Pharm Res (2013) 30:2772–2784
DOI 10.1007/s11095-013-1096-2

RESEARCH PAPER

Engineering of an Inhalable DDA/TDB Liposomal Adjuvant: A Quality-by-Design Approach Towards Optimization of the Spray Drying Process

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Received: 7 February 2013 / Accepted: 28 May 2013 / Published online: 22 June 2013

ABSTRACT

Purpose The purpose of this study was to identify and optimize spray drying parameters of importance for the design of an inhalable powder formulation of a cationic liposomal adjuvant composed of dimethyldioctadecylammonium (DDA) bromide and trehalose-6,6′-dibehenate (TDB).

Methods A quality by design (QbD) approach was applied to identify and link critical process parameters (CPPs) of the spray drying process to critical quality attributes (CQAs) using risk assessment and design of experiments (DoE), followed by identification of an optimal operating space (OOS). A central composite face-centered design was carried out followed by multiple linear regression analysis.

Results Four CQAs were identified; the mass median aerodynamic diameter (MMAD), the liposome stability (size) during processing, the moisture content and the yield. Five CPPs (drying airflow, feed flow rate, feedstock concentration, atomizing airflow and outlet temperature) were identified and tested in a systematic way. The MMAD and the yield were successfully modeled. For the liposome size stability, the ratio between the size after and before spray drying was modeled successfully. The model for the residual moisture content was poor, although, the moisture content was below 3% in the entire design space. Finally, the OOS was drafted from the constructed models for the spray drying of trehalose stabilized DDA/TDB liposomes.

Conclusions The QbD approach for the spray drying process should include a careful consideration of the quality target product profile. This approach implementing risk assessment and DoE was successfully applied to optimize the spray drying of an inhalable DDA/TDB liposomal adjuvant designed for pulmonary vaccination.

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KEY WORDS adjuvant · design of experiments · inhalation · liposomes · quality by design

ABBREVIATIONS

CPP	critical process parameter
CQA	critical quality attribute
DDA	dimethyldioctadecylammonium
DoE	Design of Experiments
FPF	fine particle fraction
MMAD	mass median aerodynamic diameter
OOS	optimal operating space
PDI	polydispersity index
QbD	Quality by Design
QTPP	quality target product profile
TDB	trehalose-6,6′-dibehenate
VMD	volumetric mean diameter

INTRODUCTION

A major pharmaceutical challenge in vaccine research is the inherent instability of many vaccines in aqueous environments, which necessitates the development of thermostable vaccine formulations (1). A possible approach towards improving vaccine stability is to dry the vaccine formulations in the presence of suitable stabilizing excipients to reduce the mobility of the formulation components (2,3). This decreases the chemical and physical degradation reaction rates, eventually resulting in an increased shelf-life of the final product. In addition, design of inhalable formulations of biologics is receiving increasing attention, and the engineering of dry vaccine formulations for airway delivery is no exception to that. Dry powder-based inhalable vaccine formulations may, in addition to obviating the need for the cold-chain during distribution, possess several advantages, as compared to the conventional vaccines administered by injection; i) they can

stimulate mucosal as well as systemic immune responses; ii) their use eliminates the risk of needle related transmission of diseases otherwise associated with injectables and iii) reduces the dependency on health care professionals during vaccination, thus making them more feasible for mass vaccination programs (3).

Spray drying and freeze drying are commonly used drying methods. Freeze drying of liposomes for inhalation has been studied previously. However, a secondary milling step has to be applied to the powder to achieve a reduction of the particle size in order for it to become suitable for inhalation (4). Spray drying has the advantage, as compared to freeze drying, that it allows for particle engineering in a single process step; Particles with customized attributes, such as the particle size and density, can be designed by adjusting the process parameters, *i.e.* the atomizing airflow, the drying temperature, the drying airflow and the feedstock concentration (5). This feature of particle engineering is of utmost importance for products intended for inhalation because the pulmonary deposition patterns of formulations are highly dependent on their particle size due to the complex anatomy of the lungs that is decisive for the particle movement patterns in the airways (6).

As a consequence of the complex branching structure of the airways, one of the most important physicochemical properties of inhalable powders is the mass median aerodynamic diameter (MMAD) (6). The aerodynamic diameter of a particle is the diameter of a unit density, spherical particle with the same air velocity and behaviour as the analysed particle, thus also taking into account the particle's density and shape (7). The deposition of dry particles or droplets in the lungs is determined by this aerodynamic diameter. In general, smaller particles are capable of penetrating deeper into the pulmonary tree than larger particles. Byron (8) modeled the deposition patterns for differentially sized particles in 1986 and suggested that maximal deposition in the alveoli is achieved with particles of an aerodynamic size in the range of 1.5–2.5 and 2.5–4 μm during slow breathing (with and without breath holding, respectively), whereas maximal tracheobronchial deposition is achieved in the 5–9 μm range (8). In the 5–9 μm size range, however, particle deposition in the throat or mouth is increased, eventually resulting in swallowing (8).

To date, there are no clinical studies reporting on the optimal deposition site in the airways for pulmonary vaccination (3). However, seeing as the conducting airways represent a true mucosal site opposed to the alveoli that is ideal for systemic drug delivery, suggests a limited potential of targeting vaccines to the alveolar areas, whereas targeting the conducting airways is much more feasible with regards to stimulating mucosal immune response (6). Furthermore, the immune cells of the alveoli are mainly scavenger macrophages, and their primary function is to ingest and destroy inert particulates without triggering an inflammatory response to keep the alveoli clean (9). The conducting airways, however, contain a tight network of airway mucosal dendritic cells with antigen presenting properties (10), which are capable of extending their dendrites into the lumen and take up foreign particulate matter (11).

In addition to the particle size of dry powder formulations, product specific formulation properties, need to be optimized (12). This, in combination with the high number of adjustable processing parameters, complicates the optimization of the operating levels with respect to the different formulation attributes. The quality by design (QbD) approach implementing risk assessment and design of experiments (DoE) is a valuable tool under these circumstances because it provides the highest amount of information from the least number of experiments. The QbD process may be divided into the following steps: i) Identification of the quality target product profile (QTPP) based on risk assessment; ii) identification of the critical quality attributes (CQA); iii) identification of possible critical process parameters (CPP) and iv) setup and execution of DoE to establish the relationship between the CQAs and the CPPs and use the information to define a process design space that will result in an end product of the desired QTPP (13,14).

