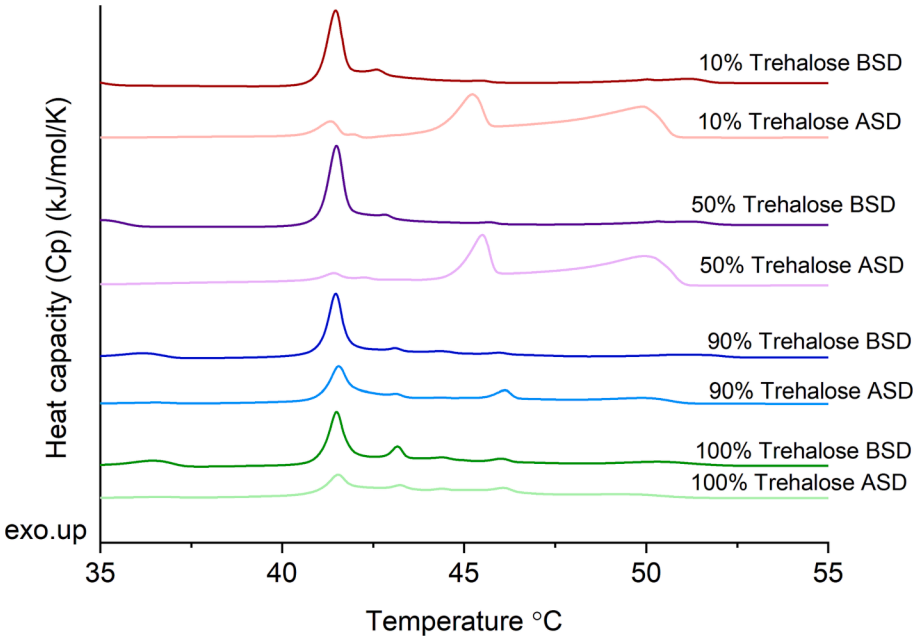

Fig. 4. Normalized thermograms of CAF04 formulations prepared with varying trehalose-dextran solutions before (BSD) and after spray drying (ASD). The scans have been displaced on the heat capacity axis for clarity. The results are representative of n = 3 individual measurements.

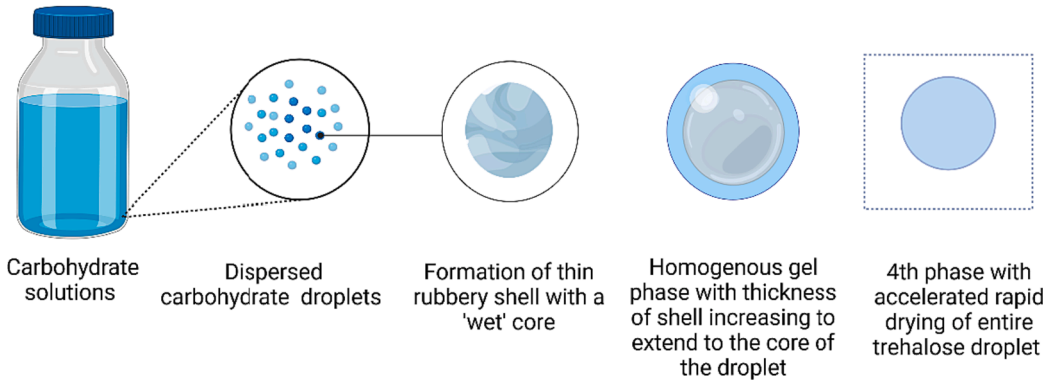

Fig. 5. Schematic of dehydration of dextran and trehalose droplets.

dextran, though not statistically significant (*p*-value greater than 0.05 – Supplementary Fig. S3). This phenomenon is hypothesized to be due to the drying kinetics of dextran solutions. During isothermal drying of dextran solutions, the evaporation rate is lowered by the formation of a rubbery thin shell on the droplet surface, followed by a homogeneous gel phase, where the shell increases in thickness due to changes (Fig. 3). Trehalose solutions have an additional step, during which the moisture in the core dries rapidly (Aksan and Toner, 2004). Likewise, trehalose-dextran solutions alter the particle drying technique due to the increased viscosity of the solutions, leading to reduced particle diffusivity. Accordingly, slower drying rates in formulations with excess dextran causes particles to retain more moisture, resulting in aggregation of liposomes when rehydrated. Furthermore, the retention of moisture in samples with excess of dextran is indicative of the varying degrees of moisture sorption by the carbohydrates, with trehalose having a lower moisture sorption capacity than dextran (Fakes et al., 2000).

To summarize, formulations with minimal dextran enrichment (90 and100% trehalose) maintained the size and PDI, the thermotropic behaviour and spherical and smooth powder particles after spray drying. Conversely, formulations with excess dextran (10 and 50% trehalose) changed drastically after spray drying, as evident in the increase in size and PDI and the shift in the phase transition temperature as well as the wrinkled powder particles. These changes indicate an occurrence of regions of unincorporated lipids, which can be attributed to inadequate protection during drying, i.e. slower drying rates. Therefore, it can be deduced that drying using trehalose-dextran precursors relies on abundance of trehalose for successful protection of liposomes.

