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Research Paper

Chitosan-Modified Dry Powder Formulations for Pulmonary Gene Delivery

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Purpose. Spray-drying is an effective process for preparing micron-dimensioned particles for pulmonary delivery. Previously, we have demonstrated enhanced dispersibility and fine particle fraction of spray-dried nonviral gene delivery formulations using amino acids or absorption enhancers as dispersibility-enhancing excipients. In this study, we investigate the use of the cationic polymer chitosan as a readily available and biocompatible dispersibility enhancer.
Methods. Lactose-lipid:polycation:pDNA (LPD) powders were prepared by spray-drying and post-mixed with chitosan or spray-dried chitosan. In addition, the water-soluble chitosan derivative, trimethyl chitosan, was added to the lactose-LPD formulation before spray-drying.
Results. Spray-dried chitosan particles, displaying an irregular surface morphology and diameter of less than 2 μm, readily adsorbed to lactose-LPD particles following mixing. In contrast with the smooth spherical surface of lactose-LPD particles, spray-dried trimethyl chitosan-lactose-LPD particles demonstrated increased surface roughness and a unimodal particle size distribution (mean diameter 3.4 μm), compared with the multimodal distribution for unmodified lactose-LPD powders (mean diameter 23.7 μm). The emitted dose and *in vitro* deposition of chitosan-modified powders was significantly greater than that of unmodified powders. Moreover, the inclusion of chitosan mediated an enhanced level of reporter gene expression.
Conclusions. In summary, chitosan enhances the dispersibility and *in vitro* pulmonary deposition performance of spray-dried powders.

KEY WORDS: chitosan; dry powder inhaler; nonviral vector; spray-drying.

INTRODUCTION

Dry powder inhalers (DPI) have shown promise for the delivery of nucleic acids to the lung (1–4). Previous studies have verified that a combination of nonviral gene therapy vector and thermoprotectant sugar carrier can maintain the structural integrity and biological activity of plasmid DNA (pDNA) following dry powder processing via lyophilization or spray-drying (5,6). Although spray-dried particles containing the well-characterized lipid:polycation:pDNA (LPD) nonviral gene vector (7) have been prepared with particle diameter within the respirable range (<5 μm), aggregation between particles provides reduced performance during the process of dose release, aerosolization, and subsequent lung deposition (5). In previous reports, amino acids, such as leucine and phenylalanine (2,3), and pulmonary absorption enhancers, particularly dimethyl-β-cyclodextrin (DMC) (4), have been shown to significantly enhance the dispersibility and *in vitro* pulmonary deposition of spray-dried sugar-LPD powders when incorporated as co-excipients. Unfortunately, many of these additives caused concomitant damage to the physical stability and biological activity of the LPD complex and consequently decreased the gene transfection and expression efficiency in cell cultures (2,3). As an exception, DMC was shown to increase the emitted dose, particle dispersibility, *in vitro* pulmonary deposition, and cell transfection efficiency of spray-dried LPD powders. Clearly, certain compounds may have the ability to enhance the dispersibility of spray-dried powders and improve the pDNA uptake and gene expression in targeted cells (4).

Chitosan (1,4-2-amino-2-deoxy-*b*-D-glucan) is a linear biodegradable polymer derived from the natural polymer of chitin, one of the most abundant polysaccharides in nature. Chitosan has been applied extensively in the agriculture, food, cosmetic, and pharmaceutical industries due to its exceptional biocompatibility, ready availability, and ease of chemical modification (8,9). The use of chitosan and its derivatives as cellular absorption enhancers has been comprehensively reported, with the results demonstrating that chitosan and its synthetic derivatives, particularly *N*-trimethyl chitosan (TMC) (10), can significantly augment the absorption of peptides, proteins, vaccines, and antibiotics *in vitro* (e.g., Caco-2, TR146) (11,12) or through various *in vivo* barriers including nasal (13), intestinal (14), urinary bladder wall (15), respiratory tract (16), ocular (17), and buccal mucosa (18).

Chitosan and its derivatives have also been widely investigated as nonviral gene carrier systems (19). For this purpose, chitosan or its derivatives are complexed with pDNA to form nanosized particulates for gene delivery to

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the target cells. It has been shown that chitosan materials can effectively condense DNA to form complexes with stable physiochemical properties (20,21). Furthermore, chitosan and its derivatives used in this way are reported to have excellent biocompatibility (22), as also observed in studies using chitosan as an absorption enhancer (23).

Although chitosan and its derivatives have been extensively studied both as absorption enhancers and as nonviral gene vectors, their exploitation in dry powder formulations specifically for the enhancement of powder dispersibility, airflow, and deposition has not been reported. In this paper, chitosan, spray-dried chitosan, and the water-soluble synthetic derivative TMC are employed as "dispersibility enhancers" in dry powder formulations, with the aim of increasing the functional lung deposition of the LPD vector. We demonstrate that post-mixing commercially available chitosan with spray-dried lactose-LPD powders, post-mixing spray-dried chitosan with lactose-LPD spray-dried powders, or, most simplistically, adding TMC into the spray-drying solution to prepare the spray-dried powders improves the dry powder capsule emission, dispersibility, and resulting *in vitro* pulmonary deposition. In addition, augmented cellular gene expression was observed in chitosan-modified preparations.

