



## Technical note

## Evaluation of the effects of spray drying parameters for producing cubosome powder precursors



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## ABSTRACT

Vaccines provide great benefit for global health, but are insufficiently distributed in developing countries due to high costs of manufacturing and limited storage stability. Spray drying formulations of peptide-based vaccines offer a promising strategy to reduce production costs and improve unrefrigerated storage stability. This design of experiments investigated how adjusting spray drying parameters (inlet temperature, atomization pressure, feed rate and aspiration rate) affects residual moisture and reconstitution properties of the powder product, and morphology and size of the rehydrated particles. An emulsion capable of forming cubosomes with the protein ovalbumin as model antigen was used as vaccine formulation and spray dried. The produced powders had low residual moisture content, independent of the spray drying parameters within the range investigated. Cryogenic transmission electron microscopy revealed that cubosomes were produced after rehydration regardless of the spray drying parameters. Furthermore, dynamic light scattering showed that the average diameter of the rehydrated particles consistently was close to 230 nm at any combination of spray drying parameters as long as the outlet temperature was kept below a threshold of 115 °C. The process thus appears to be robust allowing optimization to be focused on yield, dry particle engineering or outlet temperature considerations.

## 1. Introduction

Vaccines are a cost-effective medical intervention with great positive impact on global human health [1]. However, they remain insufficiently distributed in developing countries due to factors such as the high costs of manufacturing and the challenges of cold-chain distribution [2]. A promising method for improving vaccine stability is to use peptides instead of conventional whole organism antigens followed by drying the vaccine formulation [2,3].

To provide effective immunization, peptides need to be delivered in a particulate delivery system [1]. Cubosomes are lipid-based nanoparticles that have been shown *in vivo* to be a promising delivery system for vaccination [4,5]. They are composed of a highly twisted continuous lipid bilayer with three-dimensional long range order forming two discrete but highly tortuous water channels [5]. Cubosomes are traditionally produced as dispersions in water by fragmentation of bulk cubic phase liquid crystals (top-down) or by self-assembly of certain amphiphilic lipids upon solvent dilution in water (bottom-up) [6]. Recently, however, spray dried cubosome precursors containing the

protein ovalbumin (OVA) – a commonly used model peptide antigen – have been reported to form cubosomes in the size-range of 200–300 nm after hydration [5,7]. These cubosomes could furthermore be co-delivered with the adjuvant Quil-A with little effect on their structure and size and were able to stimulate strong humoral and cellular immune responses [5].

Spray dried vaccine formulations are easier and cheaper to transport compared to liquid formulations and can offer increased storage stability [5]. Attenuvax<sup>®</sup> is a lyophilized commercial live-attenuated measles vaccine. Its product package insert indicates that the storage stability is 24 months at 2–8 °C, but is reduced to 1 week at 37 °C and should be used within a maximum of 6 h after reconstitution [8]. Oh-take et al. reported on using spray drying to produce a powder formulation of a similar measles vaccine that was eight times more stable at 37 °C than the commercial lyophilized product [8]. Spray drying is a process in which a liquid feed is atomized into small droplets within a flow of hot air, resulting in fast evaporation of the solvent and formation of dry particles [3]. The method is simple, inexpensive and usually easy to scale up. Spray drying furthermore offers the possibility of high

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throughput production and is well suited for drying labile compounds. Therefore, it is a promising method for drying of vaccines [5,8]. However, changes in spray drying parameters aimed to optimize e.g. the yield may affect product properties such as the particle size. Similarly, up-scaling the production process to a commercial type spray drier will inevitably affect the drying parameters necessitating re-validation of the method and possibly expensive optimization work at the large scale. A nanoparticle system that is robust to changes in spray drying parameters is therefore desirable. However, comprehensive studies of the robustness of spray drying processes for pharmaceutical nanoparticulate formulations are rare.