3.2. *Increasing lipid concentration in CAF04 formulations*

Previous studies have demonstrated that the highest lipid concentration of CAF01 that can be spray dried was at 10% w/w of the total solid feedstock concentration (Ingvarsson et al., 2013a). One aim of this project was to increase the liposome concentration in the formulation, while maintaining stability after spray drying. To investigate the influence of the lipid content on the protective capacity of trehalose-dextran spray drying precursors, formulations with lipid solid content ranging from 15% w/w to 20% w/w CAF04 were prepared and spray dried.

To determine whether the particles became unstable after spray drying, the particles' light scattering was measured before and after spray drying and the measurement was correlated to their sizes. Monodisperse formulations for CAF04 liposomes with particle size of 200 ± 50 nm and PDI below 0.3 were deemed to be acceptable. It was found that formulations prepared with 15% w/w CAF04 in the

formulation did not have a significant change in their sizes after spray drying (p-value = 0.743 for 100%-trehalose, and p-value = 0.446 for 90%-trehalose) (Table 3, Supplementary Fig. S4). Conversely, formulations with 20% w/w CAF04 had significant changes in the sizes after spray drying (p-value = 0.04 for 100%-trehalose, and p-value = 0.0001 for 90%-trehalose). Additionally, PDI values of formulations with up to 15% CAF04 content were less than or equal to 0.3, while those of formulations with 20% CAF04 content increased to 0.4 and 0.5, respectively (Table 3).

Comparing the sizes of formulations with 15% w/w CAF04, there was no significant difference in formulations with 100% trehalose and 90% trehalose before and after spray drying (p-value = 0.99 and 0.86 respectively - Supplementary Fig. S4). It can be deduced that the formulations with 15% w/w CAF04 were adequately protected during drying, as the particle sizes were comparable after drying and rehydration, and the formulations were monodisperse. In contrast, for the formulations prepared with 20% w/w CAF04, the increase in size as well as the high PDI could be an indication of insufficient or unsuccessful protection during drying. Insufficient protection during drying in the formulations with 20% w/w CAF04 could be attributed to excess or unbound DDA amine groups. As stated earlier, one mechanism of protection is by forming hydrogen bonds between the trehalose head groups and the amine groups in the DDA (Christensen et al., 2007). This implies that the amount of trehalose should provide enough hydroxyl groups to engage with the lipid head groups. In the absence of sufficient hydroxyl groups, the liposomes are not fully protected which leads to merging of liposomes as water is drawn out during drying or collapsed liposomes. This will lead to aggregation which is reflected in the high PDI.

The zeta potential of liposomes reflects the vesicles surface net charge, which can influence the liposome colloidal stability and can also be used to evaluate the extent of charged lipid incorporation and head–group interaction (Davidsen et al., 2005). The zeta potential of the particles before and after spray drying was not significantly different for formulations prepared with 15% w/w CAF04-100%trehalose (p-value = 0.81). Conversely, the zeta potential for 15% w/w CAF04-90%trehalose, 20% w/w CAF04-100%trehalose and 20% w/w CAF04-90% trehalose decreased significantly (p-value = 0.02, p-value 0.0004 and p-value 0.002 respectively – Supplementary Fig. S4). Nevertheless, all formulations had zeta potential above 20 mV indicating cationic particles. The residual moisture content was not significantly different in all formulations.

DSC was applied to further characterize the redispersibility of the formulations. After spray drying, the post transition peaks appeared to be inflated in formulations with CAF04 concentration higher than 15% w/w (Fig. 6). Christensen *et al.* also demonstrated that in formulations with high lipid content, exothermic peaks usually occur after the main T_p due to DDA domains that are not easily redispersed into the vesicles, but often disappeared after dilution (Christensen et al., 2007).

The main T_p occurred at 42 °C for all formulations before spray drying (Fig. 6a). Additionally, formulations with 15% w/w CAF04 had a main T_p at 42 °C after spray drying, as well as small peaks after the main T_p (Fig. 6b). The small exothermic peaks after the main T_p in formulations with 15% w/w CAF04 were consistent with the formulations before spray drying (Fig. 6b). Therefore, based on the particle size characterization and the thermotropic phase behavior, we deduced that the liposomes were completely rehydrated and thus, adequately protected upon spray drying. After spray drying, formulations with 20% w/w CAF04 had a small T_p at 42 °C, a main T_p at 46 °C and a peak after 50 °C (Fig. 6b). The occurrence of the main T_p at 46 °C, close to the main T_p of neat DDA liposomes is consistent with the observation in the earlier section.

This reiterates the phenomenon of incomplete redispersion of lipids into the bilayer after spray drying due to inadequate protection. To achieve sufficient protection, there has to be a realistic ratio between the hydroxyl groups on trehalose and the lipid head groups. Previously, it was shown that 211 mM trehalose was required for successful protection of 3 mg/mL CAF01 upon freeze-drying (Christensen et al., 2007). Our studies showed that 60.7–67.4 mM (90–100% trehalose) could successfully protect up to 4.5 mg/mL (15% w/w) of CAF04 liposomes upon spray drying. Since more trehalose is required during freeze-drying, this discrepancy in trehalose threshold may be due to the drying method.