MATERIALS AND METHODS

Materials

1,2-Dioleoyl-3-trimethylammoniumpropane (DOTAP) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Protamine sulfate (Grade X from salmon sperm) was obtained from Sigma-Aldrich Chemicals (Poole, UK). The 4.7-kbp pEGFP-N1 plasmid construct containing the green fluorescent protein (GFP) reporter gene was propagated and purified as detailed previously (24), using kanamycin to ensure selective growth of transformed bacteria.

The following chemicals were used as received: agarose LE (Promega, Southampton, UK); ethidium bromide (EtBr) solution, sodium dodecyl sulphate (SDS) (Pharmacia Biotech, St. Albans, UK); Tris base, Luria–Bertani (LB) agar, LB broth (Sigma-Aldrich); Chitosan (Chitoclear™, ~100 kDa, Primex, Iceland). The soluble chitosan derivative trimethyl chitosan (TMC) (30% degree of substitution, ~100 kDa) was kindly donated by Dr. Maya Thanou (formerly Welsh School of Pharmacy). Cell culture plastics were purchased from Corning-Costar (High Wycombe, UK). Dulbecco's modified Eagle's medium [DMEM; 25 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES)], fetal bovine serum, penicillin–streptomycin solution, and trypsin-ethylenediaminetetraacetic acid (EDTA) solution 1× were obtained from Life Technologies (Paisley, UK). All other chemicals were obtained from Fisher Scientific UK Ltd. (Leicestershire, UK) and were of analytical grade.

Preparation of LPD Complexes

The LPD complexes were prepared as detailed previously (5). Briefly, protamine sulphate solution (1 mg/mL) and extruded DOTAP liposomes (1 mg/mL) were added sequentially, with 10-min incubation at each step, to plasmid DNA (pEGFP-N1; 1 mg/mL stock solution) to achieve an LPD complex with a 3:2:1 mass ratio. Complexes were freshly prepared before use.

Preparation of Spray-Dried Powders

(i) Lactose-LPD powders: LPD complex preparations comprising pDNA 400 μg, protamine sulphate 800 μg, and DOTAP 1200 μg were added to 3% w/v lactose solution (50 mL total volume) and spray-dried (Büchi B-191 mini spray-dryer, Büchi Labortechnik AG, Flawil, Switzerland) to produce a dry powder with a typical yield of approximately 30%. Standard operating conditions employed for spray-drying were inlet temperature 150°C (outlet temperature between 83 and 85°C), spray flow rate 600 L/h, aspirator setting 35 m^3/h, and pump setting 450 mL/h.

(ii) Chitosan: Spray-dried chitosan powders were prepared by spray-drying 0.2% chitosan solution, pH 5.0 adjustment with acetic acid, under the abovementioned standard operating conditions.

(iii) TMC-lactose-LPD powders: The chitosan derivative TMC was added at concentrations of 0.003, 0.03, 0.1, and 0.3% (w/v) to a 3% w/v lactose solution containing LPD complexes (pDNA 400 μg, protamine sulphate 800 μg, and DOTAP 1200 μg). Dry powders were prepared by spray-drying under the standard operating conditions.

(iv) Lactose-LPD spray-dried powders were post-mixed with chitosan or spray-dried chitosan in a pestle and mortar for 60 s. Initial assessments used chitosan to lactose mass ratios ranging from 0.1:1 to 1:1.

Scanning Electron Microscopy

Selected spray-dried powders were sputter-layered with gold under partial vacuum (EMScope®; EMScope Laboratories, Ashford, UK). Representative scanning electron micrographs of the powders were taken using a Philips XL20 (Philips, Eindhoven, the Netherlands) scanning electron microscope.

Particle Size Analysis

Laser diffraction (Mastersizer 2000; Malvern Instruments, Malvern, UK) was used to determine the particle size of dry powders using a dry dispersion technique in air. Approximately 200 mg powder per sample was used to achieve the required obscuration, i.e., amount of laser light lost due to the introduction of the sample within the analyzer beam, of 0.5–5%.

Agarose Gel Electrophoresis

Gel electrophoresis was used to assess the degree of physical protection afforded to pDNA during the spray-drying process through complexation with the polycationic peptide protamine and the cationic lipid DOTAP, and the influence of chitosan and its derivative on physical stability of the LPD complex. A 1% agarose gel was prepared in 0.5× Tris-borate EDTA (TBE) buffer and immersed in 0.5× TBE buffer containing 0.5 μg/mL EtBr. Dry powders were reconstituted and mixed with gel loading buffer (10× strength, blue-orange dye containing 0.25% bromophenol

Table I. Effect of Concentration of TMC on Dry Powder Yield and Emitted Dose Following Spray-Drying

	Spray-drying yield (%)		Emitted dose (%)				
Mass TMC in solution (% w/v)	Mean	S.D.	Mean	S.D.	Significant difference ($P < 0.05$)		
0	31.4	5.7	57.0	5.9	X		
0.003	35.7	4.2	63.3	5.3	X		
0.03	39.6	3.1	73.4	5.7		X	
0.1	55.3	2.8	90.8	2.7			X
0.3	54.9	3.5	90.5	3.5			X