The aim of this study was to investigate how the spray drying parameters (inlet temperature, atomization pressure, feed rate and aspiration rate) affect selected properties of a cubosomes formulated with ovalbumin. Powder product characteristics investigated in this study are the residual moisture and the powders ability to reconstitute. Following reconstitution, the size and morphology of the hydrated particles was measured. The study also evaluated two process parameters, namely the outlet temperature and the yield.

## 2. Materials and methods

A design of experiments (DOE) was constructed, comprising the main spray drying parameters (Table 1). A full factorial design (Table S1) with one replicate and a center point was created using SAS JMP® 13 (SAS Institute, Cary, NC, USA).

Dimodan® MO 90/D (monoolein) was generously provided by Danisco (Grindsted, Denmark). OVA (Grade VII, from chicken egg white) and dextran (from *Leuconostoc* spp., 40 kDa) were purchased from Sigma Aldrich (St. Louis, MO, USA). Purified water (MilliQ, Millipore) was used throughout the study. All other chemicals were of analytical grade and used as received.

A feed emulsion known to be able to generate cubosome precursors after spray drying was prepared as described previously [5]. Briefly, monoolein was dissolved at 5.33 mg/mL in ethanol. OVA and dextran were dissolved in water at 0.52 and 2.63 mg/mL and mixed with the ethanolic monoolein at a volume ratio of 3.04:1. This formed an emulsion which was spray dried using air as drying gas on a Büchi B-290 mini spray dryer (Büchi Labortechnik AG, Flawil, Switzerland). The spray dryer was operated in open-loop configuration with a B-296 dehumidifier (Büchi Labortechnik AG, Flawil, Switzerland) and a pressure nozzle with a diameter of 1.5 mm. Each formulation was spray dried according to the parameters specified by the DOE (Table S1).

Yield was evaluated as the weight of the product relative to the dry weight of the feed. Residual moisture was assessed by the weight loss of the powder after a secondary drying step at 57 °C for 24 h in a dry atmosphere. The powders ability to redispense was then evaluated by rehydrating particles at 20 mg/mL in water by vortexing and grading the dispersion by visual inspection into the categories described in Table 2. The size (average hydrodynamic diameter) of particles

**Table 1**  
Spray drying parameters (inlet temperature, atomization pressure, feed rate and aspiration rate), limits and outputs of the DOE design.

Inputs:			
Parameter	Low	High	Center-point
Inlet temperature (°C)	85	200	142.5
Atomization pressure	3.5	5	4.25
Feed rate (mL/min)	0.9	4.5	2.7
Aspiration rate (%)	60	100	80
Outputs:			
Process outputs:	Outlet temperature and yield		
Powder product outputs:	Residual moisture and ease of reconstitution to form an aqueous dispersion		
Hydrated particle outputs: (reconstituted)	Particle size and morphology		

**Table 2**  
Classification scheme for grading the reconstitution properties of spray dried powders.

Grade	Description
0	Clear and transparent liquid with large aggregates
1	Translucent dispersion with easily visible aggregates
2	Cloudy dispersion, aggregates hardly visible
3	Homogeneous milky dispersion, no visible aggregates

rehydrated at 0.1 mg/mL in water was measured by dynamic light scattering (DLS) on a Zetasizer Nano ZS (Malvern Zetasizer, NanoZS Zen 3600, Malvern, UK) equipped with a 633 nm laser and 173° detection optics at 25 °C. The size was calculated from 3 replicates using the cumulant method with a dispersant viscosity of 0.8872 cP. Particle morphology was evaluated by cryogenic transmission electron microscopy (cryo-TEM) using a Tecnai G2 20 TWIN transmission electron microscope (FEI, Hillsboro, OR, USA) as described previously [5]. The experimental results were analyzed with custom written software (MATLAB® R2018a, The MathWorks Inc., Natick, MA, U.S.A. The script is available online per Nov. 2nd, 2018: <https://doi.org/10.11583/DTU.c.4213148>) and with SAS JMP® 13 using the standard least squares approach and a significance level of 5%. Results are given as mean ± standard deviation where relevant. For DLS, the minimum mean count rate for individual measurement replicates was 100 kcps.

