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Optimization of a spray drying process to prepare dry powder microparticles containing plasmid nanocomplex

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ABSTRACT

Successful gene delivery to the lung depends not only on precise and effective design of a nanosized nucleic acid delivery system but also on well engineered liquid or solid microparticles. In present work, we tried to statistically optimize spray dried formulations of low molecular weight chitosan–plasmid nanocomplexes via a D-optimal design with respect to five critical responses: yield of the process, microparticle sizes, nanocomplex sizes, DNA stability and relative transfection efficiency. Nonocomplex formulations prepared by different amounts of solid contents and leucine ratio, and spray dried immediately with varying inlet temperature, feed rate and spray air flow rate. Mean results fitted to 2FI models except for relative transfection efficiency, which fitted in a quadratic model. According to the fitted models, the most important pure factors influencing each response determined to be feed rate for yield and DNA stability, feed fluid concentration for microparticle size, inlet temperature for nanoparticle size and leucine concentration for relative transfection efficiency. However, two-factor interactions have more important roles in microparticle size, nanocomplex size and DNA stability. It was concluded that the optimized formulation could be obtained when all the independent variables were at their maximum tested values, except for feed fluid concentration, which should be in its middle point.

1. Introduction

Pulmonary gene delivery holds promise for treatment of pulmonary diseases like asthma, lung cancer and cystic fibrosis (Gill et al., 2004) and also for pulmonary vaccination (Bivas-Benita et al., 2005). The majority of gene carriers used in pulmonary gene therapy in clinical trials are viruses (see Gene Therapy Clinical Trials Worldwide, http://www.wiley.co.uk/wileychi/genmed/clinical/). However, there is a growing interest in developing safe and effective non-viral carriers to overcome drawbacks of viral vectors such as safety issues, high production fees and limited capacity (Glover et al., 2005).

When the epithelial cells of airways or alveolus are targets of gene therapy, successful delivery of therapeutic nucleic acids depends not only on engineering of a gene delivery system but also on route of administration and formulation of the dosage form (Bragonzi et al., 2000). After intravenous administration of most non-viral gene delivery systems, lungs trap substantial amounts of the nanoparticles and show high expression rates, but this expression takes effect mostly in endothelial cells of alveolar capillary network (Li and Huang, 1997; McLean et al., 1997) which is of limited value for the treatment of diseases like cystic fibrosis. Despite the existence of obstacles against inhaled administration of gene therapy formulations, the pulmonary route has been subject of extensive investigation (Griesenbach and Alton, 2009). To date, most *in vivo* experiments regarding respiratory gene delivery systems have utilized aqueous nanosuspension formulations. Long-term storage of liquid based nanoparticle formulations is associated with extensive aggregation and in case of gene delivery; efficacy of the system will decrease substantially (Anchordoquy et al., 1998; Anchordoquy and Koe, 2000; Zelphati et al., 1998). So, even when the formulation is considered to be nebulized, injected or administered orally as a liquid dosage form, it is better to be immobilized to be stable for an economically reasonable period of time (Anchordoquy and Koe, 2000). Drying of gene delivery formulations by different techniques has been suggested as a method of

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choice, since the resulted dry powders have demonstrated long-term stability in room temperature (Anchordoquy and Koe, 2000; Talsma et al., 1997). Moreover, dried formulations can be applied directly as dry powder inhalers, which reduce the time needed for administration compared to reconstituted aqueous nebulized formulations (Colonna et al., 2008; Li and Birchall, 2006; Li et al., 2003, 2005; Mizuno et al., 2009; Mohri et al., 2010; Okamoto et al., 2003, 2005; Seville et al., 2002).

Freeze drying is a well established industrial approach for drying pharmaceuticals, but it cannot offer control over particle size and morphology, which is of vital importance in inhalable powders (Seville et al., 2002). Among other drying techniques, spray drying, supercritical fluid technology and spray-freeze-drying have been tried to obtain inhalable powders for gene delivery to the lung. Because of modifiable process variables, which can affect both particle size and morphology, spray drying is favored for particle engineering, especially in the field of respiratory drug delivery (Vehring, 2008). Besides, efforts have been made to produce dry powder inhalers with lower aerodynamic diameter and density, researchers also tried to formulate nano-sized drug carriers as microparticle formulations (Dolenc et al., 2009; El-Sherbiny and Smyth, 2010; Klingler et al., 2009; Peek et al., 2008; Sham et al., 2004; Tsapis, 2002). Formulation of a gene delivery system as inhalable microparticles is a subcategory of this field, in which a microparticulate delivery system should be designed to avoid aggregation of nano-sized particles, precipitate nucleic acid content in a targeted lung region, avoid exhalation and finally decompose to original particles (Tsapis, 2002). Succeeding in design of these microparticles requires all parameters related to the process and formulation to be tuned precisely. Li and co-workers, developed a spray drying technique to produce inhalable powders containing lipid–protein–pDNA and studied the effect of various excipients on stability of the plasmid in the process, *in vitro* gene expression and aerodynamic properties of the resulted powders (Li and Birchall, 2006; Li et al., 2003, 2005). Statistical design of experiments is a helpful technique in these situations when many parameters should be changed simultaneously and both their pure effects and/or effects of their interactions are to be measured on series of depended variables. This technique has been applied to characterize process and formulation parameters affecting physicochemical properties of microparticles obtained by spray drying process (Chawla et al., 1994; Prinn et al., 2002; Tajber et al., 2009). However, the effects of mentioned parameters on conversion of nonviral polymeric gene delivery systems to dry powders by means of spray drying process have not been studied. In this research, we tried to use a D-optimal method to optimize spray drying process over several process and formulation variables to obtain microparticles containing polyplexes prepared by binding plasmid with low molecular weight chitosan as a model polymeric gene delivery system.

2. Materials and methods

2.1. Materials

Plasmid (pEGFP-N1) was obtained from Clontech (CA, USA), propagated in *Escherichia coli* DH5α and extracted by Nucleobond™ PC 10000 plasmid DNA extraction kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Medium viscose chitosan was purchased from Primex (Siglufjordur, Iceland). Sodium nitrite, sodium hydroxide and L-leucine were obtained from Merck KGaA (Frankfurt, Germany). Lactose monohydrate was provided by DMV-Fonterra Excipients (Foxhol, Netherland) as a free sample. Cell culture media were prepared by dissolving high glucose. L-Glutamine containing DMEM powder was purchased from Invitrogen (CA, USA), in Milli-Q™ water and sterilized via filtration. Fetal Calf Serum (FCS) was also purchased from Invitrogen.

2.2. Methods

2.2.1. Preparation of low molecular weight chitosan

Low molecular weight chitosan was prepared as stated before (Gilani et al., 2011). Briefly, 2000 mg of medium viscose chitosan was dispersed in 78 ml water and then dissolved by adding 6 ml of glacial acetic acid, while stirring vigorously. The reaction was initiated by adding 16 ml of 5 mg/ml sodium nitrite solution. After 90 min, low molecular weight chitosan was precipitated by adjusting pH to 9 via addition of 4 M sodium hydroxide. The precipitate was washed by ice-cold water after centrifugation and dialyzed against 2 l of deionized water, which was being replaced every 12 h, for 48 h. The resulted solution was freeze-dried, and the powder was kept at 2–8 °C in the dark.