Fifty-milliliter solutions containing TMC (0.003–0.3% w/v) and 3% w/v lactose were spray-dried using a Büchi B-191 mini spray-dryer. Powder was recovered and the yield expressed as a percentage of initial powder mass. Emitted dose was determined gravimetrically by firing each powder from Spinhaler® into a one-stage liquid impinger. 'X' in the column indicates significantly different subsets of data ($P < 0.05$).

blue, 40% glycerol in TBE buffer) and added to the wells in the gel at a volume equivalent to 1 μg pDNA per well. Equivalent samples were incubated with a digestion buffer (100 mM NaCl, 10 mM Tris–HCl pH 8, 25 mM EDTA pH 8, 0.5% SDS, 0.1 mg/mL proteinase K) for 35 min at 50°C to liberate pDNA from the complexing moieties. The Wizard® DNA clean-up system (Promega, Madison, WI, USA) was used to separate the released pDNA, and samples equivalent to 1 μg pDNA were loaded onto the gel. The gel was run at 100 V for 1 h and then visualized under UV light. Quantification of pDNA was carried out using Molecular Analyst® software (Bio-Rad Gel Doc 1000; Bio-Rad Laboratories, Hercules, CA, USA).

In Vitro Cell Transfection Efficiency of Dry Powders

A549 human lung epithelial carcinoma cells (European Collection of Animal Cell Cultures, Salisbury, UK) were cultured in DMEM with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin 5000 IU/mL at 37°C in a humidified atmosphere at 95% air/5% CO_2. Cells were seeded onto 24-well plates (40,000 cells/cm^2) and grown to 85% confluency. For transfection, cells were washed twice with prewarmed phosphate buffered saline (PBS) and exposed to selected spray-dried powders (equivalent to 5 μg pDNA per well) reconstituted in serum-depleted DMEM. The plates were incubated at 37°C for 6 h, then the surfaces were rinsed thoroughly with PBS and the cells fed with 1 mL culture medium, and returned to the incubator for a further 42 h to allow intracellular expression of the reporter plasmid (pEGFP-N1) to proceed.

In Vitro Powder Aerosolization and Deposition

The dose emission of selected spray-dried powders was determined using a one-stage impinger consisting of a United States Pharmacopeia (USP) throat attached to a pump via a filter. Powders were loaded into size 2 gelatin capsules and placed into a Spinhaler® (Fisons, Bedford, MA, USA) dry powder inhaler, located in the mouthpiece of the USP throat. The powder was aerosolized using a flow rate of 60 L/min for 5 s, corresponding to a single deep inhalation, under a controlled environment (35% relative humidity, 20°C). The percentage of powder emitted from the capsule was determined gravimetrically as the percentage of the total loaded dose released during aerosolization.

The *in vitro* deposition performance of the powders was characterized using a Multistage Liquid Impinger (MSLI; Westech Instrument Services Ltd., Beds, UK), as outlined previously (2–4). Briefly, powders were loaded into size 2 gelatin capsules and placed into a Spinhaler® dry powder inhaler that was attached to the MSLI via a stainless steel USP throat. Each stage of the MSLI was filled with 20 mL DMEM. The powder was aerosolized at a flow rate of 60 L/min for 5 s under a controlled environment (35% relative humidity, 20°C). Ten capsules (equivalent to 133 μg pDNA) were aerosolized into the MSLI, and the DMEM at each stage was removed for analysis. The capsule and the MSLI throat and filter were washed with DMEM (20 mL respectively). A 4-mL aliquot of the collected DMEM was placed onto A549 cells at 85% confluency in six-well cell culture plates to determine gene expression. The plates were incubated at 37°C for 6 h, then the surfaces were rinsed thoroughly with PBS and the cells fed with 4 mL culture medium, and returned to the incubator for a further 42 h as above.

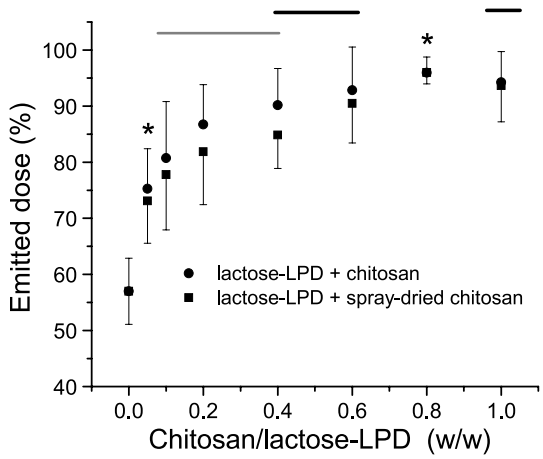

Fig. 1. Effect of chitosan concentration on the emitted dose of spray-dried lactose-LPD powders. No significant difference in emitted dose is observed between the formulations with mass ratio of chitosan:-lactose of 0.1, 0.2, and 0.4 and also for mass ratios of 0.4, 0.6, and 1. Significant difference was observed between the chitosan:lactose-LPD 0.8:1 and 0.4:1 mass ratios. Data represented as mean ± s.e.m.; $n = 8$. Statistical analysis by one-way analysis of variance and Duncan's multiple range test. Significance level, $p < 0.05$. Asterisks indicate significant difference from 0.4:1 mass ratio. Bars indicate no significant difference.