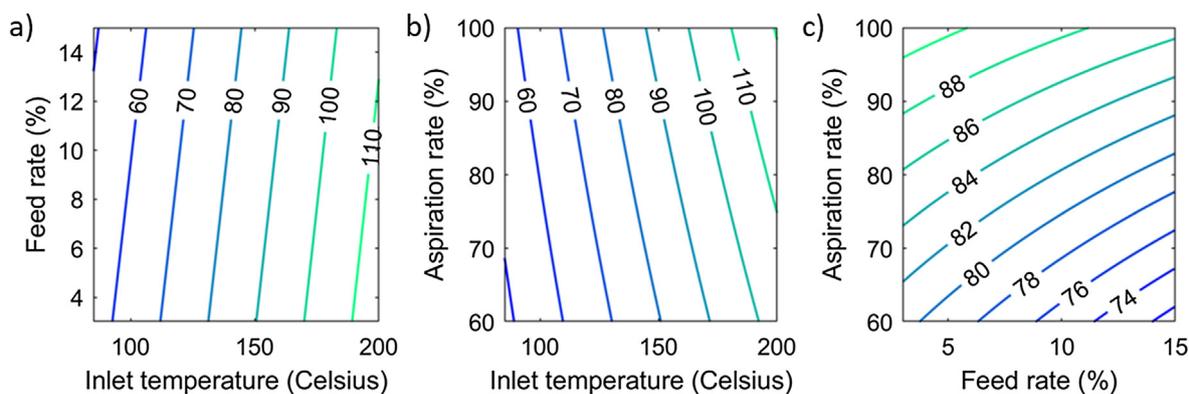
## 3. Results and discussion

Cubosomes were spray dried using different combinations of spray drying settings to evaluate the influence of these on properties of the produced powder as well as of the rehydrated particles. Models were fitted to the experimental output data to determine how each output parameter depends on the input parameters. These will be discussed below.

The outlet temperature was well explained by the model ( $r^2 = 0.96$ , Fig. S1). The inlet temperature, aspiration rate and feed rate were significant parameter effects, while their interactions were insignificant (all p-values ≤ 0.010, Table S3). To control the outlet temperature, the inlet temperature is the most important parameter, but the aspiration rate and feed rate are also influential as shown in Fig. 1. Decreasing the inlet temperature and aspiration rate while increasing the feed rate minimizes the outlet temperature.

The powder yield was also well explained by the model ( $r^2 = 0.96$ , Fig. S1d) with all parameter effects significant (p-value < 0.001) and two significant first order interactions (Table S3). The effect of the parameters are shown in Fig. 2. To obtain a high yield, a low atomization pressure and a high aspiration rate combined with a low inlet temperature and a high feed rate is optimal. Low atomization pressure gives larger droplets and should therefore give larger powder particles that are more easily caught in the cyclone. A high aspiration rate results in a larger centrifugal force in the cyclone expected to help to sediment the particles. A low inlet temperature and high feed rate can give moist powders, thus artificially increasing the yield. However, the produced powders were all dry (residual moisture =  $1 \pm 0.02\%$ , Table S2) and the model describing the residual moisture was insignificant (p-value of 0.372, Fig. S1e). The low inlet temperature and high feed rate may also give a slower drying rate thus giving more time during drying for diffusion and rearrangement of the solids dissolved in the droplet. This might result in denser powder particles [9] and consequently improved sedimentation in the cyclone giving a greater powder yield.