Weight and number average molecular weight of the resulted polymer were determined by gel permeation chromatography (Gilani et al., 2011) to be 16 and 10 kDa, respectively.

2.2.2. Design

A D-optimal design was used for optimization using design expert software (trial version, 8.0.0) to keep the number of experiments affordable. Three process and two formulation parameters were selected as independent variables, including inlet temperature (A), spray air flow rate (B), liquid feed rate (C), feed fluid concentration (D) and leucine ratio with respect to solid content (E). Independent variables and their levels, which were chosen based on previous work of the research group (data not shown), are presented in Table 1. High limit of the inlet temperature was selected by monitoring the outlet temperature (80 °C) to reduce the risk of DNA double strand denaturation. The spray air flow rate of 700 l/h was selected as the highest rate to ensure constant pressure of the nozzle air flow throughout the experiments. High limits of the leucine ratio and feed fluid concentration were estimated according to the solubility of leucine in the initial feed in the absence of heat treatment. The liquid feed rate of 9 ml/min was chosen to be the highest rate that is used to produce dry powder without formation of droplets on the wall of the drying chamber when the outlet temperature was at the lowest limit. The low limits were chosen based on the high limits and the middle points. The liquid formulations were prepared, and spray dried in a randomized manner to eliminate bias. Design table is presented in Table 2.

The measured dependent variables were process yield, size of the microparticles, size of the nanocomplexes, stability of the supercoiled plasmid in process and relative transfection efficiency. All measurements were carried out in triplicate.

2.2.3. Preparation of liquid formulations

Chitosan solution prepared in 0.005 M acetic acid, was added spontaneously to 5 ml of plasmid solution with concentration of 50 μg/ml in the same solvent to gain an N/P ratio of 10. The mixture was vortexed vigorously for 20 s and incubated in room temperature for 10 min. Afterwards excipient solutions containing various concentrations of leucine and lactose were added separately to the nanosuspensions to obtain liquid systems required by the design.

2.2.4. Spray drying

All liquid systems were spray dried immediately after preparation by a B-191 mini-spray dryer™ (BuchiLabortechnik AG, Switzerland), with conditions indicated by the design. Ethanol–water mixture was sprayed into the chamber of the spray dryer and then evaporated at 160 °C before each run to avoid contamination. The jacket of the spray nozzle was cooled by circulation

Table 1
List of independent and dependent variables used in the design.

Independent variables				
Variable	High limit (1)	Central point (0)	Low limit (−1)	Unit
Inlet temperature (A)	160	140	120	°C
Spraying air flow rate (B)	700	600	500	l/h
Feed rate (C)	9	6	3	ml/min
Feed fluid concentration (D)	10	7	4	%
Leucine ratio (E)	15	10	5	%

of ambient temperature water. At the end of the spray drying process powders were collected near flame of a bunsen burner, and the process yield was calculated. Collected powders were sealed from moisture and stored at −20 °C.

2.2.5. Characterization of microparticles

Micrometric size distribution of spray dried powders obtained by laser light scattering. Powders were dispersed in 0.01% span 20 ethyl acetate solution. The dispersions were sonicated for 2 min in an ultrasonic bath, and their sizes determined by a Mastersizer™ (Malvern Instruments, Malvern, Worcestershire, UK) particle size measurement instrument.

Scanning electron micrographs of the selected powder formulations was acquired with a Philips XL 30™ microscope (Amsterdam, Netherland), at an acceleration voltage of 17 kV, after vacuum deposition of gold (SCD 005 sputter coater, Bal-Tec, Germany).

2.2.6. Size of nanoparticles

Particle size distribution of the freshly prepared and the processed nanocomplexes was determined by dynamic light scattering technique using a Zetasizer Nano ZS™ instrument (Malvern Instruments, Malvern, Worcestershire, UK). The freshly prepared nanocomplexes and the spray-dried ones were diluted in Milli-Q™ water to concentrations of 100–200 KC/S. 700 μl of the diluted sample was transferred to a disposable cell of the instrument. The content was equilibrated to 25 °C for 2 min. Default values of viscosity and refractive index for water, stored in Zetasizer software were used to analyze the results.

2.2.7. Electrophoresis

The content of supercoiled plasmid structure was obtained by image analysis of DNA electrophoresis gel photographs. To liberate DNA from the nanocomplex, nanosuspensions were incubated with equal volumes of a 50 μg/μl solution of heparin at room temperature for 5 h. Afterward, an amount of the mixture equivalent to 0.5 μg DNA was transferred to each well of a 0.5% agarose gel containing EthBr and electrophoresed in TBE buffer (pH approximately 8) for 1 h with a potential of 100 V. Photographs of the gels acquired by a BioDoc-It™ imaging system (UVP, Upland, CA, USA) and analyzed by Image J image analysis program (Rasband, W.S., Image J, U.S. National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/, 1997–2009), where uncomplexed plasmid was used as control for unprocessed DNA.

Table 2
D-optimal design and mean values for measured responses.

Number	Design					Responses				
	A[a]	B	C	D	E	Microparticle size (μm)	Nanocomplex size (nm)	Yield (%)	DNA stability (%)	Relative transfection (%)
1	1	1	1	−1	−1	5.7	215.5	30.2	100	50.0
2	1	−1	−1	1	1	10.7	278.0	42.0	85.4	50.3
3	−1	1	−1	1	1	7.9	196.7	46.1	79.0	61.4
4	1	1	−1	1	−1	6.4	209.0	50.0	85.6	87.0
5	−1	1	1	1	−1	7.5	318.7	61.8	70.3	81.9
6	1	−1	1	1	1	9.1	149.5	37.1	96.9	101.3
7	1	−1	−1	−1	−1	6.7	183.2	47.7	81.5	104.9
8	−1	−1	1	−1	−1	10.3	260.0	42.9	76.4	139.7
9	1	−1	1	1	−1	8.6	117.4	49.0	90.7	89.7
10	1	−1	0	−1	1	9.3	120.2	25.6	93.7	50.5
11	1	1	0	1	1	11.2	301.9	38.0	93.0	91.9
12	−1	1	0	−1	−1	7.7	246.6	39.2	76.8	99.2
13	0	−1	0	0	0	11.8	350.0	38.9	72.7	111.6
14	−1	0	0	0	0	5.4	368.6	37.5	69.4	80.7
15	0	0	0	1	0	11.4	267.0	29.3	63.5	64.3
16	1	1	−1	−1	0	6.6	177.9	48.5	82.8	73.3
17	−1	−1	0	1	1	8.3	160.2	51.6	93.6	88.1
18	−1	1	1	−1	1	3	290.9	49.7	60.6	69.0
19	−1	−1	−1	1	−1	6.5	119.9	47.1	74.8	71.4
20	−1	−1	−1	−1	0	5.3	196.7	50.2	84.7	78.3
21	0	1	−1	−1	1	10.7	343.0	45.4	94.2	144.6
22	1	−1	1	−1	−1	2.8	141.9	45.5	83.0	141.3
23	1	−1	−1	1	−1	4.5	117.0	44.7	79.2	86.5
24	−1	1	−1	1	−1	8.5	84.8	54.0	94.5	125.7
25	−1	−1	1	1	−1	8.5	122.5	43.3	79.6	69.3
26	1	1	1	1	−1	2.5	255.0	36.8	67.1	75.4
27	0	0	0	1	0	5.9	173.7	42.5	72.4	109.3
28	0	−1	0	0	0	2.9	203.1	57.5	69.0	80.4
29	−1	0	0	0	0	6.5	174.5	55.2	70.0	126.7
30	0	1	−1	−1	1	2.9	274.5	43.4	72.4	84.4
31	−1	1	1	−1	1	3.4	127.1	46.6	94.1	70.4

[a] (A) Inlet temperature, (B) spray air flow rate, (C) feed rate, (D) feed fluid concentration, and (E) leucine ratio with respect to solid content.