Flow Cytometric Quantification of Gene Expression

Following surface rinsing with PBS, transfected cells were trypsinized with 0.3 mL trypsin-EDTA solution and resuspended in 0.6 mL culture media (DMEM/10% FBS/ antibiotics). The percentage of cells displaying GFP-associated fluorescence (FL1-H) was quantified by flow cytometry (FACScan; Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) with analysis by WinMDI Software (Joseph Trotter, The Scripps Institute, La Jolla, CA, USA), as described previously (2,5).

RESULTS

Yield of Spray-Dried Lactose-LPD, Chitosan, and TMC-Lactose-LPD Powders

Following spray-drying, the processed powders were collected from the receptor and lower section of the spray-dryer separator. The spray-dried lactose-LPD powder provided a yield of 31.4%, with a high proportion of particles being collected from the upper section of separator. Spray-dried chitosan particles, demonstrating a lower cohesion to the separator surface, were primarily deposited in the receptor, producing an elevated yield of 68%. The yield of spray-dried TMC-lactose-LPD was closely related to the content of TMC in the formulation (Table I). The yield increased with increasing concentration of TMC in the spray-drying solution to a maximum of 55.3% at 0.1% (w/v).

Dose Emission of Dry Powder Formulations

The emitted dose is defined as the percentage of powder loaded into the capsule that is emitted during aerosolization. The influence of addition of the chitosan derivative, TMC, on the emitted dose of the powders was investigated. Lactose-LPD capsule emission was inefficient following aerosolization, with an emitted dose of only 57.0%. In contrast, all powders containing TMC achieved an improved level of

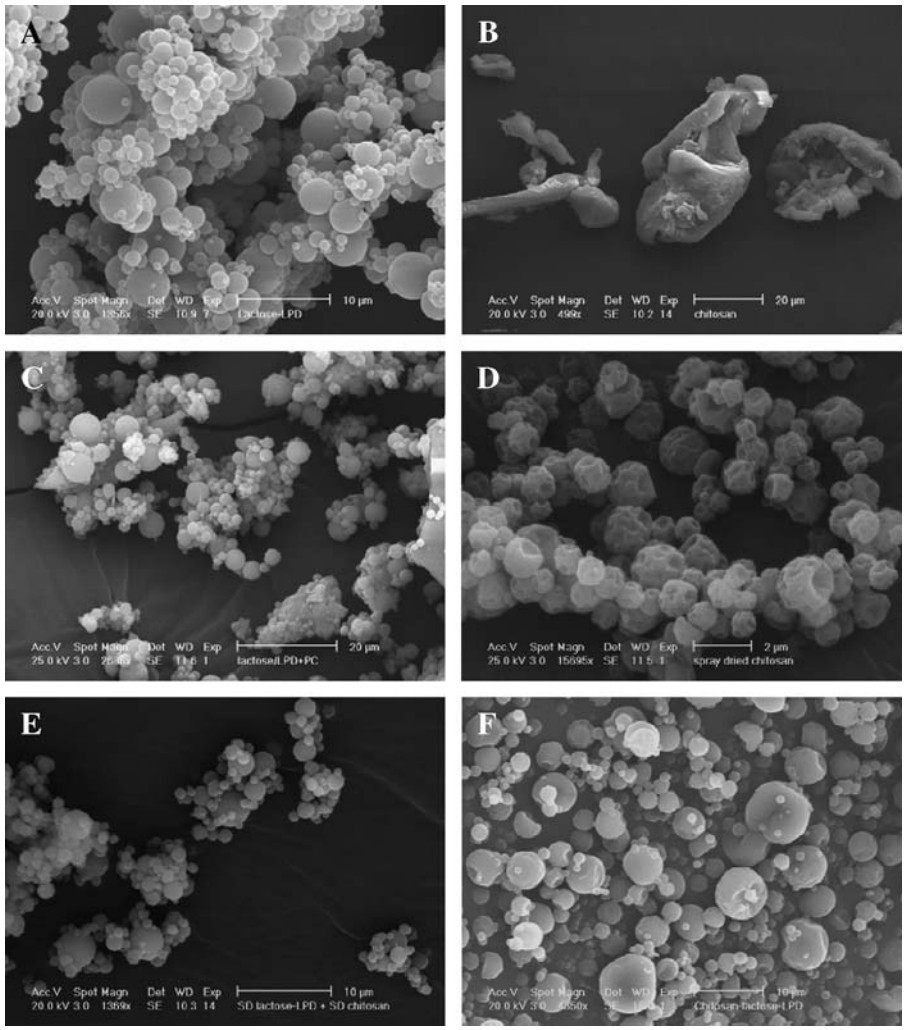

Fig. 2. Visualisation of particle size and surface morphology by scanning electron microscopy (SEM). (A) Lactose-LPD spray-dried powders; (B) commercial grade chitosan; (C) lactose-LPD spray-dried powders post-mixed with chitosan (1:0.8 w/w); (D) spray-dried chitosan; (E) lactose-LPD spray-dried powders post-mixed with spray-dried chitosan (1:0.8 w/w); (F) spray-dried TMC-lactose-LPD (TMC:lactose 1:30 w/w).

powder emission, with formulations containing 0.1% (w/v) TMC producing an optimal emitted dose of 90.8%. Consequently, this ratio of TMC to lactose (mass ratio 1:30) was employed in subsequent studies.