To further confirm whether the liposomes were rehydrated sufficiently after spray drying, cryoTEM imaging was performed. Evidently, lipid redispersion was attained for all samples, with samples containing 100% trehalose occasionally exhibiting multilamellar vesicles. (Fig. 7, Supplementary Fig S2.) and those enriched with dextran showing unilamellar vesicles. The sizes of the liposomes were estimated from the cryoTEM micrographs of 15 different particles and the sizes appeared significantly lower than sizes from the DLS measurements (Table 3 and Supplementary Table 2). This difference can be explained by the two different techniques. In DLS, Brownian motion is applied to determine the particle size, while in cryoTEM the samples are analyzed in 2D. Also, the DLS is a scattering method, using laser diffraction to estimate the size. Therefore, larger particles tend to scatter more, and presence of aggregates often results in larger hydrodynamic sizes and the particle sizes could be exaggerated.

4. Conclusion

The combination of cryoTEM and thermotropic methods were complimentary in characterizing the liposomes rehydration because thermotropic methods gave an insight into the behavior of the liposomes in relation to lipid equilibrium, while the cryoTEM method confirmed the presence and appearance of liposomes after spray drying. Although, it was predicted that trehalose and dextran are not soluble on the molecular scale, it was deduced that the solubility of the two carbohydrates did not hinder protection of liposomes upon drying, as evident in the cryoTEM images showing presence of liposomes across all formulations. Conclusively, it was demonstrated that trehalose fortified with small amounts of dextran adequately protects CAF04 liposomes during spray drying.

Additionally, a 2.4-fold increase in lipid concentration (3 mM vs 7.245 mM) for spray dried CAF04 liposomes was achieved. This can influence the dosing of CAF04-based formulations, as the amount of active ingredient could be higher. Further studies such as formulation with antigens or drugs will be insightful in the future in understanding the quality of liposomes after spray drying with the trehalose-dextran matrix. Ultimately, it is clear that trehalose is essential as the main

Table 3
Characterization of formulations with increased lipid content before (BSD) and after (ASD) spray drying. (Mean ± SD, $n = 3$).

Formulation		15% w/w CAF04 – 100% trehalose	15% w/w CAF04 – 90% trehalose	20% w/w CAF04 – 100% trehalose	20% w/w CAF04 – 90% trehalose
Size (d.nm)	BSD	177 ± 26	192 ± 17	178 ± 22	181 ± 18
	ASD	192 ± 21	228 ± 6	335 ± 30	710 ± 150
PDI	BSD	0.2	0.3	0.3	0.3
	ASD	0.2	0.2	0.4	0.7
Zeta potential (mV)	BSD	48 ± 3	52 ± 4	51 ± 2	52 ± 2
	ASD	48 ± 3	43 ± 0	40 ± 5	41 ± 2
% Process yield		58 ± 3	61 ± 3	58 ± 4	52 ± 5
% Moisture content		2.7 ± 1.5	3.4 ± 1.4	3.5 ± 1.4	3.4 ± 0.7