The powders were found to reconstitute and form a dispersion more easily when increased inlet temperature and aspiration rates were used. The model describing this was significant and explained 79% of the variation in the data (p-value < 0.001 and  $r^2 = 0.79$ , Fig. S1f). The inlet temperature, aspiration rate and their interaction effect were the



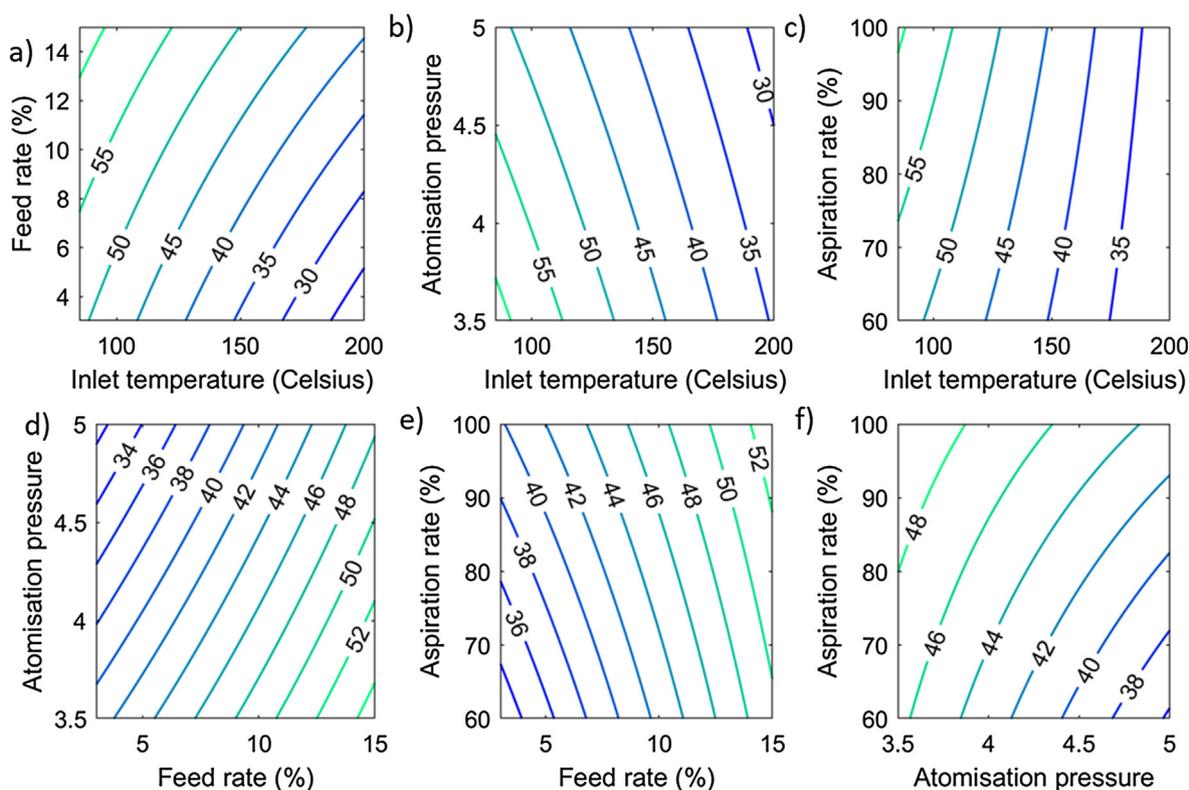
**Fig. 1.** Interactions between parameter effects with significant influence on the outlet temperature. In each figure, the effect of two parameters is shown in the case where all other parameters at kept constant at the center-point value of the DOE. The numbers in the lines indicate the predicted outlet temperature (°C). The interaction effects are not significant (Table S3).

only significant parameters (p-value < 0.001 for both factors and a p-value of 0.016 for their interaction effect, Table S3). The dependence of the reconstitution properties on these parameters is shown in Fig. 3. The ease of reconstitution of cubosomes precursors is likely related to a homogeneous distribution of the steric stabilizer (dextran) on the particles [6]. It may therefore be speculated that the fast drying rate resulting from a high inlet temperature and aspiration rate [9] would improve homogeneous distribution of dextran on the particles.