The effect of post-mixing either chitosan or spray-dried chitosan with the lactose-LPD spray-dried powders is shown in Fig. 1. These data demonstrate that dry powder release from the capsule increases with increasing concentration of chitosan in the formulation. The formulation comprising a chitosan to lactose ratio of 0.8:1 showed the statistically (one-way analysis of variance, $P < 0.05$) optimal capsule release with an emitted dose of 96%. The relationship between improved emitted dose and chitosan concentration was consistent whether or not the chitosan additive was spray-dried.

Particle Morphology

Scanning electron microscopy (SEM) was used to visualize the size, shape, and surface morphology of the powders used in this study (Fig. 2). As observed previously (3), spray-dried lactose-LPD particles appear as perfect spheres with a particle diameter of less than 10 μm (Fig. 2A). Commercial chitosan contains particles of different sizes and variant morphology (Fig. 2B). When spray-dried lactose-LPD particles are post-mixed with chitosan, the smaller lactose-LPD powders seem to adsorb to the surface of chitosan particles to form large discrete aggregates (Fig. 2C). This observation may account for the improved capsule emission of these binary systems.

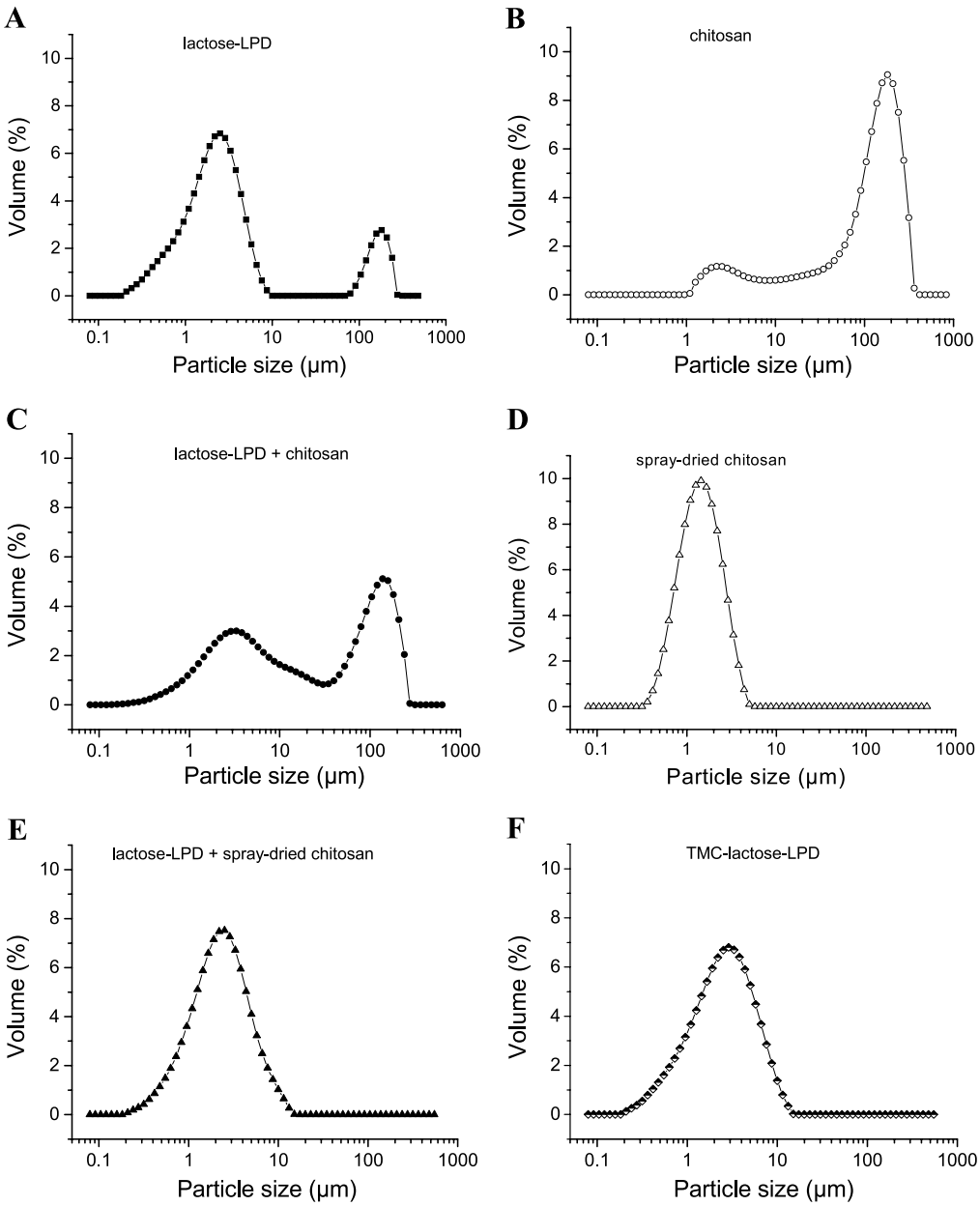

Fig. 3. Particle size distribution of dry powders analyzed by laser diffraction. (A) Lactose-LPD spray-dried powders; (B) commercial-grade chitosan; (C) lactose-LPD spray-dried powders post-mixed with chitosan (1:0.8 w/w); (D) spray-dried chitosan; (E) lactose-LPD spray-dried powders post-mixed with spray-dried chitosan (1:0.8 w/w); (F) spray-dried TMC-lactose-LPD (TMC:lactose 1:30 w/w).