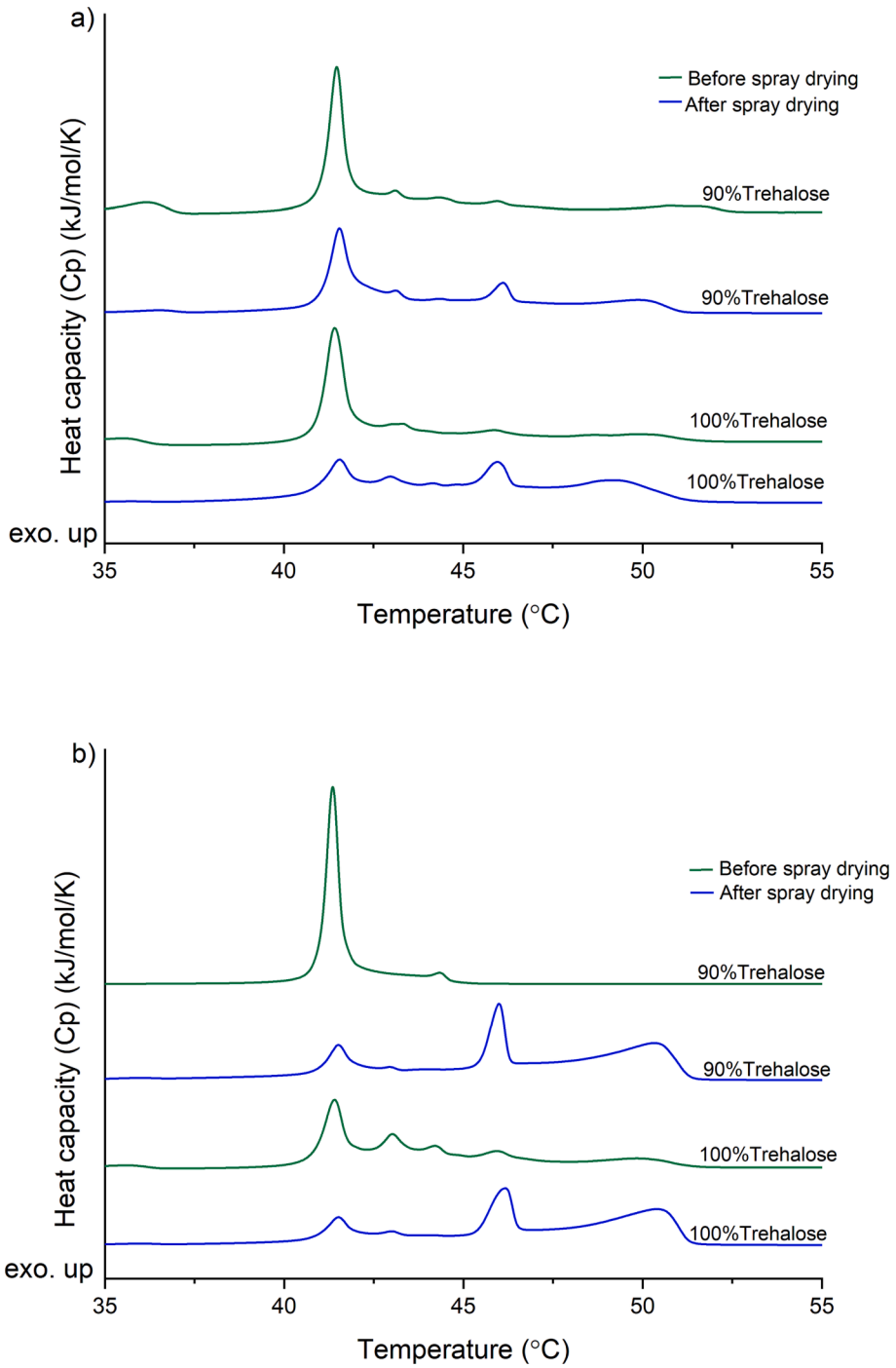

Fig. 6. Normalized DSC thermograms of CAF04 liposomes in increased lipid content before and after spray drying. a)15% w/w CAF04, b) 20% w/w CAF04.The scans have been displaced on the heat capacity axis for clarity.

component during drying of CAF04 liposomes, and a small amount of dextran can be incorporated to act as a vitrifier, and to control the particles' drying rate.

CRediT authorship contribution statement

Anitta Lutta: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **Matthias M. Knopp:** Writing – review & editing, Validation, Formal analysis, Data curation. **Matteo Tollemeto:** . **Gabriel K. Pedersen:** . **Signe T. Schmidt:** Writing – review & editing, Supervision, Project administration, Investigation, Conceptualization. **Holger Grohganz:** . **Line Hagner Nielsen:** Conceptualization, Supervision, Writing - review and editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: AL, GKP, STS are employed at Statens Serum Institut, which is a non-profit government research facility and holds patents on the CAF®-based adjuvants. All other authors declare that there are no competing interests.

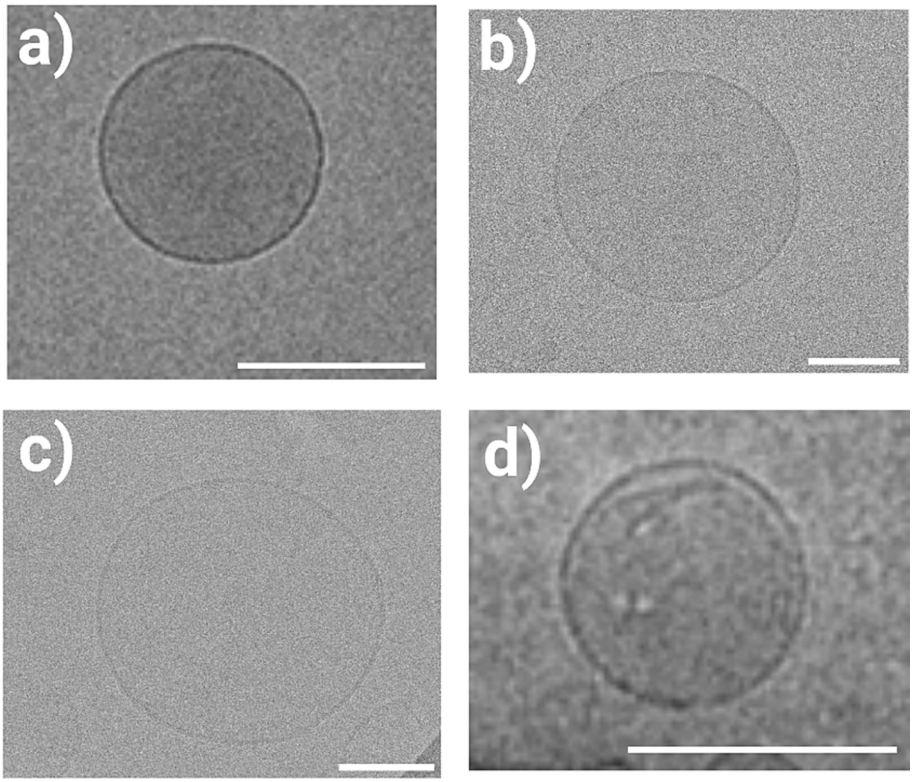

Fig. 7. CryoTEM images of 15% w/w CAF04 formulations after spray drying, with varying ratios of trehalose and dextran. a) 10% trehalose, b) 50% trehalose, c) 90% trehalose, d) 100% trehalose. Scale bar – 200 nm.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2024.123798.