Table 3
Data for fitted models onto data obtained for measured responses.

Term	Yield		Microparticle size		*Z*-average particle size		Supercoiled structure percent		Relative transfection	
	Coefficient	*F*-value	Coefficient	*F*-value	Coefficient	*F*-value	Coefficient	*F*-value	Coefficient	*F*-value
Intercept	45.28	–	7.13	–	214.97	–	81.1	–	90.42	–
A[a]	2.82	17.32	−0.64	4.41	−33.43	16.11	4.91	28.98	–	–
B	−0.71	1.19	–	–	24.72	9.62	−1.94	4.78	6.43	4.88
C	−5.3	48.93	1.44	17.2	–	–	6.29	39.34	−9.57	8.61
D	−1.79	8.24	1.49	25.68	–	–	0.73	0.71	0.93	0.11
E	1.7	5.48	1.31	16.51	3.79	0.2	2.78	8.57	19.5	39.37
D^2	–	–	–	–	–	–	–	–	−27.61	7.54
E^2	–	–	–	–	–	–	–	–	33.89	18.96
AB	2.08	8.35	–	–	–	–	–	–	–	–
AC	–	–	−2.1	35.48	–	–	–	–	–	–
AD	2.15	9	−0.79	5.86	–	–	–	–	–	–
AE	–	–	−0.92	7.49	−58.05	38.16	–	–	–	–
BC	–	–	–	–	–	–	–	–	5.36	2.93
BD	3.84	32.58	–	–	–	–	–	–	–	–
BE	–	–	–	–	−58.15	42.58	8.51	78.87	–	–
CD	−2.56	11.96	–	–	–	–	1.99	–	–	–
CE	1.98	5.68	0.68	3.58	–	–	5.08	21.7	–	–
DE	–	–	0.66	4.41	–	–	–	–	4.97	2.63
R^2	0.8858		0.8259		0.793		0.8859		0.8042	

[a] (A) Inlet temperature, (B) spray air flow rate, (C) feed rate, (D) feed fluid concentration, and (E) leucine ratio with respect to solid content.

2.2.8. Transfection

To assess biological activity of the processed nanocomplexes, *in vitro* transfection experiments were carried out on A549 cells. Twenty-four hours before transfection, 25,000 cells were seeded in a 24 well cell culture plate containing 1 ml of D-MEM culture media supplemented with 10% FCS. The freshly prepared nanocomplexes or the spray-dried powders (reconstituted in 100 μl deionized water), equivalent to 2 μg DNA, were incubated with cells for 8 h the day after. Seventy-two hours post-transfection, cells were trypsinized and analyzed by flow cytometry (CyFlow™, Partec GmbH, Münster, Germany) to obtain the ratio of transfected population. The ratio of transfected population measured for the spray-dried nanocomplexes divided by that of freshly prepared nanocomplexes to obtain relative transfection efficiency.

3. Results

Data obtained as results for five measured responses are presented in Table 2. Table 3 contains equations and other data for corresponding curves, which were fitted to the data.

3.1. Process yield

Data obtained for process yield was significantly fitted into a 2FI model ($p < 0.0001$). All selected independent variables had a significant effect on the process yield except for the spray air flow rate. The feed rate (C) had the strongest effect among the main effects. Furthermore, there were several interactions between these variables. As it can be seen in the equation of the model, the interaction between spray air flow rate and feed fluid concentration (BD) was the most important interaction. All sentences of the equation that has included inlet temperature (A) have positive signs. Feed rate had the most powerful negative effect on the yield of the process, as can be seen by its' coefficient in the equation. In addition, this negative effect was posed by interaction of this parameter with feed concentration, but not with the leucine ratio. Fig. 1 is a graphical representation of the effect of spray air flow rate, feed fluid concentration and feed rate on the process yield, keeping inlet temperature and leucine ratio at their highest levels. As it appears in this figure, the highest yield was achievable when values for inlet temperature, spray air flow rate, feed fluid concentration and leucine ratio were set to be at their high limit, while feed rate was minimum.

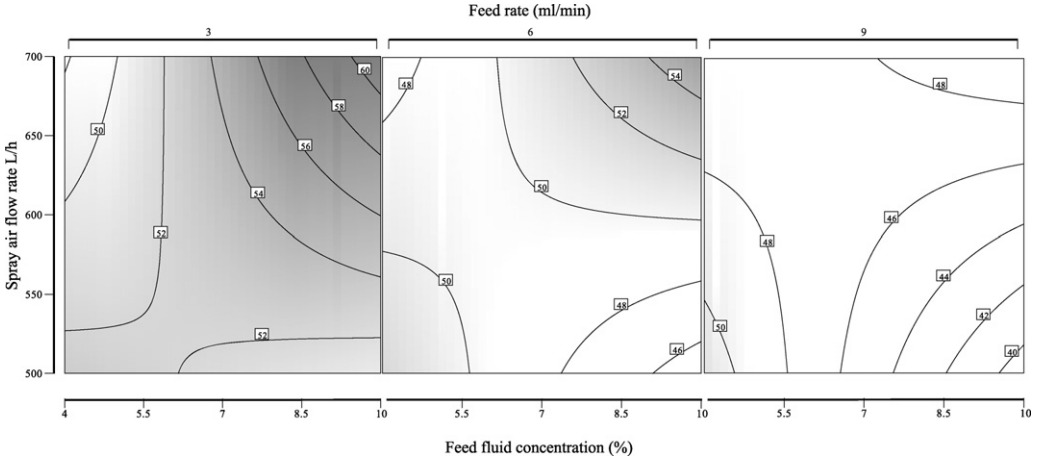

Fig. 1. Contour plots presenting variation of the process yield in response to spray air flow rate, feed fluid concentration and feed rate. Inlet temperature: 160 °C. Leucine ratio: 15%.