Spray-dried chitosan powders displayed a small particle diameter and irregular surface morphology (Fig. 2D). On mixing these particles with spray-dried lactose particles, relatively small discrete aggregates are formed (Fig. 2E). In common with the chitosan/lactose aggregates (Fig. 2C), these aggregates are segregated and are suggestive of improved powder flow properties.

In contrast with the spherical lactose-LPD particles, spray-dried TMC-lactose-LPD particles demonstrated altered surface morphology. The particles contained surface depressions and increased surface roughness (Fig. 2F), consistent with powders prepared using certain amino acids (3) and absorption enhancers (4) as co-excipients.

Particle Size Distribution of Dry Powders

The particle size distribution of the spray-dried powders was determined using laser diffraction (Fig. 3). The particle size analysis parameters are summarized in Table II. The data are expressed in terms of the particle diameter at 10, 50, and 90% of the volume distribution ($d[v,10]$, $d[v,50]$, and $d[v,90]$, respectively). The span of the volume distribution, a measure of the width of the volume distribution relative to the median diameter ($d[v,50]$), was derived from $(d[v,90] - d[v,10]) / d[v,50]$. In addition, the mean diameter over the volume distribution ($D[4,3]$) is reported. The particle size distribution for lactose-LPD powders was bimodal (mean diameter of 23.7 μm and span of 43.7), comprising a population of particles with mean diameter less than 10 μm and another with mean diameter in excess of 100 μm. This second particle size population is indicative of aggregated lactose-LPD particles that are unable to sufficiently disperse in the instrument airflow, implying a reduced dispersibility and subsequent lower respirable fraction during inhalation. In contrast, spray-dried TMC-lactose-LPD particles demonstrated no significant aggregation, with a subsequent unimodal particle size distribution (mean diameter of 3.4 μm and span 2.2). Post-mixed powders of spray-dried lactose-LPD with spray-dried chitosan also displayed a unimodal size distribution (mean diameter of 3.0 μm and span of 2.1), suggesting improved dispersibility in the airflow. As expected, spray-dried lactose-LPD powders post-mixed with chitosan had a bimodal particle size distribution attributable to the larger size of the chitosan raw material as observed under SEM (Fig. 2B). Clearly, the TMC-lactose-LPD powders and the post-mixed formulation of spray-dried chitosan with spray-dried lactose-LPD powders are more suitable for pulmonary deposition than unmodified lactose-LPD powders, with particles of diameter less than 7 μm generally being appropriate for deposition in the respiratory tract.

Gel Electrophoresis

An EtBr exclusion assay was used to determine whether the inclusion of chitosan or TMC adversely affected the structural integrity of the LPD complex and the enclosed pDNA. Equivalent samples of each dry powder formulation were analyzed by gel electrophoresis and EtBr staining (Fig. 4). Lanes 1, 3, 5, and 7 relate to the unmodified spray-dried lactose-LPD powder, spray-dried lactose-LPD powder post-mixed with chitosan, spray-dried lactose-LPD powder post-mixed with spray-dried chitosan powder, and TMC-lactose-LPD spray-dried powder, respectively. Charge-mediated pDNA condensation by protamine sulphate prevents intercalation of EtBr with the entrapped pDNA; therefore, no fluorescent signal is observed in these lanes. The lack of detectable fluorescent signal for the modified dry powder formulations suggests that the degree of complexation of pDNA by protamine and DOTAP has not been adversely affected by the addition of the supplementary charged species, chitosan.

To further investigate the effects of chitosan on pDNA structural integrity, equivalent LPD powder samples were incubated with digestion buffer to liberate pDNA from the condensing moieties, and the purified extracts were also analyzed by gel electrophoresis. The fluorescent bands corresponding to released pDNA fell in the linear response range of the gel documentation software (Molecular Analyst®, Bio-Rad Laboratories) allowing semiquantification. The fluorescent signal in lane 2, attributed to pDNA released from the unmodified lactose-LPD spray-dried powder following digestion and purification (Wizard® DNA clean-up system), was approximately 80% (densitometric analysis) of the fluorescent signal associated with pDNA liberated from non-spray-dried LPD complexes (not shown). This reduction in pDNA signal, also observed in our previous experiments (2–5), implies that the elevated processing temperature (spray-dryer inlet temperature, 150°C; outlet temperature, 83–85°C) causes the damage to the pDNA that thereafter partially loses its biological activities. Lanes 4 and 6 show pDNA signal, following digestion and purification, of post-mixed formulations of chitosan with lactose-LPD spray-dried powders and spray-dried chitosan with lactose-LPD spray-dried powders, respectively. As with the spray-dried lactose-LPD powders, approximately 80% of the fluorescent signal is preserved, suggesting that further mixing with chitosan does

Table II. Particle Size Distribution Data of Dry Powders Analyzed by Laser Diffraction

	$d[v,10]$ (μm)	$d[v,50]$ (μm)	$d[v,90]$ (μm)	Span	$D[4,3]$ (μm)
Spray-dried lactose-LPD	0.87	2.71	119.50	43.70	23.72
Chitosan	5.48	140.11	269.73	1.89	140.43
Spray-dried lactose-LPD + chitosan	1.58	18.58	182.08	9.71	64.52
Spray-dried chitosan	0.75	1.50	2.92	1.44	1.69
Spray-dried lactose-LPD + spray-dried chitosan	0.86	2.40	5.99	2.14	3.02
Spray-dried TMC-lactose-LPD	0.82	2.70	6.89	2.25	3.37

Note: $d[v,\chi]$, particle diameter at χ% of the volume distribution; span, width of the volume distribution relative to the median diameter (found from $(d[v,90] - d[v,10]) / d[v,50]$); $D[4,3]$, volume-weighted mean diameter.