The purpose of this study was to adapt the QbD approach to the process of formulating inhalable dry powders constituting the liposome-based cationic adjuvant formulation 01 (CAF01, Statens Serum Institut, Denmark), *via* spray drying. The liposomal adjuvant is composed of the cationic surfactant dimethyldioctadecylammonium (DDA) bromide and α,α′-trehalose-6,6′-dibehenate (TDB) (15). Previous studies have shown that CAF01 is a versatile adjuvant system capable of eliciting both humoral and cell-mediated immune responses against co-administered antigens (15–17), and it is currently in clinical trials with antigens against tuberculosis (NCT00922363) and HIV (NCT01141205; NCT01009762). Both freeze drying and spray drying of CAF01 with trehalose as the stabilizing excipient under optimal processing conditions result in a final product with preserved adjuvant activity, as compared to aqueous formulations (18,19). Sucrose (18) and mannitol (19) have been shown to be less efficient stabilizers with regards to conservation of the liposome size during drying of CAF01, as compared to trehalose, whereas lactose has similar stabilizing capabilities as trehalose (19). However, its reducing nature limits its use to the stabilization of the liposomes only and not a liposome-antigen mixture due to the high probability for interaction between the reducing sugar and the protein. Trehalose was therefore selected for the current experiment. Based on prior research, we identified CQAs and CPPs more systematically and performed a DoE to identify a process design space that met the criteria for all CQAs of an inhalable powder formulation of CAF01 applying trehalose as stabilizing excipient.

MATERIALS AND METHODS

Materials

DDA was purchased from Avanti Polar Lipids (Alabaster, AL, USA) and TDB was synthesized by Clausen-Kaas A/S (Farum, Denmark). Methanol and chloroform (extra pure) were purchased from VWR (Leuven, Belgium) and Merck (Darmstadt, Germany), respectively. Trehalose dihydrate (≥ 99%) was from Sigma-Aldrich (St. Louis, MO, USA). Purified water of Milli-Q quality was used to prepare all solutions.

The QbD Process: Risk Assessment and Experimental Design

Possible CQAs and CPPs in the spray drying process were defined. The risk assessment using an Ishikawa diagram was applied at two different levels; i) a patient-oriented risk overview for inhalable products aiming at identifying the QTTP, followed by ii) an engineering overview of the spray drying process. The experiment was subsequently designed and set up using the program Modde 9.0 (Umetrics AB, Umeå, Sweden). A DoE can be executed according to different models, depending on the desired depth of information to be obtained. An optimization model was chosen for the present study (a central composite face (CCF) centered fractional factorial design) in order to explore the effects of the primary, the secondary and the interactional terms. Five factors were varied at a low (−1), center (0) and high (+1) level (Table I). A total of 29 experimental runs were conducted, of which 16 experiments represented a two-level fractional factorial design (2^{5-1}), three experiments were center points and 10 experiments were star points, *i.e.* four factors were kept at a center level, whereas the fifth was varied to positive and negative values, respectively.

Preparation of Liposomes

The liposomes were prepared essentially as described previously (15). In brief, weighed amounts of DDA and TDB were dissolved in chloroform:methanol (9:1, v/v) and mixed in a round bottomed flask resulting in a final DDA:TDB molar ratio of 89:11. The solvents were evaporated under vacuum and at constant rotation. The lipid films were stripped twice with ethanol at 1 h intervals to remove trace amounts of the organic solvents, after which they were dried under vacuum overnight. The lipid films were rehydrated with the respective trehalose solutions in MilliQ water, resulting in a constant lipid to total solid percentage of 10.9% (w/w), but at different total concentrations (feedstock concentration, Table I).

Spray Drying

Spray drying was carried out using a co-current Büchi B-290 spray dryer coupled to a Dehumidifier B-296 (both from Büchi Labortechnik, Flawil, Switzerland). The spray drying settings (Table I) were varied with respect to feedstock concentration (9.1–45.7 mg/ml), feed flow rate (0.3–1.5 ml/min), atomizing airflow (436–601 l/h), drying airflow rate (aspirator rate 80–100% of total capacity) and outlet temperature (75–95°C). Stable outlet temperature was reached using pure water and the inlet temperature adjusted accordingly. The outlet temperature was in this study chosen as a controlled variable as an alternative to the inlet temperature due to the fact that the temperature of the droplets/particles never exceeds the value of the outlet temperature (20). It may thus be more important to control the outlet temperature during drying of thermolabile structures such as liposomes. In addition, it has been shown previously that the process yield when spray drying trehalose formulations decreases above an outlet temperature of 105°C as a result of increased adhesion of the particles to the wall of the cyclone (21). To prevent this, the outlet temperature was kept at a maximum of 95°C.

Aerodynamic Particle Size and Volumetric Mean Diameter

A time-of-flight principle was used to measure the aerodynamic particle size using an Aerodynamic Particle Size Spectrometer® 3321 equipped with a Small-Scale Powder Disperser® (both TSI, Shoreview, MN, USA). A few milligrams of sample were examined by continuous analysis for 20 s. The particle size distribution was converted into MMAD using the Aerosol Instrument Manager® Software (TSI). The raw data was furthermore transferred to GraphPad Prism v. 5 (Systat Software, San Jose, CA, USA), fitted to an asymmetric five parameter curve, and the best fit was used to calculate i) the mass percentage of particles with an aerodynamic diameter smaller than 5 μm (the fine particle fraction, FPF) and ii) the particle span. The Stoke's correction factor, which accounts for the influences of the geometrical diameter on the particle acceleration, was not applied. The volumetric mean diameter (VMD) was determined by scanning electron microscopy (SEM) by using a JSM-5200 Scanning Microscope (JEOL, Tokyo, Japan). The powders were sprinkled onto a stub covered with double-adhesive carbon tape, and sputter coated with gold by using an E5200 Auto Sputter Coater (Biorad, Watford, UK). Examination was performed at an acceleration voltage of 25 kV and a working distance of 20 mm. Three images for each sample were analysed, and the diameter of 150 easily distinguishable and randomly selected particles on each image was measured by using Motic Images Plus 2.0 (Motic Deutschland, Wetzlar, Germany) followed by plotting of the cumulative volume percentage

Table I Process Design Space

Factor	Abbreviation	Low level (−1)	Center level (0)	High level (+1)
Aspirator (%)	Asp	80	90	100
Outlet temperature (°C)	Out	75	85	95
Feedstock concentration (mg/ml)	FeC	9.1	27.4	45.7
Feed flow (ml/min)	FeF	0.3	0.9	1.5
Atomizing airflow (l/h)	Ato	436	519	601

against the particle diameter to determine the mean VMD (D_{50}).