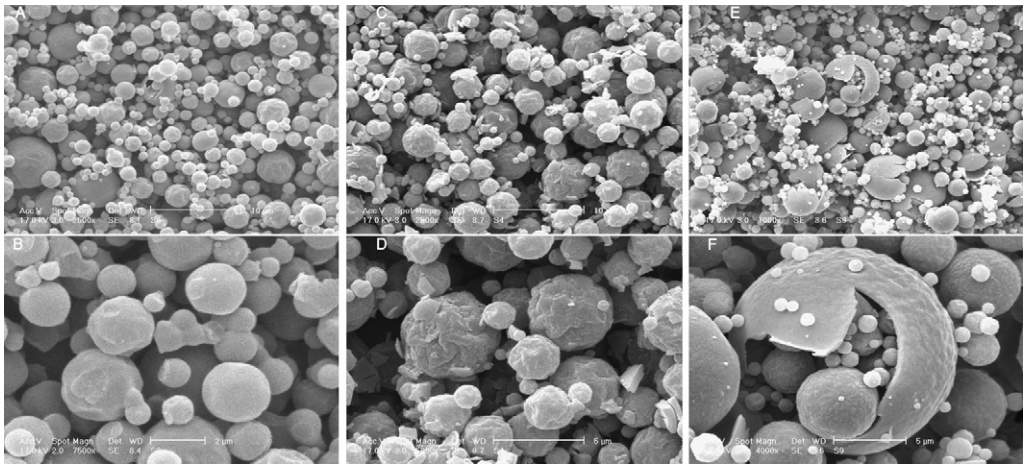

Fig. 2. SEM images of formulations number 18, 27 and 6.

3.2. Properties of microparticles

Fig. 2 illustrates SEM micrographs of three samples, which exhibited different particle sizes and surface morphologies. Particle sizes of spray dried powders ranged from 2.5 to 11.7 μm. Mean size of the microparticles was also fitted to a 2FI model ($p = 0.0002$). Pure effects in order of importance were solid content of feed fluid, feed rate and leucine ratio, all having an increasing effect on particle size. In addition, there were significant interactions between factors. The inlet temperature and feed rate interaction (AC) was the most powerful one, posing a negative effect. Fig. 3 shows the effect of inlet temperature, feed rate and leucine ratio on the size of microparticles, while feed fluid concentration was kept at the minimum. As it can be seen, at the lower temperature limit and at various leucine ratios, increased feed rate led to increased size of the microparticles. However, at the high temperature limit, the particle size was not changed significantly through modification of the other parameters.

3.3. Nanoparticle size distribution

Z-average particle sizes ranged from 84.8 to 368.6 nm. Inlet temperature and spray air flow rate were two main effects, which significantly controlled the size of nanocomplexes entrapped in the spray dried powders. However, their interactions with the leucine ratio (i.e. AE and BE) showed more powerful effects on size, although the effect of the leucine ratio was not significant as a pure factor. Fig. 4 presents effect of inlet temperature, spray air flow rate and leucine ratio on *Z*-average size of the spray dried nanocomplexes. According to the fitted model and Fig. 4, minimum nanoparticle size could be obtained in two extreme regions, first where the inlet temperature, spray air flow rate and leucine ratio were set at their maxima, second when all these parameters were minimum.

3.4. DNA stability

Percent of supercoiled structure band intensity in acquired photographs of gel electrophoresis was considered as DNA stability. Results, which ranged from 60.6 to 100% of intensity of un-bonded DNA, were fitted in a 2FI model ($p < 0.0001$). Inlet temperature and feed rate were two significant pure factors affecting DNA stability, both having a positive effect. Two significant interaction effects were also observed. First, the interaction between spray air flow rate and leucine ratio (BE), and second the interaction between the feed rate and leucine ratio (CE). Fig. 5 presents the interaction between leucine ratio and spray air flow rate, which is the most powerful term in the equation (*F* value: 78.9). The model predicted the highest DNA stability when inlet temperature, spray air flow rate and feed rate were at their high limits.

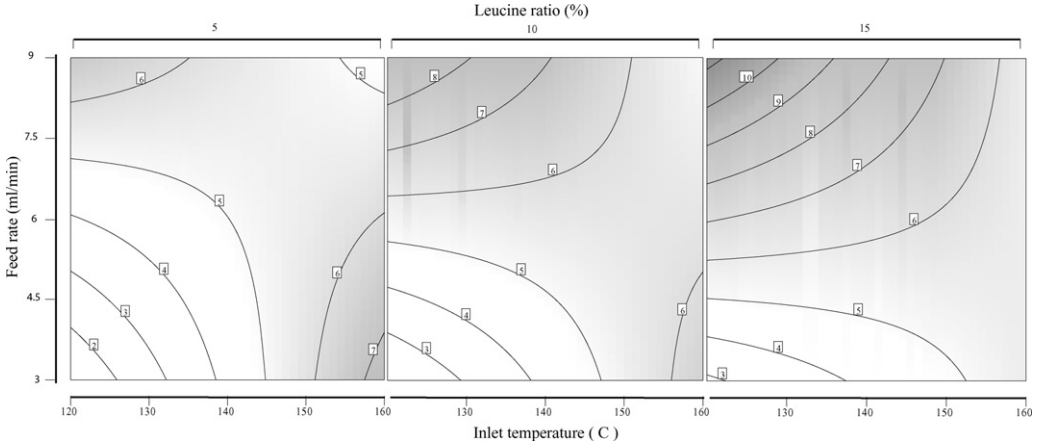

Fig. 3. Effect of inlet temperature, feed rate and leucine ratio on the size of microparticles. Feed fluid concentration: 4%.

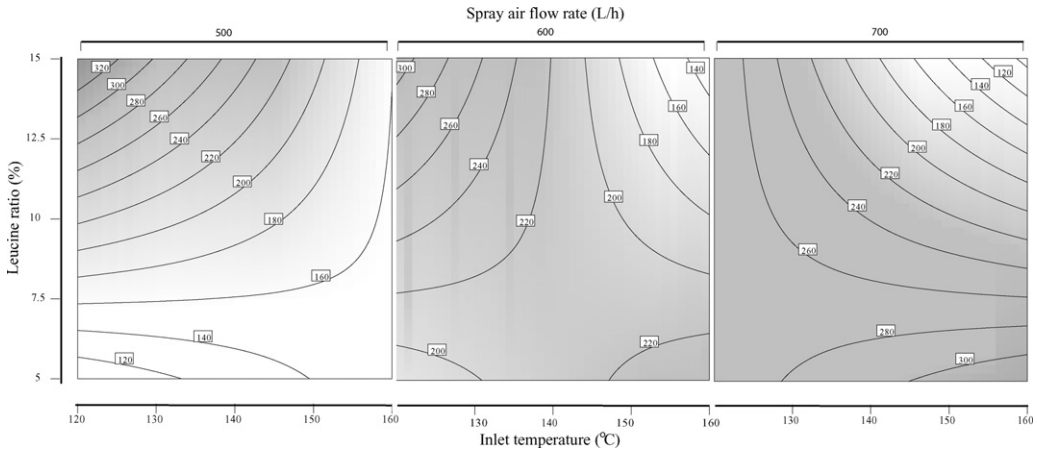

Fig. 4. Variation of *Z*-average nanocomplex size in response to inlet temperature, spray air flow rate and leucine ratio.