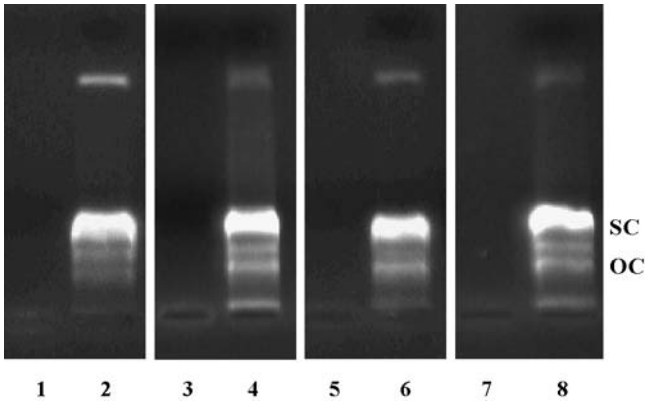

Fig. 4. Determination of pDNA structural integrity through intercalation of ethidium bromide. Spray-dried powders containing pEGFP-N1 were analyzed before (lanes 1, 3, 5, and 7) or after (lanes 2, 4, 6, and 8) incubation with digestion buffer, with subsequent purification using the Wizard® DNA clean-up system. Lanes 1 and 2, spray-dried lactose-LPD; lanes 3 and 4, spray-dried lactose-LPD post-mixed with chitosan; lanes 5 and 6, spray-dried lactose-LPD post-mixed with spray-dried chitosan; lanes 7 and 8, spray-dried TMC-lactose-LPD. SC, supercoiled pDNA; OC, open circular pDNA.

not diminish the stability of the LPD complex. Addition of the soluble chitosan derivative TMC into the spray-drying solution also did not adversely affect stability of the LPD complex, with a retained fluorescent signal of 82% (lane 8).

In Vitro Transfection Efficiency

In vitro transfection of human lung epithelial carcinoma (A549) cells was used to investigate the influence of chitosan or chitosan derivative, post-mixed with lactose-LPD or added into the spray-drying solution, on the biological functionality of the lipid/polycation complexed pDNA. Figure 5 shows that transfection with spray-dried lactose-LPD powder results in approximately 40% of cells showing GFP-associated fluorescence (FL1-H) arising from the pEGFP-N1 expression product. When post-mixed with commercially available chitosan, the gene expression efficiency of the LPD complex remains statistically (one-way analysis of variance, $P < 0.05$) consistent. Interestingly, when spray-dried chitosan particles or soluble TMC is added to the formulation, the number of cells displaying reporter gene expression is significantly ($P < 0.05$) increased, i.e., 58.7 and 50.6% positive cells, respectively.

In Vitro Pulmonary Deposition

The *in vitro* deposition of the dry powder dispersions was determined using the Multistage Liquid Impinger (MSLI), a standard European and US Pharmacopeia method to test respirable particles containing pharmaceutically active agents (25). The dry powders were aerosolized from the gelatin capsules through a USP throat and deposited in the device stages. Deposited powders from each stage and capsule and throat washings were collected in DMEM and placed on A549 cells to determine gene expression (Fig. 6).

Following aerosolization of spray-dried lactose-LPD powders, maximum gene expression was observed in cells treated with capsule washings, confirming significant retention of lactose-LPD powder in the capsule following inhalation (Fig. 1). Subsequent deposition of the aerosolized lactose-LPD powders was mainly restricted to the throat and upper stages of the MSLI (corresponding to deposition sites in the upper airway), with insignificant powder deposition in stages 3 and 4 and in the device filter (related to the secondary bronchi, terminal bronchi, and alveoli regions). Dry powder formulations of spray-dried lactose-LPD containing chitosan, spray-dried chitosan, or TMC were not significantly (one-way analysis of variance, $P < 0.05$) retained in the capsule following aerosolization, confirming fundamentally enhanced dose emission. The reporter gene expression from the formulation containing spray-dried lactose-LPD powders post-mixed with chitosan was statistically consistent