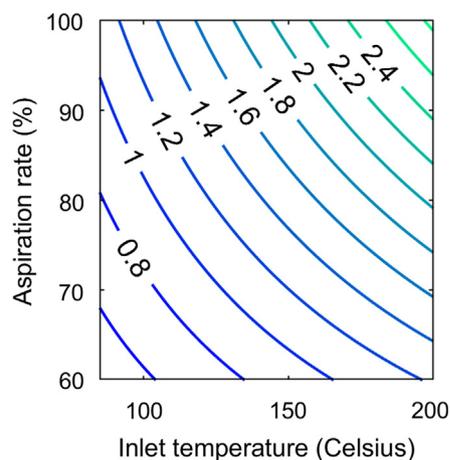
Reconstitution of powders in water resulted in dispersions of particles. The size (average hydrodynamic diameter) of the rehydrated particles was approximately 230 nm in 28 of the total 34 measurements and above 300 nm for the remaining 6 measurements (Fig. 4). The model for particle size had inlet temperature, feed rate and aspiration rate as significant parameter effects (p-value of 0.006, 0.032 and 0.043, respectively, Table S3). However, the model appeared to be based on an

artefact introduced by the great leverage on the model of the above mentioned 6 apparently outlying size measurements (Fig. S1b). The effects of the model parameters on the particle size are shown in Fig. 5. Interestingly, in all cases with large particle sizes, high outlet temperatures (123–132 °C) were also recorded. Indeed, the same parameter effects that were significant in the model for outlet temperature were also significant in the model for particle size. The outlet temperature showed a threshold at approximately 115 °C in relation to the hydrated particle size (Fig. 6): Below 115 °C, the particle size was  $231 \pm 20$  nm (mean  $\pm$  SD) while above 115 °C, the size was much larger and highly variable ( $506 \pm 253$  nm, mean  $\pm$  SD). To keep the particle size of the cubosomes low and controlled, the outlet temperature should therefore be below 115 °C.

Particles morphology can influence the immunogenicity of particulate vaccines [10]. Cubosomes with the model antigen ovalbumin



**Fig. 2.** Interactions between parameter effects with significant influence on powder yield. In each figure, the effect of two parameters is shown in the case where all other parameters at kept constant at the center-point value of the DOE. The numbers in the lines indicate the predicted powder yield (%). The interaction effects in (a) and (c) are significant (p-value of 0.004 and 0.015, respectively, Table S3), all others are not.



**Fig. 3.** Interactions between parameter effects with significant influence on the ease of reconstitution of the powder. The effect of the two parameters is shown in the case where all other parameters are kept constant at the center-point value of the DOE. The numbers in the lines indicate the predicted powder reconstitution grade. The interaction effect is significant ( $p$ -value of 0.016).

and the adjuvants monophosphoryl lipid A and imiquimod have been reported to stimulate stronger immune responses than similarly adjuvanted liposomes [4]. To evaluate the cubosome content in the dispersions formed by reconstitution of the powders in water, cryo-TEM was employed. Cryo-TEM revealed three types of particles: Dispersed cubosomes, agglomerated cubosomes and dispersed vesicles (Fig. 7a–c, respectively). Cubosomes were present in all samples and most samples also contained varying amounts of vesicles. The proportion of the total amount of particles that were cubosomes rather than vesicles was estimated, and is referred to as the cubosome content. The average estimated cubosome content found with cryo-TEM was  $86 \pm 17\%$  (Table S2). The only significant parameter effect on the determined cubosome content was the inlet temperature ( $p$ -value of 0.019, Table S3). An elevated inlet temperature resulted in a slightly higher cubosome content. However, the model was insignificant ( $p$ -value of 0.206) explaining only 39% of the sample variation (Fig. S1a). In addition, all

samples were replicated once and for every sample with cubosome content below 75%, the replicate had more than 75% cubosomes. The variation thereby seems associated with the inherently limited particle numbers visualized by cryo-TEM rather than a dependence of particle morphology on spray drying parameters.