Liposome Size and Polydispersity

The mean particle diameter (Z-average) and polydispersity index (PDI) of the liposomes were determined by dynamic light scattering by using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) equipped with a 633 nm laser and 173° detection optics. Malvern DTS v 6.30 software (Malvern Instruments) was used for data acquisition and analysis. All samples were measured at a lipid concentration of 0.1 mg/ml and a trehalose concentration identical to concentration of the solutions used to hydrate the lipid films. The powder samples were rehydrated to a lipid concentration of 1 mg/ml. Following addition of the rehydration medium, the vials were shaken gently and rotated vertically for 15 min and subsequently diluted 10-fold in the respective trehalose solution. The data acquisition software was used to adjust for different viscosity values. A Nanosphere™ Size Standard (220 ± 6 nm, Duke Scientific, Palo Alto, CA, USA) was used to verify the performance of the instrument. The particle size distribution was reflected in the PDI, which ranges from 0 for a monodisperse to 1.0 for an entirely heterodisperse system.

Moisture Content

Thermogravimetric analysis (TGA) was used to determine the residual moisture content in the samples after drying. The samples were heated from 20–160°C at a rate of 5°C/min with nitrogen purging, and the weight loss in percent from 20–150°C was recorded by using a TGA 7 (Perkin Elmer, Waltham, MA, USA).

Yield

The yield was measured as the percentage of input material deposited in the collection flask after spray drying. A sample vial was weighed before and after addition of the collected dry powder, the yield in percent was calculated as the weight difference divided by the initial batch size.

Statistics

All statistical analyses of the DoE were performed using Modde 9.0 (Umetrics AB, Umeå, Sweden). Models were fitted with multiple linear regression (MLR) and adjusted by removing non-significant model terms.

RESULTS

Identification of QTPP and CQAs

The most important QTPP of an aerosol formulation is the delivery of the optimal dose to the right segment of the lungs in order to achieve the desired response. Therefore, the MMAD was considered to be one of the main physical CQAs for an inhalable vaccine product (Table II). In addition, we identified i) the liposome stability during processing; ii) the yield of the spray drying process and iii) the moisture content of the resulting dry powder as CQAs (Fig. 1a). The liposome stability may include both the chemical and the physical stability, such as oxidation of carbon chains, fusion and aggregation of liposomes (2) and changes in the thermotropic phase behavior (18,19). In this case, the size stabilization was chosen as the most important liposome stability factor. A proper liposome size stabilization during processing may be essential as subcutaneous immunizations with sub-optimally stabilized liposomes have implied a reduced efficacy (19). Optimization of the process yield is highly feasible from a production and economical perspective, in particular for the processing of expensive biologics like subunit vaccines. The moisture

Table II Quality Target Product Profile (QTPP) for Inhalable Dry Powder Liposomes

Response	Target	Reason
MMAD (μm)	4.5–5.5	5 μm represents the lower limit for optimizing deposition in the conducting airway (8)
Liposome size (nm)	Size reduction	Marker of stabilization(19)
Relative moisture content (%)	Minimize	Decreasing the moisture content increases the Tg of trehalose (22)
Yield (%)	>50%	Economic feasibility

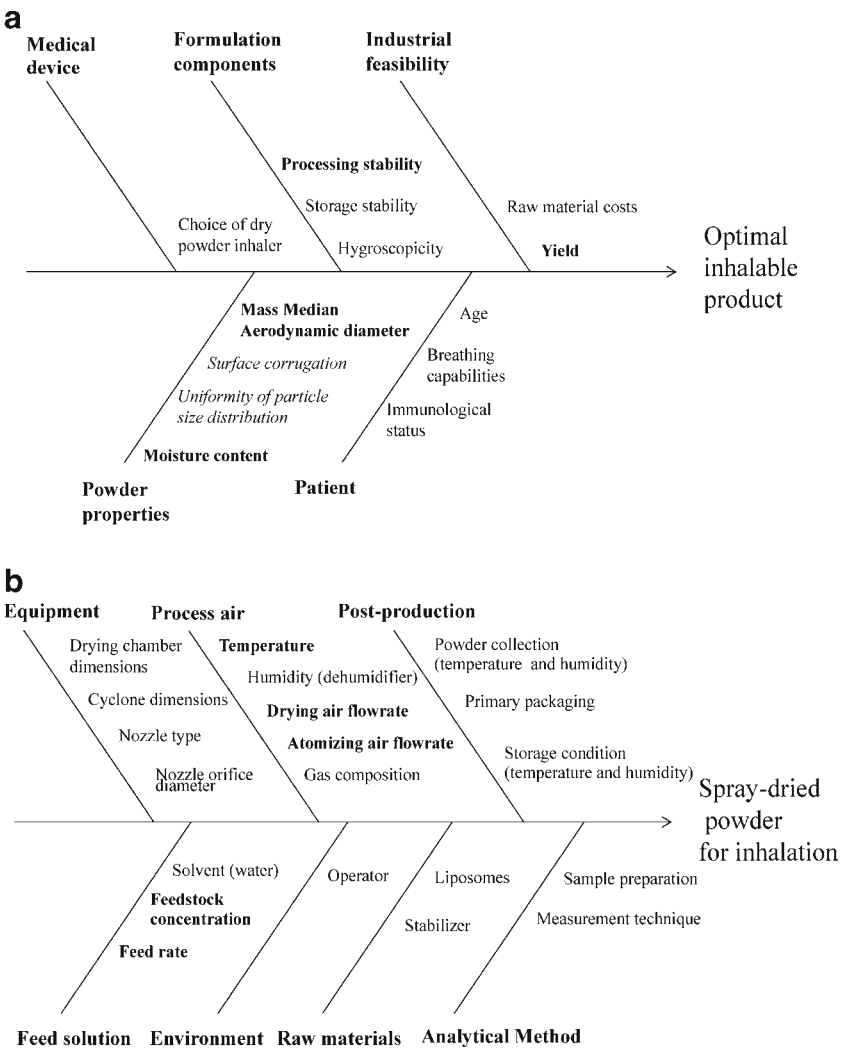

Fig. 1 Ishikawa diagram of (**a**) the factors affecting the clinical outcome of the formulation. The primary CQAs are highlighted in *bold* and the additional CQAs tested in italic. Quality attributes and biological factors not tested in this study are not highlighted; (**b**) the spray drying process. The five processing factors examined in the present study are highlighted in *bold*.

content of the final product may influence the stability of the formulation because the moisture content affects the glass transition temperature (Tg) of the system, which is decisive for the molecular mobility in the final product. Trehalose, which was selected as the stabilizing excipient for the present study, has a relatively high Tg even at a high moisture content (22), and the Tg is above room temperature at a moisture content as high as 6% (w/w), [calculated from the curve-fitting equation in reference (22)]. Finally, the surface corrugation (morphology) and the particle size range (span) were identified as additional CQA related to the particle properties and examined during the course of the study. However, they were not listed as primary CQAs (Fig. 1a).