References

Adali, M.B., Barresi, A.A., Boccardo, G., Pisano, R., 2020. Spray freeze-drying as a solution to continuous manufacturing of pharmaceutical products in bulk. In Processes 8 (6). https://doi.org/10.3390/pr8060709.

Ajitha, A.R., Thomas, S., 2020. Chapter 1 - Introduction: Polymer blends, thermodynamics, miscibility, phase separation, and compatibilization - Compatibilization of Polymer Blends. Elsevier, pp. 1–29. https://doi.org/10.1016/B978-0-12-816006-0.00001-3.

Aksan, A., Toner, M., 2004. Isothermal Desiccation and Vitrification Kinetics of Trehalose−Dextran Solutions. Langmuir 20 (13), 5521–5529. https://doi.org/10.1021/la0355186.

Alhajj, N., O'Reilly, N.J., Cathcart, H., 2021. Designing enhanced spray dried particles for inhalation: A review of the impact of excipients and processing parameters on particle properties. Powder Technol. 384, 313–331. https://doi.org/10.1016/j.powtec.2021.02.031.

Allison, S.D., Manning, M.C., Randolph, T.W., Middleton, K., Davis, A., Carpenter, J.F., 2000. Optimization of Storage Stability of Lyophilized Actin Using Combinations of Disaccharides and Dextran. J. Pharm. Sci. 89 (2), 199–214. https://doi.org/10.1002/(SICI)1520-6017(200002)89:2<199::AID-JPS7>3.0.CO;2-B.

Aroffu, M., Manca, M. L., Pedraz, J. L., & Manconi, M. (n.d.). Liposome-based vaccines for minimally or noninvasive administration: an update on current advancements. Expert Opin. Drug Deliv, 1–21. https://doi.org/10.1080/17425247.2023.2288856.

Barbălată, C.-I., Porfire, A.S., Ambrus, R., Mukhtar, M., Farkas, Á., Tomuță, I., 2023. Process development of inhalation powders containing Simvastatin loaded liposomes using spray drying technology. J. Liposome Res., just-accepted 1–20.

Baryakova, T.H., Pogostin, B.H., Langer, R., McHugh, K.J., 2023. Overcoming barriers to patient adherence: the case for developing innovative drug delivery systems. Nat. Rev. Drug Discov. 22 (5), 387–409. https://doi.org/10.1038/s41573-023-00670-0.

Blandamer, M.J., Briggs, B., Cullis, P.M., Green, J.A., Waters, M., Soldi, G., Engberts, J.B. F.N., Hoekstra, D., 1992. Differential scanning microcalorimetric study of vesicles in aqueous solutions formed by dimethyldioctadecylammonium bromide. J. Chem. Soc. {,} Faraday Trans. 88 (23), 3431–3434. https://doi.org/10.1039/FT9928803431.

Both, E.M., Siemons, I., Boom, R.M., Schutyser, M.A.I., 2019. The role of viscosity in morphology development during single droplet drying. Food Hydrocoll. 94, 510–518. https://doi.org/10.1016/j.foodhyd.2019.03.023.

Carlsen, P.H.R., Kjeldsen, R.B., Pedersen, G.K., Christensen, D., Nielsen, L.H., Boisen, A., 2023. Oral vaccination using microdevices to deliver α-GalCer adjuvanted vaccine afford a mucosal immune response. J. Control. Release 353, 134–146. https://doi.org/10.1016/j.jconrel.2022.11.015.

Chowdhary, P., Mahalakshmi, L., Dutta, S., Moses, J.A., Anandharamakrishnan, C., 2023. Chapter 11 - Strategies for stabilization and preservation of liposomes - Liposomal encapsulation in Food Science and Technology. Elsevier, pp. 223–237. https://doi.org/10.1016/B978-0-12-823935-3.00014-X.

Christensen, D., Foged, C., Rosenkrands, I., Nielsen, H.M., Andersen, P., Agger, E.M., 2007. Trehalose preserves DDA/TDB liposomes and their adjuvant effect during freeze-drying. *Biochimica et Biophysica Acta (BBA)* - Biomembranes 1768 (9), 2120–2129. https://doi.org/10.1016/j.bbamem.2007.05.009.

Costa, C., Nobre, B., Matos, A.S., Silva, A.S., Casimiro, T., Corvo, M.L., Aguiar-Ricardo, A., 2021. Inhalable hydrophilic molecule-loaded liposomal dry powder formulations using supercritical CO2 – assisted spray-drying. J. CO2 Util. https://api.semanticscholar.org/CorpusID:240532001.