3.5. Relative transfection

The relative transfection efficiency of polyplexes measured after processing by spray dryer. Freshly prepared polyplexes were used as control. The results ranged from 50 to 144.6% of primary transfection efficiencies. This mild enhancement of relative transfection after freeze drying or spray drying has been reported in previous works (Seville et al., 2002; Talsma et al., 1997). Unlike other responses, quadratic model was fitted significantly into the results ($p < 0.0001$). The leucine ratio and its square were the most influential factors in the model. Fig. 6 shows the effect of this factor along with feed fluid concentration on relative transfection efficiency of the polyplexes. As it is appeared in the figure, the highest relative transfection was observed at the high limit of leucine ratio and middle range of feed fluid concentration. The highest relative transfection was predicted by the model where feed rate was at its minimum value, the leucine ratio was high and feed fluid concentration was in the middle point.

4. Discussion

4.1. Process yield

As stated before, spray air flow rate and feed fluid concentration both had negative effects on the process yield. However, interestingly their interaction with each other poses the most powerful positive effect on the response.

It has been reported that spray drying conditions that produce powders with lower moisture content give higher process yields because of their higher cohesive nature (Broadhead et al., 1994). Therefore, spray drying conditions that is more likely to give particles with low moisture content may have better yields. Here, using lower feed rate and higher inlet temperature during spray drying process can be assumed to dry nanosuspension droplets more efficiently and so increase the yield. Tajber et al. (2009) have reported a statistically insignificant effect of inlet temperature on the yield of spray drying process of a budesonide and formoterol fumarate mixture. The liquid phase in their work was mainly ethanol, which

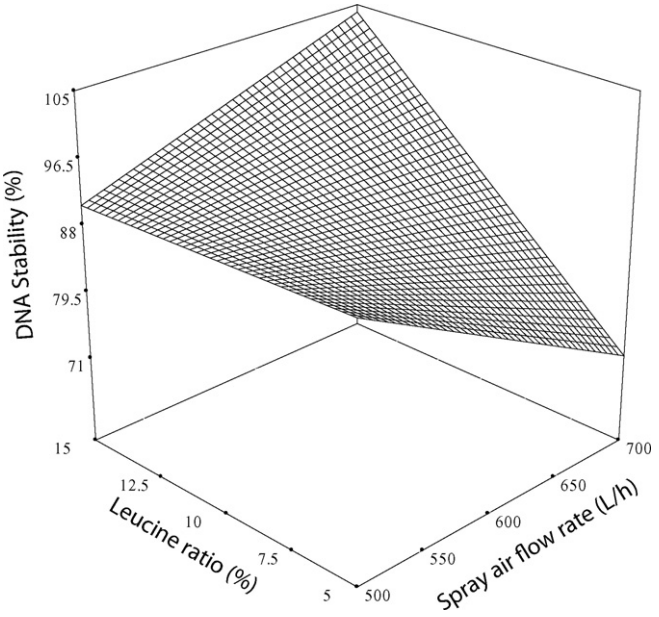

Fig. 5. Effect of interaction between leucine ratio and spray air flow rate on the intensity of supercoiled plasmid band in gel electrophoresis.

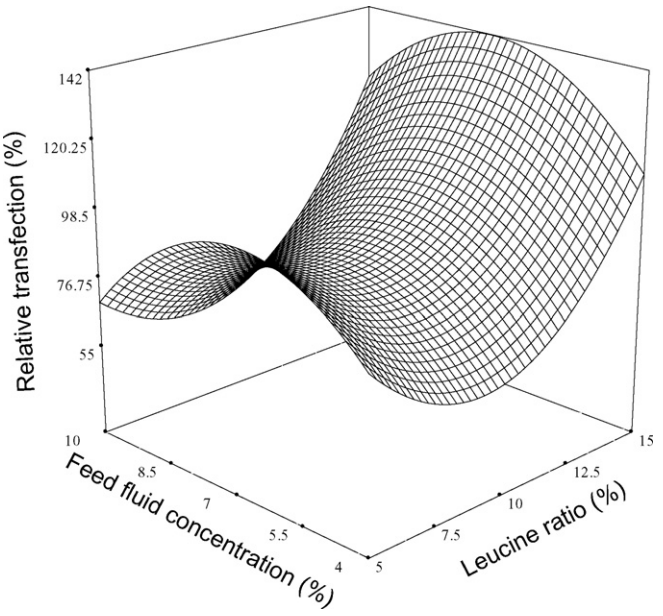

Fig. 6. Effect of the interaction between leucine ratio and feed fluid concentration on relative transfection efficiency.

is more volatile than water; therefore, the range of inlet temperature that they have used in their work was above the critical limit for drying of the formulations.

Prinn et al. (2002) and Chawla et al. (1994) reported feed fluid concentration as the most important factor determining process yield. Contrary to their results, we observed feed rate to be the most determinant variable. The concentration of bovine serum albumin in the work has been done by Prinn et al. was ranged from 2 to 100 mg/ml (0.2–10%) which was a 50-fold increase in concentration, but in this study we had a 2.5-fold increase in concentration. Chawla et al. has also used a concentration ranged from 10 to 20% of salbutamol sulphate in an aqueous feed fluid, which was higher than the concentration range we applied. In addition, the difference in the nature of the materials, which have been used in these works, might be considered as a source of contradiction.

4.2. Properties of microparticle

The size of microparticle is related to droplet size by:

$$D_{particle} \cong D_{droplet} \times \sqrt[3]{C \times \frac{\rho_{droplet}}{p_{particle}}} \quad (1)$$

In which D is the diameter of particle or droplet, C is the concentration of solids in feed fluid and ρ is the density of droplet or particle. Diameter of droplet, generated by a two fluid nozzle, is also determined by the equation developed by Nukiyama and Tansawa (1939).

$$D_{droplet} = \frac{585}{V_{rel}}\sqrt{\frac{\sigma}{\rho_l}} + 597\left(\frac{\mu_l}{\sqrt{\rho_l \sigma}}\right)^{0.45}\left(1000\frac{Q_a}{Q_l}\right)^{1.5} \quad (2)$$

where V_{rel} is the relative velocity of air to fluid, σ is surface tension, ρ_l is the density of liquid, μ is viscosity and Q is volumetric flow rates of air and liquid. It can be concluded from Eq. (2) that feed rate strongly affects the particle size by controlling V_{rel} and Q_a/Q_l. This conclusion is in accordance with our results. The size of particle is also related to the concentration of solids in feed fluid (Eq. (1)). It was reported that the solid content of bovine serum albumin (Prinn et al., 2002) or salbutamol sulphate (Chawla et al., 1994) in feed fluid has a strong effect on the particle size of spray dried powders. Our results were also showed the strongest pure effect on particle size for the feed fluid concentration. Eqs. (1) and (2) suggest that the spray air flow rate can affect V_{rel} and Q_a/Q_l, therefore, it can influence the particle size via altering the diameter of atomized droplets. The spray air flow rate was found to be the most important factor affecting the size of spray dried particles of budesonide/formoterol fumarate mixture (Tajber et al., 2009). Contrarily, the effect of this factor on size of the microparticles containing the plasmid nanocomplexes was not significant.