Risk Overview

Constructing an Ishikawa diagram (also called a fishbone diagram) is one of the risk assessment methods recommended in the International Conference on Harmonization (ICH) guidelines (12). Applying this method enables the identification of CPPs *via* a structured overview of relevant factors. The proposed Ishikawa diagram is partially based on studies by Baldinger *et al.* (23) and divides the possible causes for experimental failure into seven groups, which can be further divided into subgroups (Fig. 1b). Five factors were chosen for investigation, where three factors are related to the process air (the temperature, the drying air flow rate and the atomizing air flow rate) and two factors are related to the feed solution (the feedstock concentration and the feed rate). Additional factors, such as the spray drying equipment, the solvent, the drying air humidity and the raw materials were kept constant.

Furthermore, additional factors were identified that might affect the clinical outcome of the product (listed together with the CQAs in Fig. 1a). These were not tested because they were considered irrelevant for the overall scope of the present study (*e.g.* the choice of inhaler device and the patient population) and have to be tested in clinical studies.

Setup of the DoE

A DoE was carried out in order to link and quantify the effects of the potential CPPs identified in the risk analysis to the CQAs. An inherited limitation of conventional optimization methods, where one factor is optimized at a time, is that it does not take into account the interactions between the factors, thus implying the risk of overlooking the optimal combination (24). The use of DoE overcomes this problem by systematically varying the factors thus evaluating simultaneously the effects of multiple variables. The results from the DoE are summarized in Tables III and IV, and below is discussed the influence of the factors on the chosen responses, followed by a definition of the optimal operating space (OOS) suitable for the production of an end product with the desired CQAs.

The CQAs of the Dry Powder Formulation Can be Modeled with DoE

After model adjustments that included removing non-significant factors, both the MMAD and the VMD were modeled with high accuracy: The values predicted according to the model correlated closely with the measured values (Fig. 2a), and the MMAD had an R^2 (fraction of the response variation explained by the model) of 0.979 and a Q^2 (fraction of response variation predicted according to a cross validation of the model) of 0.916 (Table III). For the VMD, the R^2 value was 0.945 and the Q^2 was 0.872. The experimental range of the responses for the MMAD was from 4.51 to 7.89 μm, whereas the responses for the VMD spanned a larger range (4.37–10.0 μm). The influences of the primary factors were predominantly a positive effect of the feedstock concentration and a negative effect of the atomizing airflow for both responses, although both factors showed inverted quadratic effects on the MMAD (Table III), suggesting that the effects are diminished at higher values. The drying airflow rate influenced negatively the MMAD as well as the VMD, whereas the outlet temperature had a positive effect on the MMAD and a negative effect on the VMD. However, there was a correlation of approximately 0.7 between the two responses (Table V).

As expected, there was a strong, negative correlation between the MMAD and the FPF (−0.988, Table V), and the

Table III Adjusted Model for Dry Powder Characteristics

	MMAD[a]		FPF		VMD		Yield		Moisture content	
	Coefficient	*P*-value	Coefficient	*P*-value	Coefficient	*P*-value	Coefficient	*P*-value	Coefficient	*P*-value
Asp	−0.0206	≤0.0001	0.0338	≤0.0001	−0.369	≤0.01	6.31	≤0.0001	*−0.00758*	0.64
Out	0.0102	≤0.01	*−0.0108*	0.053	−0.215	≤0.05	*−0.473*	*0.68*	−0.0478	≤0.01
FeC	**0.0505**[b]	**≤0.0001**	**−0.0874**	≤0.0001	**1.00**	**≤0.0001**	9.28	≤0.0001	−0.0360	≤0.05
FeF	*−0.00181*[c]	*0.53*	0.0121	≤0.05	0.318	≤0.01	*−1.47*	*0.21*	0.0364	≤0.05
Ato	−0.0413	≤0.0001	0.0753	≤0.0001	−0.852	≤0.0001	**−10.7**	**≤0.0001**	*0.0179*	0.28
Asp*Asp					−0.857	≤0.01				
Out*Out										
FeC*FeC	−0.0203	≤0.05	0.0444	≤0.01			−10.1	≤0.0001		
FeF*FeF	−0.0173	≤0.05	0.0302	≤0.05	0.723	≤0.01			***0.0687***	0.078
Ato*Ato	*0.0146*	*0.056*	−0.0280	≤0.05					*−0.0497*	0.19
Asp*Out	*0.00579*	*0.070*	−0.0173	<0.01			*−1.90*	*0.13*		
Asp*FeC							*−1.79*	*0.15*	−0.0482	≤0.05
Asp*FeF	−0.0111	≤0.01	0.0140	≤0.05	0.218	≤0.05				
Asp*Ato			*−0.0111*	0.060	−0.371	≤0.01	4.13	≤0.01		
Out*FeC					−0.322	≤0.01				
Out*FeF	*−0.00329*	*0.28*					−3.00	≤0.05		
Out*Ato			*0.00873*	0.13			*−1.82*	*0.14*	*0.0177*	0.31
FeC*FeF	−0.0124	≤0.001	0.0217	≤0.01	0.256	≤0.05			*0.0184*	0.29
FeC*Ato							4.78	≤0.001		
FeF*Ato	0.00998	≤0.01	−0.0136	≤0.05	*0.139*	*0.19*				
R^2	0.979		0.979		0.945		0.944		0.657	
Q^2	0.916		0.914		0.872		0.812		0.298	

[a] Underlined: Log-transformed responses

[b] Bold: Factors with the largest influence on the given response

[c] Italic: Non-significant parameters kept in the model to either conserve the hierarchy or the predictability of the model

Table IV Adjusted Model for Liposome Characteristics

	Liposome size before[a]		Liposome size after		Liposome size ratio	
	Coefficient	*P*-value	Coefficient	*P*-value	Coefficient	*P*-value
Asp			15.8	<0.05	0.0344	<0.05
Out			*−4.63*	*0.44*	*−0.0100*	*0.47*
FeC	**0.0404**	**<0.0001**	*5.16*	*0.39*	−0.0533	<0.01
FeF			*−6.98*	*0.25*	*−0.0239*	*0.097*
Ato			−20.0	<0.01	−0.0750	<0.0001
Asp*Asp			**38.4**	**<0.05**	**0.0808**	**<0.05**
Out*Out			*−13.1*	*0.41*		
FeC*FeC			38.3	<0.05	*0.0608*	*0.069*
FeF*FeF						
Ato*Ato			*−13.5*	*0.39*		
Asp*Out			*11.6*	*0.081*	*0.0175*	*0.24*
Asp*FeC			*−11.4*	*0.087*	*−0.0113*	*0.44*
Asp*FeF					*0.0250*	*0.10*
Asp*Ato						
Out*FeC			*−11.5*	*0.083*	−0.0513	<0.01
Out*FeF					*−0.0300*	*0.054*
Out*Ato						
FeC*FeF			*7.96*	*0.22*	*0.0138*	*0.35*
FeC*Ato			*5.68*	*0.37*		
FeF*Ato			14.0	<0.05	*0.0163*	*0.27*
R^2	0.444		0.846		0.894	
Q^2	0.36		0.513		0.757	