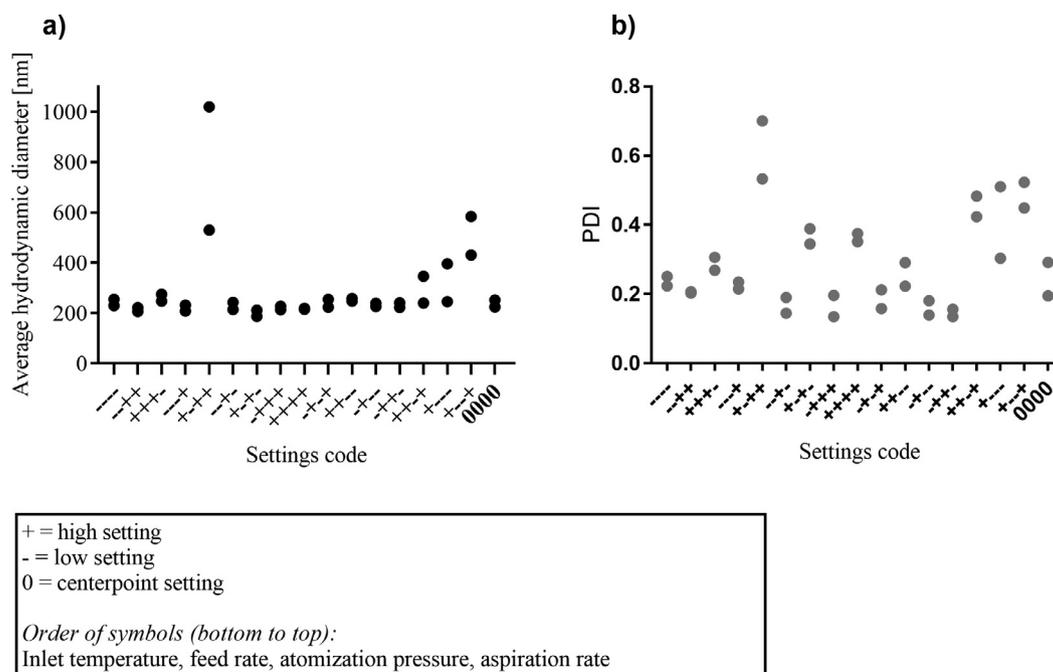
In total, 19 significant parameter effects and interaction effects were found in this DOE study (excluding the intercepts): Three for the outlet temperature, six for the powder yield, zero for the residual moisture content of the powders, three for the ease of powder reconstitution, six for the average size of rehydrated particles and one for the cubosome content. The calculated false positive discovery rate [11] amongst the effects found to be significant was 1.4. It may therefore be expected that 1 or 2 of the found significant effects is a false positive discovery.

In conclusion, spray drying of cubosomes is a robust method giving low residual moisture, high cubosome contents and consistent size of rehydrated particles within a wide range of parameter settings as long as the outlet temperature is kept below  $115^\circ\text{C}$ . Settings can thus be optimized with regards to obtaining high powder yields, improved reconstitution properties, reduced outlet temperatures or with regards to dry particle engineering considerations.