As it appears from Fig. 2, the surface roughness of microparticles was increased by increasing leucine ratio. This result has been witnessed in previous works (Feng et al., 2011; Gervelas et al., 2007). Large hollow microparticles was also observed more common in powder samples produced with higher leucine ratio, feed fluid concentration and feed rate (Fig. 2E and F), which according to the model was more likely to give higher particle sizes.

4.3. Nanoparticle size distribution

To our knowledge, the effect of formulation and process variables on size distribution of spray-dried nucleic acid nanocomplexes has not been studied yet.

As it can be seen in Table 3 inlet temperature had a strong reducing effect on the nanocomplex size. This effect can be justified with a view of optimal conditions of freeze drying of nanoparticles. Reduction of time needed for freezing of nanoparticles, via a radical decrease in temperature have been demonstrated to give better preservation of particle size in freeze drying process (Abdelwahed et al., 2006; Anchordoquy and Koe, 2000; Seville et al., 2002). The mechanism of this effect was suggested to be reduced time, given to nanoparticles to concentrate in the non-frozen state during formation of ice crystals by trapping them in amorphous structure of snap frozen water. It seems that using low inlet temperature in spray drying process nanoparticles have enough time to concentrate inside drying droplets. Therefore, increased inlet temperature can reduce the drying time and inhibit particle aggregation.

Our results showed a positive effect for spray air flow rate as a pure effect, which might be owing to increased collisions between particles. However, interaction of this factor with the inlet temperature and leucine ratio in the formulations had a more influential negative effect.

Mechanism of the effect of the leucine ratio in reducing the size via interaction with inlet temperature is unknown to the researchers. However, this effect might be due to competition of leucine with nanoparticles for occupying positions on shrinking surface of a drying droplet, which reduces chance for particle aggregation by keeping surface concentration of nanoparticles low.

The chance for a particle or molecule to accumulate on the surface of a droplet is calculated by Peclet number, P_e (Vehring, 2008).

$$P_e = \frac{\kappa}{8D} \quad (3)$$

In which κ is the evaporation rate and D is the diffusion coefficient for that particle or molecule. Leucine molecules and nanoparticles experienced similar drying conditions so the difference in their Peclet numbers arises from their different diffusion coefficients. To calculate the diffusion coefficient of nanoparticles or leucine molecules one can use Stokes–Einstein's equation (Tsapis, 2002).

$$D = \frac{k_B T}{6\pi\eta R_H} \quad (4)$$

where k_B is Boltzman's constant, T is temperature, η is viscosity and R_H is the hydrodynamic diameter of particle or molecule. Regarding to this equation Peclet number for leucine molecules is much less than the one for nanoparticles. However, former studies have shown a high percent of surface accumulation for leucine (Lucas et al., 1999; Najafabadi et al., 2004). Because of special properties of leucine Stokes–Einstein's equation is not enough for modeling behavior of this molecule during spray drying process. Recently, Feng et al. (2011) have suggested a mechanism to explain this behavior. The time needed for leucine to reach surface saturation is lower than the time needed for droplet drying, so this difference gives time to leucine molecules to crystallize. R_H of formed crystals is high enough to give low diffusion coefficient and high Peclet number so that they can efficiently occupy surface of formed particles. This mechanism does not include surface activity of leucine, which has been suggested to be important in formation of hollow particles by surface active molecules (Tsapis, 2002). However, it successfully explains the ability of leucine and its derivatives to gain high surface concentration during spray drying process.

As it can be seen in Fig. 4, at the low limit of inlet temperature and the high limit of the leucine ratio, the size of nanoparticles was increased. It seemed that at the low limit of inlet temperature, where Peclet number and surface enrichment of nanoparticles is lower, negative charge of leucine (Li et al., 2003) reduces zeta potential of nanoparticles and promotes their aggregation. On the other hand, at the high limit of temperature, Peclet number is high and nanoparticles tend to concentrate at the surface of drying droplet. In these conditions, the ability of leucine in surface enrichment might be a possible mechanism involved in size reducing effect of this material.

There was also a strong interaction between leucine ratio and spray air flow rate, which was the most powerful term determining particle size. A plausible mechanism for the effect of leucine on preservation of size distribution of the nanocomplexes might be suggested by considering surface activity of leucine. At the time of breakdown of the liquid stream to fine droplets, amphiphile small leucine molecules can easily diffuse (Eq. (4)) to the surface of the droplets and prevent nanocomplexes with a high hydrodynamic diameter to become concentrated at the air-liquid interface. Despite high positive zeta potential of low molecular weight chitosan–plasmid polyplexes, it has been suggested that they include areas with hydrophobic properties in their structure (Koping-Hoggard et al., 2001) and thus can behave like polypeptides, which can go through extreme aggregation upon a tremendous increase in surface area during atomization (Wang, 2005). Aggregation of polypeptides during processes that increase air liquid interface is also inhibited by addition of surface-active agents (Bam et al., 1998; Maa et al., 1998; Webb et al., 2002).

4.4. DNA stability

The most important destabilizing factor in spray drying of nucleic acids is shear rate, which is applied to convert feed fluid to aerosol droplets. Referring to Eq. (2), one can conclude that increased spray air flow rate and decreased feed rate can decrease droplet size via increasing V_{rel}, both increase shear stress and can cause destabilization of plasmid DNA. Here negative sign of spray air flow rate and positive sign of feed rate reflect this fact.

Increased inlet temperature had a strong positive effect on the stability of plasmid DNA. Covalent bond cleavage in DNA due to shear stress has been subject of several investigations, and some equations have been developed (Lengsfeld and Anchordoquy, 2002). According to our knowledge, the effect of temperature on shear induced breakdown of complexed DNA has not been studied before. It can be suggested that the effect of temperature might be through the reduction of viscosity of water as a Newtonian liquid. Lower viscosity will increase Reynold's number and enhance the turbulent flow in the nozzle. The unsteady flow causes free flexible plasmid to oscillate between compact and extended conformation (Hur et al., 2001), and degrade to higher extend. However, complexed plasmid is less vulnerable to these deformations. On the other hand, higher turbulent flow will also decrease the velocity gradient and thus the strain rate (Lengsfeld and Anchordoquy, 2002) and might result in lower degradation of plasmid as witnessed in this work.

Although a previous work by Li et al. (2003) has declared a destabilization effect for leucine on a lipid–protein based gene delivery system in spray drying, our results showed that in the low limit of spray air flow rate leucine poses a practically insignificant destabilization effect of 8% reduction in intensity of supercoiled band (Fig. 5). However, in the high limit of spray air flow rate the fitted model showed a strong stabilization for leucine. The mechanism of this shift is again unknown to the researchers, but it might be explained by surface activity of leucine. Referring to Eq. (2), it can be concluded that increased surface tension increases droplet size of atomized liquid, so surface active agents are valuable in accelerating atomization of liquids in pneumatic nozzles (Ruiz et al., 1993). On the other hand, generally accepted physical mechanism for aerosol formation in 2-fluid nozzles is two-stage instability, in which the liquid stream is distorted primarily by shear instability flowed by Rayleigh–Taylor's destabilization of liquid tongues (Aliseda et al., 2008; Varga et al., 2003). Thus, during this process the majority of shear stress is applied to surface of the fluid stream. This destabilization effect on nucleic acids can be avoided by reducing the concentration of these biopharmaceuticals on the surface. Considering this mechanism true, at the low limit of spray air flow rate the effect of leucine is practically insignificant because shear stress generated by the air stream in the nozzle is low and protection of plasmid is mainly carried out by binding polymer. On the other hand, at the high limit of spray air flow rate, where shear stress is high, protecting effect of leucine becomes significant.