[a] Log-transformed responses

[b] Bold: Factors with the largest influence on the given response

[c] Italic: Non-significant parameters kept in the model to either conserve the hierarchy or the predictability of the model

factors influencing the FPF were largely the reciprocal of the factors influencing the MMAD (Fig. 2b): The larger the MMAD, the smaller the mass percentage was contained within the FPF. No major differences were observed in the morphology of the resulting particles, except for distinguishable differences in the fractions of small *versus* large particles (Fig. 2c). Modeling of the particle span resulted in a poor model validity (results not shown), indicating statistically significant model problems, and the span was therefore excluded from further analysis.

The average diameter of the liposomes after drying resulted in a poor model, mainly reflected by a poor predictability (R^2 = 0.846, Q^2 = 0.513), suggesting either that the liposome size is poorly controllable within the design space, or that additional external factors affect the response. Ten of the 15 factors, which the model was based on, were also non-significant (Table IV). The results were improved significantly by taking into account the batch-to-batch variation of the liposome average diameter before spray drying by applying the ratio between the liposome diameter before and after drying for modeling (R^2 = 0.894, Q^2 = 0.757). This shows the importance of a standardized liposome preparation procedure. The PDI measurements were analyzed in a similar manner that resulted in a poor model (results not shown), presumably due to the presence of bimodal size distributions for some of the samples, increasing the PDI substantially.

The yield ranged from 16 to 85%, and only four experiments resulted in yields below 50% (Fig. 2d, top). The response was modeled with high accuracy (R^2 = 0.944, Q^2 = 0.812) and three primary factors affecting the yield were identified, *i.e.* the atomization airflow with a negative effect and the aspirator rate and the feedstock concentration both with positive effects, respectively. The feedstock concentration also had large negative quadratic effects (Table III) suggesting that there is a nonlinear relationship between the feedstock concentration and the yield (Fig. 2d, bottom). Neither the aspirator rate nor the atomizing airflow showed significant quadratic terms, indicating linear effects of these parameters within the entire process design space.

The moisture content for all the batches ranged between 1.5 and 3%. However, the modeling was rather poor (R^2 = 0.657, Q^2 = 0.298), and the center points showed moisture contents in a range from 1.8–2.4%, which accounts for more than one third of the total response range. These large experimental differences do most probably reflect variations caused by external factors that are not accounted for in the model, *e.g.* the relative humidity during powder collection (Fig. 1b).

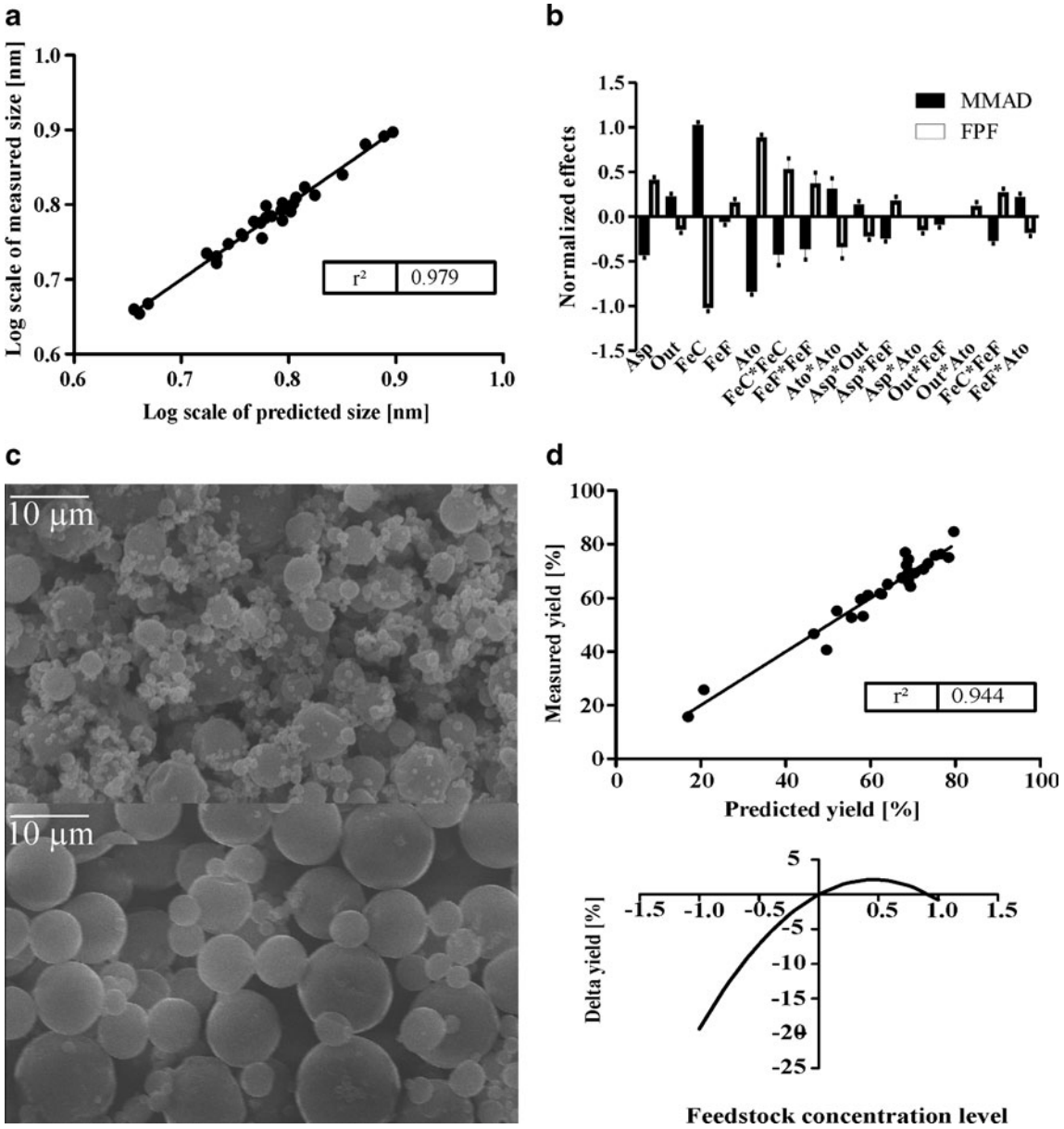

Fig. 2 (**a**) Correlation between the measured and the predicted MMAD after establishment of the model; (**b**) Normalized effects of process parameters on the MMAD and FPF; (**c**) Representative SEM images of samples exhibiting the lowest (*top*) and the highest (*bottom*) MMAD; (**d**) (*top*) Correlation between the measured percentage for the yield (y-axis) against the values predicted by using the established model (x-axis) and (*bottom*) plot of the non-linear effects of the feedstock concentration on the yield, ranging from −1, representing the low level feedstock concentration, to 1 representing the high level feedstock concentration.