Crowe, J.H., Oliver, A.E., Hoekstra, F.A., Crowe, L.M., 1997. Stabilization of Dry Membranes by Mixtures of Hydroxyethyl Starch and Glucose: The Role of Vitrification. Cryobiology 35 (1), 20–30. https://doi.org/10.1006/cryo.1997.2020.

Danaei, M., Dehghankhold, M., Ataei, S., Hasanzadeh Davarani, F., Javanmard, R., Dokhani, A., Khorasani, S., Mozafari, M.R., 2018. Impact of Particle Size and Polydispersity Index on the Clinical Applications of Lipidic Nanocarrier Systems. Pharmaceutics 10 (2). https://doi.org/10.3390/pharmaceutics10020057.

Davidsen, J., Rosenkrands, I., Christensen, D., Vangala, A., Kirby, D., Perrie, Y., Agger, E. M., Andersen, P., 2005. Characterization of cationic liposomes based on dimethyldioctadecylammonium and synthetic cord factor from M. tuberculosis

(trehalose 6,6′-dibehenate)—A novel adjuvant inducing both strong CMI and antibody responses. Biochimica et Biophysica Acta (BBA) - Biomembranes 1718 (1), 22–31. https://doi.org/10.1016/j.bbamem.2005.10.011.

Dieplinger, J., Pinto, J.T., Dekner, M., Brachtl, G., Paudel, A., 2023. Impact of Different Saccharides on the In-Process Stability of a Protein Drug During Evaporative Drying: From Sessile Droplet Drying to Lab-Scale Spray Drying. Pharm. Res. 40 (5), 1283–1298. https://doi.org/10.1007/s11095-023-03498-w.

Ekdahl, A., Mudie, D., Malewski, D., Amidon, G., Goodwin, A., 2019. Effect of Spray-Dried Particle Morphology on Mechanical and Flow Properties of Felodipine in PVP VA Amorphous Solid Dispersions. J. Pharm. Sci. 108 (11), 3657–3666. https://doi.org/10.1016/j.xphs.2019.08.008.

Fakes, M., Dali, M., Haby, T., morris, kenneth, Varia, S. A., & Serajuddin, A. (2000). Moisture Sorption Behavior of Selected Bulking Agents Used in Lyophilized Products. PDA J. Pharm. Sci. Technol./PDA, 54, 144–149.

Fan, Y., Marioli, M., Zhang, K., 2021. Analytical characterization of liposomes and other lipid nanoparticles for drug delivery. J. Pharm. Biomed. Anal. 192, 113642 https://doi.org/10.1016/j.jpba.2020.113642.

Feitosa, E., Barreleiro, P.C.A., Olofsson, G., 2000. Phase transition in dioctadecyldimethylammonium bromide and chloride vesicles prepared by different methods. Chem. Phys. Lipids 105 (2), 201–213. https://doi.org/10.1016/S0009-3084(00)00127-4.

Franzé, S., Selmin, F., Samaritani, E., Minghetti, P., Cilurzo, F., 2018. Lyophilization of liposomal formulations: still necessary, still challenging. Pharmaceutics 10 (3). https://doi.org/10.3390/pharmaceutics10030139.

Giordani, S., Marassi, V., Zattoni, A., Roda, B., Reschiglian, P., 2023. Liposomes characterization for market approval as pharmaceutical products: Analytical methods, guidelines and standardized protocols. J. Pharm. Biomed. Anal. 236, 115751 https://doi.org/10.1016/j.jpba.2023.115751.

Giuffrida, S., Cottone, G., Cordone, L., 2017. The water association band as a marker of hydrogen bonds in trehalose amorphous matrices. Phys. Chem. Chem. Phys. 19 (6), 4251–4265. https://doi.org/10.1039/C6CP06848K.

Guimarães, D., Noro, J., Silva, C., Cavaco-Paulo, A., & Nogueira, E. (2019). Protective Effect of Saccharides on Freeze-Dried Liposomes Encapsulating Drugs. Front. Bioeng. Biotechnol. (Vol. 7). https://www.frontiersin.org/articles/10.3389/fbioe.2019.00424.

Haeuser, C., Goldbach, P., Huwyler, J., Friess, W., Allmendinger, A., 2020. Impact of dextran on thermal properties, product quality attributes, and monoclonal antibody stability in freeze-dried formulations. Eur. J. Pharm. Biopharm. 147, 45–56. https://doi.org/10.1016/j.ejpb.2019.12.010.

Imamura, K., Ogawa, T., Sakiyama, T., Nakanishi, K., 2003. Effects of Types of Sugar on the Stabilization of Protein in the Dried State. J. Pharm. Sci. 92 (2), 266–274. https://doi.org/10.1002/jps.10305.

Ingvarsson, P.T., Yang, M., Nielsen, H.M., Rantanen, J., Foged, C., 2011. Stabilization of liposomes during drying. Expert Opin. Drug Deliv. 8 (3), 375–388. https://doi.org/10.1517/17425247.2011.553219.