4.5. Relative transfection

Fitted model on relative transfection data differed from other responses, which can be attributed to more complicated nature of this response. *Z*-average particle size and supercoiled structure content theoretically should be correlated with relative transfection of the nanocomplexes, but here we failed to find a correlation between these responses and transfection. This might be owing to strong effect of the leucine ratio and its square on relative transfection, which needs to be further investigated. An ANOVA test, followed by a Duncan post hoc analysis showed that powders containing 15% leucine with respect to dry weight of formulation have significantly ($p < 0.05$) higher transfection efficiencies regardless of particle size or supercoiled structure content, compared to those containing 5 or 10% leucine. Li et al. (2003) found that powders containing 10% leucine with regard to lactose weight in formulation showed 60% of transfection obtained by powders without leucine. They also observed that leucine had a negative effect on the stability of plasmid and attributed this effect to negative charge of this amino acid, which can loosen the electrostatic bond between plasmid and the lipid vector. Observed inconsistency between the results of present work with those of Li and co-workers might be rising from different applied gene delivery systems. Here, we applied a polymeric gene delivery system, which is generally proven to condense DNA more efficiently than lipid gene delivery systems (Ernst et al., 1999; Lengsfeld and Anchordoquy, 2002). However, the enhancing effect of leucine has to be studied in future works.

5. Conclusion

In this research, we tried to optimize a low molecular weight chitosan based gene delivery system, regarding most important process and formulation parameters, to produce a potentially inhalable formulation with minimum loss in activity. We used lactose and leucine as two popular excipients in production of dry powder inhalers. Results showed strong dependency of all measured responses on the process and formulation parameters. Results obtained for four out of five responses were fitted in a two-factor interaction model, but a quadratic model showed the lowest *p*-value fitted on data obtained for relative transfection. Leucine showed desirable effects on size distribution of microparticles and nanoparticles, supercoiled structure of plasmid and transfection via unknown mechanisms, which needs further investigations to be truly understood. High inlet temperature led to higher process yield, lower microparticle and nanoparticle size and higher DNA stability during the process, but had no significant effect on relative transfection efficiency. Another important variable was shown to be feed rate, which had an undesirable effect on yield, microparticle size and relative transfection efficiency but desirable effect on DNA stability.

Finally, the optimized conditions regarding all five responses at their best levels were determined to be when all independent variables are at their high limit, except for feed fluid concentration, which should be in the middle point.

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References

Abdelwahed, W., Degobert, G., Stainmesse, S., Fessi, H., 2006. Freeze-drying of nanoparticles: formulation, process and storage considerations. Adv. Drug Deliv. Rev. 58, 1688–1713.

Aliseda, A., Hopfinger, E., Lasheras, J., Kremer, D., Berchielli, A., Connolly, E., 2008. Atomization of viscous and non-Newtonian liquids by a coaxial, high-speed gas jet. Experiments and droplet size modeling. Int. J. Multiphas. Flow 34, 161–175.

Anchordoquy, T.J., Girouard, L.G., Carpenter, J.F., Kroll, D.J., 1998. Stability of lipid/DNA complexes during agitation and freeze–thawing. J. Pharm. Sci. 87, 1046–1051.

Anchordoquy, T.J., Koe, G.S., 2000. Physical stability of nonviral plasmid-based therapeutics. J. Pharm. Sci. 89, 289–296.

Bam, N.B., Cleland, J.L., Yang, J., Manning, M.C., Carpenter, J.F., Kelley, R.F., Randolph, T.W., 1998. Tween protects recombinant human growth hormone against agitation-induced damage via hydrophobic interactions. J. Pharm. Sci. 87, 1554–1559.

Bivas-Benita, M., Ottenhoff, T.H., Junginger, H.E., Borchard, G., 2005. Pulmonary DNA vaccination: concepts, possibilities and perspectives. J. Control. Release 107, 1–29.

Bragonzi, A., Dina, G., Villa, A., Calori, G., Biffi, A., Bordignon, C., Assael, B.M., Conese, M., 2000. Biodistribution and transgene expression with nonviral cationic vector/DNA complexes in the lungs. Gene Ther. 7, 1753–1760.

Broadhead, J., Rouan, S.K., Hau, I., Rhodes, C.T., 1994. The effect of process and formulation variables on the properties of spray-dried beta-galactosidase. J. Pharm. Pharmacol. 46, 458–467.

Chawla, A., Taylor, K.M.G., Newton, J.M., Johnson, M.C.R., 1994. Production of spray dried salbutamol sulphate for use in dry powder aerosol formulation. Int. J. Pharm. 108, 233–240.

Colonna, C., Conti, B., Genta, I., Alpar, O.H., 2008. Non-viral dried powders for respiratory gene delivery prepared by cationic and chitosan loaded liposomes. Int. J. Pharm. 364, 108–118.

Dolenc, A., Kristl, J., Baumgartner, S., Planinsek, O., 2009. Advantages of celecoxib nanosuspension formulation and transformation into tablets. Int. J. Pharm. 376, 204–212.

El-Sherbiny, I.M., Smyth, H.D., 2010. Biodegradable nano-micro carrier systems for sustained pulmonary drug delivery. I: self-assembled nanoparticles encapsulated in respirable/swellable semi-IPN microspheres. Int. J. Pharm. 395, 132–141.

Ernst, N., Ulrichskotter, S., Schmalix, W.A., Radler, J., Galneder, R., Mayer, E., Gersting, S., Plank, C., Reinhardt, D., Rosenecker, J., 1999. Interaction of liposomal and polycationic transfection complexes with pulmonary surfactant. J. Gene Med. 1, 331–340.

Feng, A.L., Boraey, M.A., Gwin, M.A., Finlay, P.R., Kuehl, P.J., Vehring, R., 2011. Mechanistic models facilitate efficient development of leucine containing microparticles for pulmonary drug delivery. Int. J. Pharm.

Gervelas, C., Serandour, A.L., Geiger, S., Grillon, G., Fritsch, P., Taulelle, C., Le Gall, B., Benech, H., Deverre, J.R., Fattal, E., Tsapis, N., 2007. Direct lung delivery of a dry powder formulation of DTPA with improved aerosolization properties: effect on lung and systemic decorporation of plutonium. J. Control. Release 118, 78–86.

Gilani, K., Moazeni, E., Ramezanli, T., Amini, M., Fazeli, M.R., Jamalifar, H., 2011. Development of respirable nanomicelle carriers for delivery of amphotericin B by jet nebulization. J. Pharm. Sci. 100, 252–259.

Gill, D.R., Davies, L.A., Pringle, I.A., Hyde, S.C., 2004. The development of gene therapy for diseases of the lung. Cell. Mol. Life Sci. 61, 355–368.