Application of DoE to Model an Optimal Operating Space

Modeling an OOS is based on overlaying Contour plots for all CQAs, applying restrictions for each plot to comply with the acceptance limits. Based on the present data, a design space was plotted with the restrictions that the MMAD should be within the range of 4.5–5.5 µm, the liposome size ratio within the range of 0.4–0.6 and the yield should be higher than 50% (Fig. 3). The moisture content was omitted from the modeling due to a poor model and acceptably low values for all the experiments. A four-dimensional plot was then created with the feed flow rate at a maximum level as it had the smallest effect of the tested CPPs on the CQAs (Fig. 3). In addition, the feedstock concentration and the atomizing airflow were located on the inner x- and y-axes since they are more difficult to control than the temperature and the drying airflow rate. This method allowed for the identification of the OOS with a high feed flow rate (1.5 ml/min), a low outlet temperature (75°C), a medium aspirator rate (90%) and in the area of low feedstock concentration and high atomizing airflow (Fig. 3, bottom, center).

Table V Estimated Correlation Between the Responses. Correlations Larger than 0.5 are Highlighted in Bold

	MMAD	VMD	LiB	LiA	Moi	Inl	Yie	LiR	FPF
MMAD									
VMD	0.688								
LiB	0.296	0.220							
LiA	0.072	0.273	0.020						
Moi	-0.405	-0.020	0.004	0.158					
Inl	0.134	-0.063	0.034	-0.236	-0.251				
Yie	0.592	0.468	0.122	0.189	-0.344	-0.227			
LiR	-0.131	0.057	-0.553	0.798	0.165	-0.203	0.060		
FPF	-0.988	-0.661	-0.275	-0.076	0.407	-0.054	-0.633	0.116	

MMAD mass median aerodynamic diameter; *VMD* volumetric mean diameter; *LiB* liposome size before drying; *LiA* liposome size after drying; *Moi* moisture content; *Inl* inlet temperature; *Yie* yield; *LiR* liposome size ratio, before and after drying; *FPF* fine particle fraction

Further analysis of how each response was influenced within the design space was performed by preparing individual Contour plots for the responses within the suggested OOS (lower level outlet temperature, center aspirator level, higher level feed flow rate) (Fig. 4). The MMAD was the response restricting the entire space to the low feedstock concentration and the high atomizing airflow (Fig. 4, top), whereas both the liposome size ratio and the yield met their criteria in a larger space. The liposome ratio restricted the OOS to higher level atomizing airflow (Fig. 4, center), and the yield was decreased below the criteria of 50% at the combination of a low feedstock concentration and a high atomizing airflow (Fig. 4, bottom).

DISCUSSION

This study illustrates the feasibility of the QbD approach for modeling a design space to optimize the processing of liposome-adjuvanted vaccines into dry powder formulations *via* spray drying for pulmonary delivery.

All the primary factors influencing the MMAD were statistically significant, except for the feed flow rate. The feedstock concentration and the atomizing airflow had the largest influence on the MMAD, but in a reciprocal manner. The positive effect of the feedstock concentration is a result of the increased solid content in each droplet generated during atomization, eventually resulting in an increased particle size. The negative effect of the atomizing airflow can be explained by the larger energy input applied to atomize the feed dispersion into smaller droplets for drying, eventually resulting in a reduced particle size, as reported previously in the literature (25,26). The negative effect of the drying airflow rate on the particle size is the result of an improved separation of the smaller particles in the cyclone at higher air flows, increasing the yield of the smaller-sized subsets (27).

The explanation for the positive influence of the outlet temperature is less evident. However, similar results have been reported in the literature: Maury *et al.* observed a higher yield upon increasing the outlet temperature (21). This was explained by an increased drying rate at higher temperatures eventually resulting in less impaction and deposition of insufficiently dried droplets on the walls of the drying chamber (21). Since larger droplets have a smaller surface area per volume available for evaporation of water, this effect is thus expected to improve the collection efficiency of the larger-sized particles to a greater extent than of the smaller-sized particles. The negative effect of the outlet temperature on the VMD is on the other hand intriguing, as an increased outlet temperature should in theory cause an increased evaporation rate and thus lead to shell formation at an earlier stage of the drying process (reviewed by Vehring (28)). Hence, each particle should constitute a larger volume. With respect to the feed flow rate, increasing the feed flow rate translates in theory to more dispersion per unit kinetic energy used for atomization (as opposed to increasing the atomizing airflow). This should result in larger droplets, and hence, larger particles, given that each droplet formed during atomization results in one particle (27). This lack of influence (or small influence) of the feed flow on the particle size has been observed previously for similar experiments (23,26) and Maury *et al.* measured as little as a 12% increase in the droplet size as a response to more than a three-fold increase in the feed flow rate (21). We therefore suggest screening a larger design space if the particle size should be engineered by adjusting the feed flow rate.