Ingvarsson, P.T., Schmidt, S.T., Christensen, D., Larsen, N.B., Hinrichs, W.L.J., Andersen, P., Rantanen, J., Nielsen, H.M., Yang, M., Foged, C., 2013a. Designing CAF-adjuvanted dry powder vaccines: Spray drying preserves the adjuvant activity of CAF01. J. Control. Release 167 (3), 256–264. https://doi.org/10.1016/j.jconrel.2013.01.031.

Ingvarsson, P.T., Yang, M., Mulvad, H., Nielsen, H.M., Rantanen, J., Foged, C., 2013b. Engineering of an Inhalable DDA/TDB Liposomal Adjuvant: A Quality-by-Design Approach Towards Optimization of the Spray Drying Process. Pharm. Res. 30 (11), 2772–2784. https://doi.org/10.1007/s11095-013-1096-2.

Jójárt-Laczkovich, O., Katona, G., Aigner, Z., Szabó-Révész, P., 2016. Investigation of recrystallization of amorphous trehalose through hot-humidity stage X-ray powder diffraction. Eur. J. Pharm. Sci. 95, 145–151. https://doi.org/10.1016/j.ejps.2016.08.003.

Kim, J.-S., 2016. Liposomal drug delivery system. J. Pharm. Investig. 46 (4), 387–392. https://doi.org/10.1007/s40005-016-0260-1.

Knopp, M.M., Tajber, L., Tian, Y., Olesen, N.E., Jones, D.S., Kozyra, A., Löbmann, K., Paluch, K., Brennan, C.M., Holm, R., Healy, A.M., Andrews, G.P., Rades, T., 2015. Comparative Study of Different Methods for the Prediction of Drug-Polymer Solubility. Mol. Pharm. 12 (9), 3408–3419. https://doi.org/10.1021/acs.molpharmaceut.5b00423.

Larsen, B.S., Skytte, J., Svagan, A.J., Meng-Lund, H., Grohganz, H., Löbmann, K., 2019. Using dextran of different molecular weights to achieve faster freeze-drying and improved storage stability of lactate dehydrogenase. Pharm. Dev. Technol. 24 (3), 323–328. https://doi.org/10.1080/10837450.2018.1479866.

Li, W., Yun, L., Zhao, Y., Zhi, Z., Muhindo, E.M., Geng, X., Liu, R., Wu, T., Sui, W., Zhang, M., 2022. Effect of water sorption on glass transition and microstructural variation of dextran & sugar mixtures. Carbohydr. Polym. 290, 119505 https://doi.org/10.1016/j.carbpol.2022.119505.

Liu, P., Chen, G., and Zhang, J. (2022). A Review of Liposomes as a Drug Delivery System: Current Status of Approved Products, Regulatory Environments, and Future Perspectives. Molecules (Basel, Switzerland), 27(4). https://doi.org/10.3390/molecules27041372.

Marasini, N., Ghaffar, K. A., Skwarczynski, M., and Toth, I. (2017). Chapter Twelve - Liposomes as a Vaccine Delivery System. In M. Skwarczynski & I. B. T.-M. and N. in V. D. Toth (Eds.), Micro and Nano Technologies (pp. 221–239). William Andrew Publishing. https://doi.org/10.1016/B978-0-323-39981-4.00012-9.

Marsac, P.J., Shamblin, S.L., Taylor, L.S., 2006. Theoretical and Practical Approaches for Prediction of Drug-Polymer Miscibility and Solubility. Pharm. Res. 23 (10), 2417–2426. https://doi.org/10.1007/s11095-006-9063-9.

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

Moreno Raja, M., Lim, P.Q., Wong, Y.S., Xiong, G.M., Zhang, Y., Venkatraman, S., Huang, Y., 2019. Chapter 18 - Polymeric Nanomaterials: Methods of Preparation and Characterization. In: Thomas (Ed.), Micro and Nano Technologies. Elsevier, pp. 557–653. https://doi.org/10.1016/B978-0-12-814033-8.00018-7.

Mohammadi, M., Hamishehkar, H., Ghorbani, M., Shahvalizadeh, R., Pateiro, M., Lorenzo, J.M., 2021. Engineering of liposome structure to enhance physicochemical properties of Spirulina plantensis protein hydrolysate: Stability during spray-drying. Antioxidants 10 (12), 1953.

Moura, C., Casimiro, T., Costa, E., Aguiar-Ricardo, A., 2019. Optimization of supercritical CO2-assisted spray drying technology for the production of inhalable composite particles using quality-by-design principles. Powder Technol. 357, 387–397. https://doi.org/10.1016/j.powtec.2019.08.090.

Newman, A., Zografi, G., 2020. What We Need to Know about Solid-State Isothermal Crystallization of Organic Molecules from the Amorphous State below the Glass Transition Temperature. Mol. Pharm. 17 (6), 1761–1777. https://doi.org/10.1021/acs.molpharmaceut.0c00181.