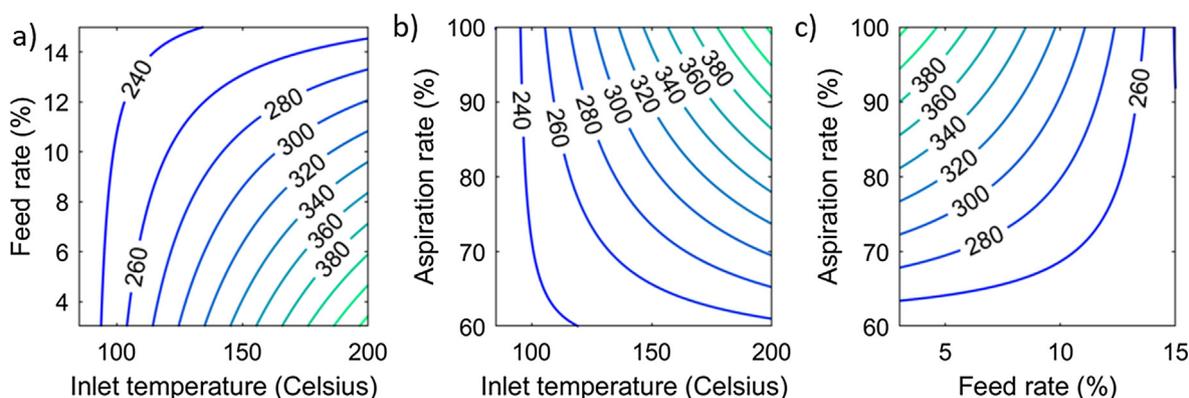
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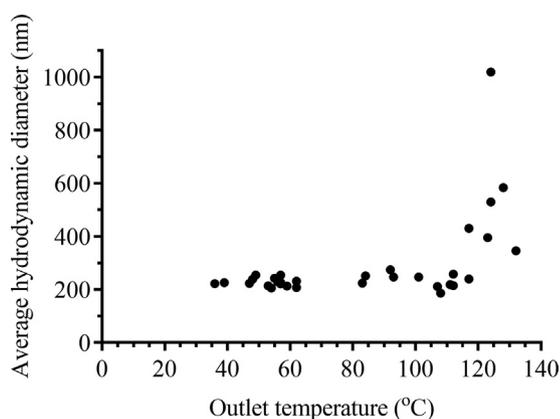
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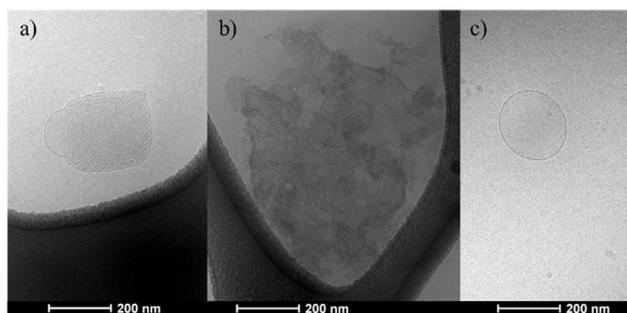
**Fig. 4.** (a) Average hydrodynamic diameter and (b) PDI of particles obtained with each DOE setting as measured by DLS after rehydrating the powder precursors in water at  $0.1\text{ mg/mL}$ .



**Fig. 5.** Interactions between parameter effects with significant influence on the size of the rehydrated particles. In each figure, the effect of two parameters is shown in the case where all other parameters are kept constant at the center-point value of the DOE. The labelled lines indicate the predicted outlet temperature ( $^{\circ}\text{C}$ ) on (a) inlet temperature and feed rate, (b) inlet temperature and aspiration rate and (c) feed rate and aspiration rate. The interaction effects are all significant ( $p$ -value of 0.021 (a), 0.022 (b) and 0.033 (c) (Table S3)).



**Fig. 6.** Average hydrodynamic diameter of particles measured by DLS after rehydrating the powder precursors in water to 0.1 mg/mL vs. outlet temperature of the spray drying process.



**Fig. 7.** Cryo-TEM images showing the variety of particle morphologies obtained. (a) dispersed cubosome, (b) agglomerated cubosomes, (c) vesicle.

#### Conflicts of interest

The authors have no conflicts of interest to declare.

#### Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ejpb.2018.12.008>.

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