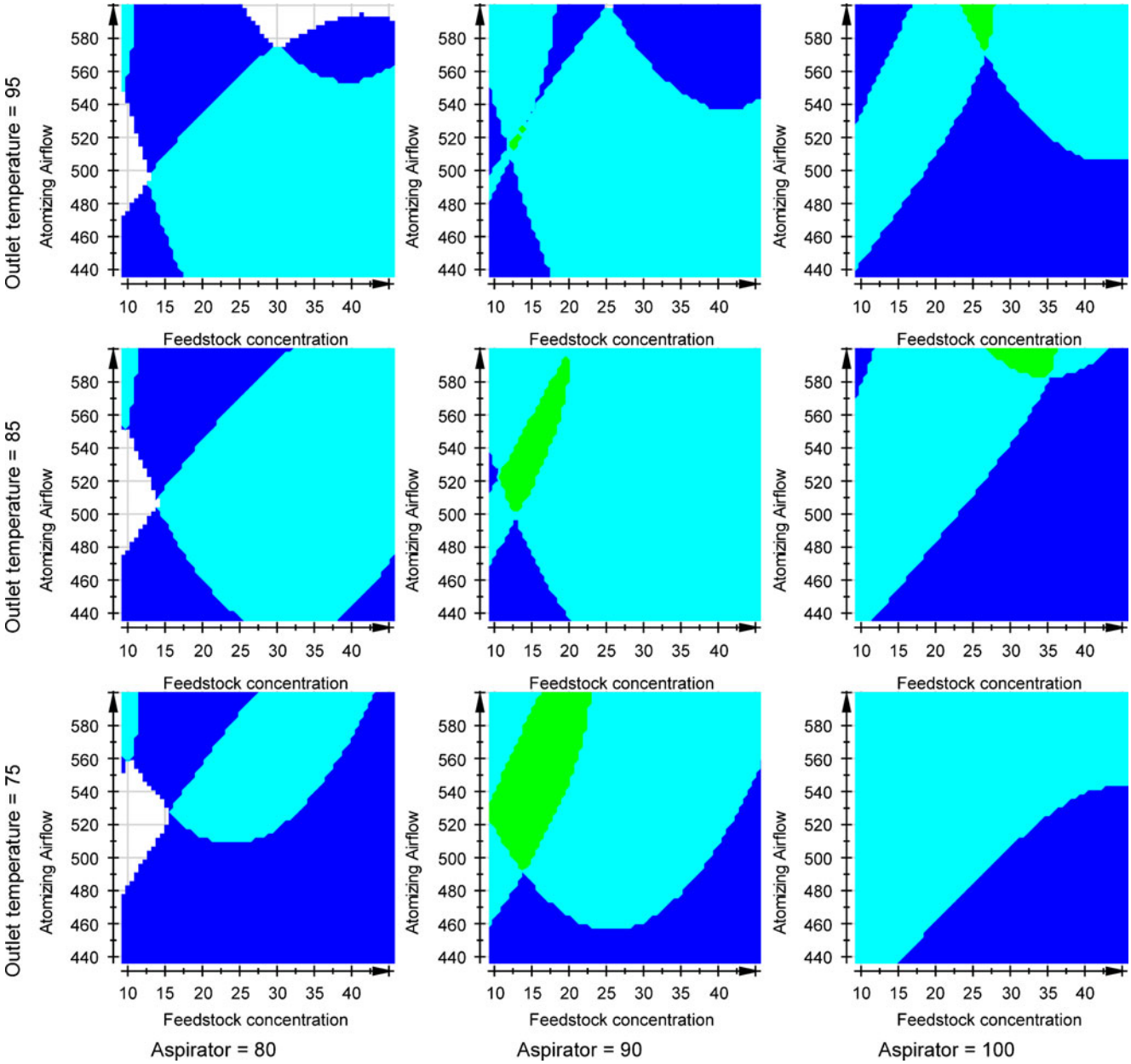

Fig. 3 A four-dimensional modeling of the OOS. Restrictions were set for an MMAD of 4.5–5.5 μm, the liposomes size ratio of 0.4–0.6 and the yield above 50%. Color code: *white* (no restriction met), *dark blue* (one restriction met), *light blue* (two restrictions met) and *green* (all restriction met). The largest OOS with respect to feedstock concentration and atomizing airflow appears as a *green* area in the lower center figure.

The smallest MMAD achiavable within the present design space was approximately 4.5 µm, thus revealing that the current experimental design is optimal for the preparation of powders engineered to deposit in the conducting airways and not in the deep lungs. In fact, one of the main reasons for the lack of marketed powders for inhalation prepared by using spray drying is that the 1–5 µm size range for deep lung delivery is at the limit of size capability afforded by the method, thus requiring intense optimization with regard to both the drying and the collection of particles (7).

As noted, some of the responses showed an inter-correlation dependency. In addition to the particle size correlations, a correlation of nearly 0.6 was evident between the MMAD and the yield. These results are in disagreement with the work of Lebrun *et al.* (29) that reported a high correlation (0.83) between the inhalable fraction (1–5 µm particles) and the yield, but somewhat in agreement with the study of Maltesen *et al.* (26) that showed a positive correlation between the MMAD and the yield by using a principle component analysis (PCA). However, a positive correlation between the particle size and the yield would be expected, based on the physics behind the cyclone