Nowak, D., Jakubczyk, E., 2020. The Freeze-Drying of Foods-The Characteristic of the Process Course and the Effect of Its Parameters on the Physical Properties of Food Materials. Foods (Basel, Switzerland) 9 (10). https://doi.org/10.3390/foods9101488.

Pedersen, G.K., Andersen, P., Christensen, D., 2018. Immunocorrelates of CAF family adjuvants. Semin. Immunol. 39, 4–13. https://doi.org/10.1016/j.smim.2018.10.003.

Penzol, G., Armisén, P., Fernández-Lafuente, R., Rodés, L., Guisán, J.M., 1998. Use of dextrans as long and hydrophilic spacer arms to improve the performance of immobilized proteins acting on macromolecules. Biotechnol. Bioeng. 60 (4), 518–523. https://doi.org/10.1002/(sici)1097-0290(19981120)60:4<518::aid-bit14>3.0.co;2-d.

Pollard, A.J., Bijker, E.M., 2021. A guide to vaccinology: from basic principles to new developments. Nat. Rev. Immunol. 21 (2), 83–100. https://doi.org/10.1038/s41577-020-00479-7.

Rask, M.B., Knopp, M.M., Olesen, N.E., Holm, R., Rades, T., 2018. Comparison of two DSC-based methods to predict drug-polymer solubility. Int. J. Pharm. 540 (1), 98–105. https://doi.org/10.1016/j.ijpharm.2018.02.002.

Romhányi, V., Kun, D., Pukánszky, B., 2018. Correlations among Miscibility, Structure, and Properties in Thermoplastic Polymer/Lignin Blends. ACS Sustain. Chem. Eng. 6 (11), 14323–14331. https://doi.org/10.1021/acssuschemeng.8b02989.

Song, E.-H., Shang, J., Ratner, D.M., 2012. *9.08 - Polysaccharides* -Polymer Science A Comprehensive Review. Elsevier, pp. 137–155. https://doi.org/10.1016/B978-0-444-53349-4.00246-6.

Sosnik, A., Seremeta, K.P., 2015. Advantages and challenges of the spray-drying technology for the production of pure drug particles and drug-loaded polymeric carriers. Adv. Colloid Interface Sci. 223, 40–54. https://doi.org/10.1016/j.cis.2015.05.003.

Susa, F., Bucca, G., Limongi, T., Cauda, V., Pisano, R., 2021. Enhancing the preservation of liposomes: The role of cryoprotectants, lipid formulations and freezing approaches. Cryobiology 98, 46–56. https://doi.org/10.1016/j.cryobiol.2020.12.009.

Taddio, A., Ipp, M., Thivakaran, S., Jamal, A., Parikh, C., Smart, S., Sovran, J., Stephens, D., Katz, J., 2012. Survey of the prevalence of immunization non-compliance due to needle fears in children and adults. Vaccine 30 (32), 4807–4812. https://doi.org/10.1016/j.vaccine.2012.05.011.

Thakur, A., Xu, Y., Cano-Garcia, G., Feng, S., Rose, F., Gerde, P., Andersen, P.M., Christensen, D., Foged, C., 2022. Optimizing the design and dosing of dry powder inhaler formulations of the cationic liposome adjuvant CAF®01 for pulmonary immunization. Front. Drug Deliv. https://api.semanticscholar.org/CorpusID:252093506.

Trenkenschuh, E., Friess, W., 2021. Freeze-drying of nanoparticles: How to overcome colloidal instability by formulation and process optimization. Eur. J. Pharm. Biopharm. 165, 345–360. https://doi.org/10.1016/j.ejpb.2021.05.024.

Tretiakova, D.S., Vodovozova, E.L., 2022. Liposomes as Adjuvants and Vaccine Delivery Systems. *Biochemistry (Moscow)*. Suppl. Series A: Membr. Cell Biol. 16 (1), 1–20. https://doi.org/10.1134/S1990747822020076.

Ubbink, J., 2016. Structural and thermodynamic aspects of plasticization and antiplasticization in glassy encapsulation and biostabilization matrices. Adv. Drug Deliv. Rev. 100, 10–26. https://doi.org/10.1016/j.addr.2015.12.019.

Vasanthavada, M., Tong, W.-Q., Joshi, Y., Kislalioglu, M.S., 2004. Phase Behavior of Amorphous Molecular Dispersions I: Determination of the Degree and Mechanism of Solid Solubility. Pharm. Res. 21 (9), 1598–1606. https://doi.org/10.1023/B:PHAM.0000041454.76342.0e.

Yu, J.Y., Chuesiang, P., Shin, G.H., Park, H.J., 2021. Post-Processing Techniques for the Improvement of Liposome Stability. Pharmaceutics 13 (7). https://doi.org/10.3390/pharmaceutics13071023.

Zhang, H. (2017). Thin-Film Hydration Followed by Extrusion Method for Liposome Preparation BT - Liposomes: Methods and Protocols (G. G. M. D'Souza (ed.); pp. 17–22). Springer New York. https://doi.org/10.1007/978-1-4939-6591-5_2.