Glover, D.J., Lipps, H.J., Jans, D.A., 2005. Towards safe, non-viral therapeutic gene expression in humans. Nat. Rev. Genet. 6, 299–310.

Griesenbach, U., Alton, E.W., 2009. Gene transfer to the lung: lessons learned from more than 2 decades of CF gene therapy. Adv. Drug Deliv. Rev. 61, 128–139.

Hur, J.S., Shaqfeh, E.S.G., Babcock, H.P., Smith, D.E., Chu, S., 2001. Dynamics of dilute and semidilute DNA solutions in the start-up of shear flow. J. Rheol. 45, 421.

Klingler, C., Muller, B.W., Steckel, H., 2009. Insulin-micro- and nanoparticles for pulmonary delivery. Int. J. Pharm. 377, 173–179.

Koping-Hoggard, M., Tubulekas, I., Guan, H., Edwards, K., Nilsson, M., Varum, K.M., Artursson, P., 2001. Chitosan as a nonviral gene delivery system. Structure–property relationships and characteristics compared with polyethylenimine in vitro and after lung administration in vivo. Gene Ther. 8, 1108–1121.

Lengsfeld, C.S., Anchordoquy, T.J., 2002. Shear-induced degradation of plasmid DNA. J. Pharm. Sci. 91, 1581–1589.

Li, H.Y., Birchall, J., 2006. Chitosan-modified dry powder formulations for pulmonary gene delivery. Pharm. Res. 23, 941–950.

Li, H.Y., Neill, H., Innocent, R., Seville, P., Williamson, I., Birchall, J.C., 2003. Enhanced dispersibility and deposition of spray-dried powders for pulmonary gene therapy. J. Drug Target 11, 425–432.

Li, H.Y., Seville, P.C., Williamson, I.J., Birchall, J.C., 2005. The use of absorption enhancers to enhance the dispersibility of spray-dried powders for pulmonary gene therapy. J. Gene Med. 7, 1035–1043.

Li, S., Huang, L., 1997. In vivo gene transfer via intravenous administration of cationic lipid–protamine–DNA (LPD) complexes. Gene Ther. 4, 891–900.

Lucas, P., Anderson, K., Potter, U.J., Staniforth, J.N., 1999. Enhancement of small particle size dry powder aerosol formulations using an ultra low density additive. Pharm. Res. 16, 1643–1647.

Maa, Y.F., Nguyen, P.A., Hsu, S.W., 1998. Spray-drying of air–liquid interface sensitive recombinant human growth hormone. J. Pharm. Sci. 87, 152–159.

McLean, J.W., Fox, E.A., Baluk, P., Bolton, P.B., Haskell, A., Pearlman, R., Thurston, G., Umemoto, E.Y., McDonald, D.M., 1997. Organ-specific endothelial cell uptake of cationic liposome–DNA complexes in mice. Am. J. Physiol. 273, H387–H404.

Mizuno, T., Mohri, K., Nasu, S., Danjo, K., Okamoto, H., 2009. Dual imaging of pulmonary delivery and gene expression of dry powder inhalant by fluorescence and bioluminescence. J. Control. Release 134, 149–154.

Mohri, K., Okuda, T., Mori, A., Danjo, K., Okamoto, H., 2010. Optimized pulmonary gene transfection in mice by spray-freeze dried powder inhalation. J. Control. Release 144, 221–226.

Najafabadi, A.R., Gilani, K., Barghi, M., Rafiee-Tehrani, M., 2004. The effect of vehicle on physical properties and aerosolisation behaviour of disodium cromoglycate microparticles spray dried alone or with L-leucine. Int. J. Pharm. 285, 97–108.

Nukiyama, s., Tansawa, Y., 1939. Experiments on the atomization of liquids in an atomized jet. Trans. Soc. Mech. Eng. Japan 5, 62–67.

Okamoto, H., Nishida, S., Todo, H., Sakakura, Y., Iida, K., Danjo, K., 2003. Pulmonary gene delivery by chitosan–pDNA complex powder prepared by a supercritical carbon dioxide process. J. Pharm. Sci. 92, 371–380.

Okamoto, H., Sakakura, Y., Shiraki, K., Oka, K., Nishida, S., Todo, H., Iida, K., Danjo, K., 2005. Stability of chitosan–pDNA complex powder prepared by supercritical carbon dioxide process. Int. J. Pharm. 290, 73–81.

Peek, L.J., Roberts, L., Berkland, C., 2008. Poly(D,L-lactide-co-glycolide) nanoparticle agglomerates as carriers in dry powder aerosol formulation of proteins. Langmuir 24, 9775–9783.

Prinn, K.B., Costantino, H.R., Tracy, M., 2002. Statistical modeling of protein spray drying at the lab scale. AAPS PharmSciTech 3, E4.

Ruiz, A.I., Canals, A., Hernandis, V., 1993. Effect of surfactants in flame atomic absorption spectrometry with pneumatic nebulization: influence of hydrophobic chain length. J. Anal. Atom. Spectrom. 8, 109.

Seville, P.C., Kellaway, I.W., Birchall, J.C., 2002. Preparation of dry powder dispersions for non-viral gene delivery by freeze–drying and spray-drying. J. Gene Med. 4, 428–437.

Sham, J.O., Zhang, Y., Finlay, W.H., Roa, W.H., Lobenberg, R., 2004. Formulation and characterization of spray-dried powders containing nanoparticles for aerosol delivery to the lung. Int. J. Pharm. 269, 457–467.

Tajber, L., Corrigan, O.I., Healy, A.M., 2009. Spray drying of budesonide, formoterol fumarate and their composites. II: statistical factorial design and in vitro deposition properties. Int. J. Pharm. 367, 86–96.

Talsma, H., Cherng, J.Y., Lehrmann, H., Kursa, M., Ogris, M., Hennink, W.E., Cotten, M., Wagner, E., 1997. Stabilization of gene delivery systems by freeze-drying. Int. J. Pharm. 157, 233–238.

Tsapis, N., 2002. Trojan particles: large porous carriers of nanoparticles for drug delivery. Proc. Natl. Acad. Sci. U.S.A. 99, 12001–12005.

Varga, C.M., Lasheras, J.C., Hopfinger, E.J., 2003. Initial breakup of a small-diameter liquid jet by a high-speed gas stream. J. Fluid Mech. 497, 405–434.

Vehring, R., 2008. Pharmaceutical particle engineering via spray drying. Pharm. Res. 25, 999–1022.

Wang, W., 2005. Protein aggregation and its inhibition in biopharmaceutics. Int. J. Pharm. 289, 1–30.

Webb, S.D., Cleland, J.L., Carpenter, J.F., Randolph, T.W., 2002. A new mechanism for decreasing aggregation of recombinant human interferon-gamma by a surfactant: slowed dissolution of lyophilized formulations in a solution containing 0.03% polysorbate 20. J. Pharm. Sci. 91, 543–558.

Zelphati, O., Nguyen, C., Ferrari, M., Felgner, J., Tsai, Y., Felgner, P.L., 1998. Stable and monodisperse lipoplex formulations for gene delivery. Gene Ther. 5, 1272–1282.