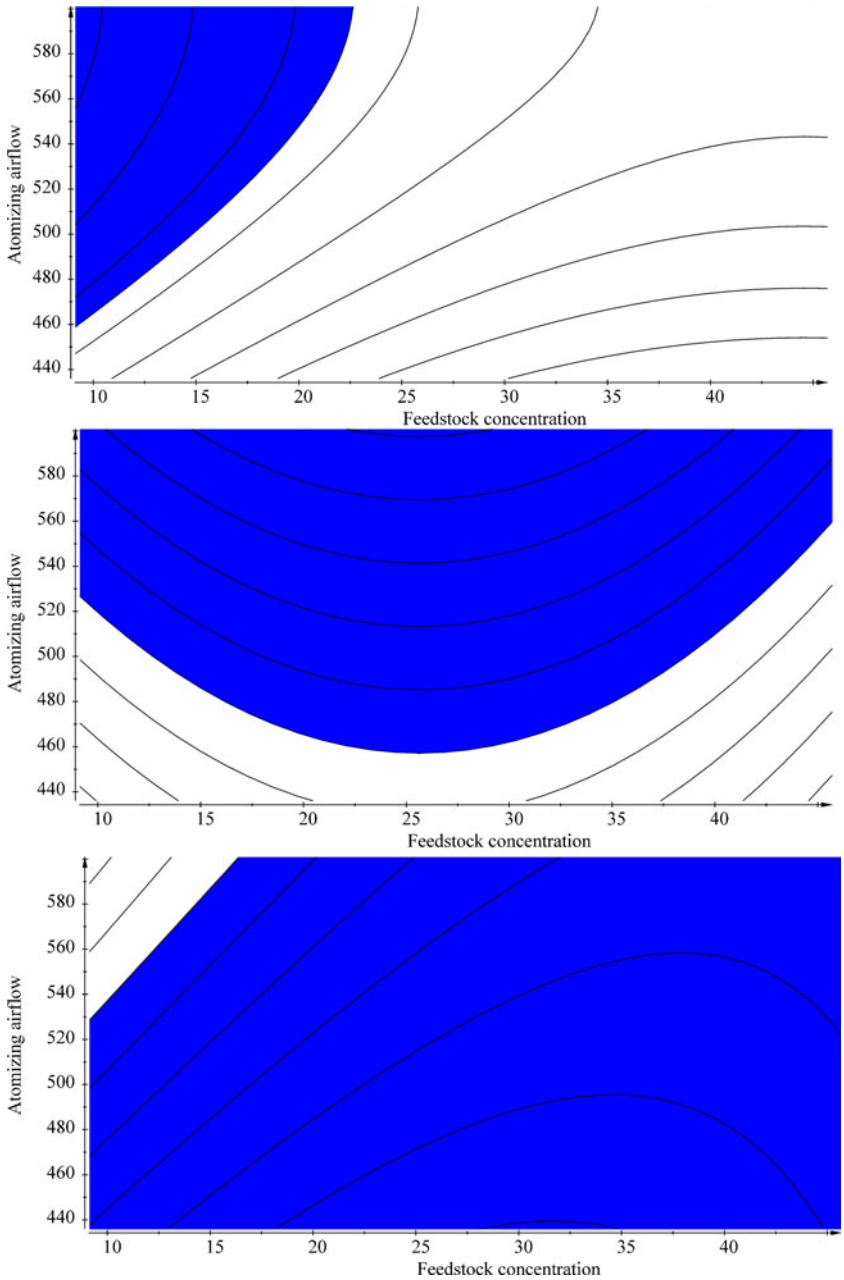

Fig. 4 Two-dimensional contour plots of the three CQAs as projections of the feedstock concentration (x-axis) and the atomizing airflow (y-axis), keeping a low level outlet temperature, center aspirator level and a high level feed flow rate. MMAD (*top*), liposome ratio (*center*) and yield (*bottom*). The *blue* color represents areas within the design space where the restrictions, MMAD of 4.5–5.5 μm, liposome ratio of 0.4–0.6 and the yield above 50%, are met.

efficiency, as smaller particles are more difficult to collect than larger particles (27).

The poorly controllable moisture content is not genuine and has been noted previously (29). The most likely explanation is that the surroundings influence the process to a large extent, even though a dehumidifier is used to remove the water vapor from the drying air, and a fully humidity-controlled working room is required to study and control this parameter further. The moisture content of the spray-dried product is known to influence the liposome stability (30). This is caused by the plasticizer effect of water, eventually resulting in a reduced Tg of the stabilizer and an increased molecular mobility of the system. Sun *et al.* (30) investigated this systematically for dried liposomes composed of egg-phosphatidylcholine with sucrose as the stabilizing excipient and showed that an increased moisture content was accompanied by a decreased Tg of the system. In addition, storage close to the Tg resulted in liposome fusion. The moisture content did not exceed 3% for any of the formulations in the present study, and it is therefore unlikely to affect the stabilizing properties of the sugar matrix at room temperature (22).

The successful modeling of the CQAs allowed for identification of an OOS within the design space where the criteria for the MMAD, the liposome size stability and the yield were met. As the B-290 spray dryer is a laboratory scale equipment, the results cannot be translated directly to a production scale

spray drying equipment of non-comparable dimensions. However, these results can provide the basis for a full-scale spray drying OOS by applying computational fluid dynamics simulations for scale-up purposes (31).

The implementation of the QbD process has not only allowed for the successful modeling of the CQAs and given a more thorough understanding of the effects of the CPPs, but also emphasized where additional processing optimization is possible, that eventually might result in a product with a more constant quality profile. The superiority of the model for the liposome size ratio, as compared to the model for the liposome size after drying, implies that a constant liposome size before spray drying would be beneficial for future use. Numerous methods exist in order to achieve more constant liposome size after rehydration, mainly extrusion and probe sonication, the latter already being studied by our group to achieve smaller sized liposomes following rehydration (32).

The criterion for the liposome size ratio was set at 0.4–0.6 during the identification of the OOS. The tested formulations fulfilling this had a corresponding average size in the range of 196–287 nm after processing. The tested formulations that did not fulfill the criterion had a liposome size ratio between 0.62–0.89 and an average liposome size in the range of 247–354 nm after processing (results not shown). As mentioned above, it has been shown previously that a reduction of the liposome size during the spray drying process may be beneficial for conserving the adjuvant activity (19). However, it was not examined how much size reduction was needed or whether this was due to the resulting liposome size or due to the size reduction by itself. The criterion set here may thus be too narrow and the whole design space may thus result in liposomes with conserved adjuvanticity. The only way to examine this further is to construct a design space based on immunological data.

The present study has focused solely on the liposome adjuvant system. However, the inclusion of a protein antigen in the formulations is needed for designing future inhalable vaccines. As proteins can have markedly different properties (33), their effects on the CQAs would be expected to be highly protein-specific. This study has established the relationships between parameters of the spray drying process and the resulting attributes of the dry powders. Furthermore, applying the QbD process has emphasized where a stringent control is needed to achieve a product of high quality. However, in light of the highly unique protein behavior, optimization would be expected to be required for each specific antigen and the OOS identified in the present study may thus serve as a valid starting point. We are currently studying the effects of including different types of antigens and model proteins. Furthermore, a question that needs further elucidation is the rehydration of the formulations following administration to the airways. A controlled rehydration process was applied in the present study, and all samples were rehydrated in a simple aqueous medium before liposome size measurements. However, future studies should address the rehydration of the formulations in the pulmonary lining fluid within the airways.

Another aspect of QbD is the implementation of process analytical technology (PAT) to enable a constant process control (34), thereby reducing the need for end product testing and increasing the understanding of the root cause for failure (14). A few in-line analytical methods have been explored for the spray drying process. An example is the application of in-line laser diffraction to measure the particle size between the drying chamber and the cyclone, as examined by Chan *et al.* (35), however concluding that poor dispersion within the spray dryer and therefore the measurement of agglomerates hindered the measurement of the particle size. Maltesen *et al.* (26) on the other hand found a correlation between the droplet size from the atomizer and the MMAD of the particles, which could be exploited by measuring the droplet size after atomization using light scattering techniques (36). In that way, a real time monitoring would allow for immediate interruption of the process in case of a non-constant droplet size. In a similar manner, near infrared spectroscopy (NIR) has been used to measure the water content and the aerodynamic particle size of the spray dried powders (37) and could thus as well serve as an in-line measurement. These techniques, combined with temperature, airflow and feed flow monitoring could thus be used to build up fast control loops enabling feedback on deviations from the constructed design space and avoid an out-of-specification product.

CONCLUSIONS

This study has successfully implemented the QbD approach to construct a design space available for manufacturing of a liposome based inhalable dry vaccine formulation. Execution of risk assessment and DoE allowed for identification and quantification of the effects of process parameters on the critical responses.

ACKNOWLEDGMENTS AND DISCLOSURES

Jukka Rantanen and Camilla Foged share senior authorship. The authors would like to acknowledge Dorthe Kyed Ørbæk (University of Copenhagen) for assistance with SEM and the Centre for Nano-Vaccines (CNV) and its members for the collaboration. The authors are grateful to The Danish Council for Strategic Research for funding the project (PTI). The funding source had no involvement in the study or the decision to submit the results for publication.

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