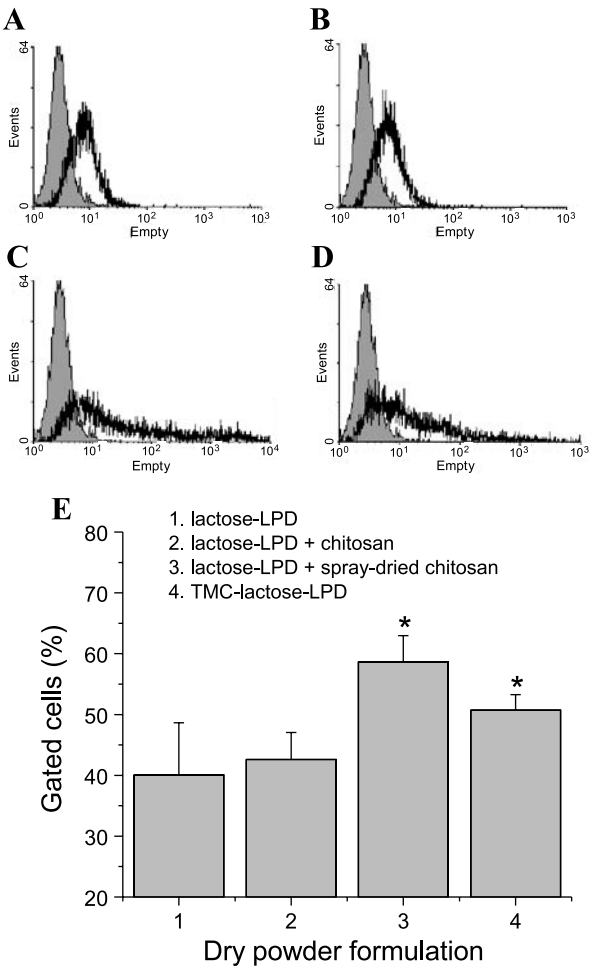

Fig. 5. Green fluorescent protein (GFP) reporter gene expression of reconstituted dry powders. The percentage of A549 cells displaying GFP-associated fluorescence (FL1-H) was quantified by flow cytometry. Cells were exposed to spray-dried powders reconstituted in DMEM (equivalent to 5 μg pDNA per well). A fluorescence region was established containing 1% of the population of untreated control cells (gray filled histogram), and the percentage of cells moving into this marked region for treated cells (black unfilled histogram) was calculated. (A) Spray-dried lactose-LPD; (B) spray-dried lactose-LPD post-mixed with chitosan; (C) spray-dried lactose-LPD post-mixed with spray-dried chitosan; (D) spray-dried TMC-lactose-LPD. (E) Data represented as mean ± s.e.m.; $n = 6$. Statistical analysis by one-way analysis of variance and Duncan's multiple range test. Significance level, $p < 0.05$. Asterisks indicate significant difference from spray-dried lactose-LPD powder.

through MSLI stages 1 to 4, with approximately 10% of cells showing reporter gene expression at stage 4, equating to a particle diameter cut-off of 3.1 μm and predicted deposition in the respiratory region of secondary bronchi and below. When spray-dried lactose-LPD powders are post-mixed with spray-dried chitosan, cellular gene expression efficiency in stage 4 is increased to 20%. Spray-dried TMC-lactose-LPD powders also demonstrate improved deposition over unmodified lactose-LPD powders, with the greatest gene expression observed in stage 3, equating to the tracheal and bronchial region of the lung. All chitosan-modified formulations demonstrated enhanced deposition and subsequent gene expression in the lower stages of the MSLI, i.e., up to an 8-fold increase at stage 4.

Fine particle fraction (FPF), defined as the cumulative gene expression in stage 3, stage 4, and filter (particle size <6.8 μm) divided by the total gene expression (from capsule to device filter), provides a tangible analysis of the dispersibility of the dry powder formulations and their utility for inhalation. Figure 7A shows an FPF of 12.2% for spray-dried lactose-LPD powders. Dry powder formulations enriched with chitosan, spray-dried chitosan, or soluble chitosan derivative had significantly ($P < 0.05$) increased FPF, i.e., 38% for post-mixed formulations and 35% for spray-dried TMC-lactose-LPD.

The total gene expression collected from the capsule to the device filter for each formulation was also calculated (Fig. 7B). The cumulative gene expression data follow the sequence of spray-dried lactose-LPD < spray-dried lactose-LPD and chitosan < spray-dried TMC-lactose-LPD ≪ spray-dried lactose-LPD and spray-dried chitosan and are in close agreement with the *in vitro* transfection data from Fig. 5, indicating that the inhalation process did not adversely effect the biological activity of the LPD dried powders.

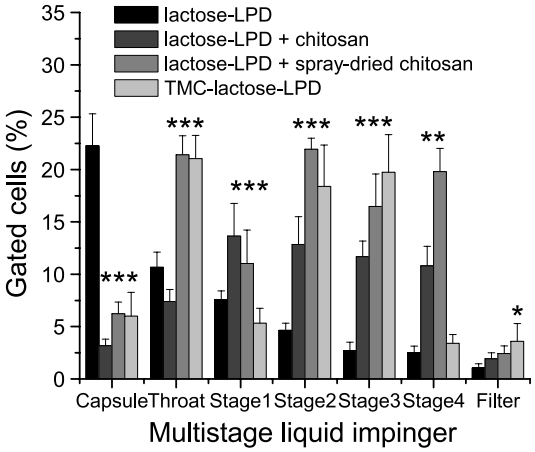

Fig. 6. *In vitro* deposition and gene expression of spray-dried powders. Powders were loaded into gelatin capsules and aerosolized using a Spinhaler® dry powder inhaler into a Multistage Liquid Impinger (MSLI) containing four individual stages (stage 1, 2, 3, and 4). A 4-mL aliquot of DMEM (20 mL) containing dry powder from capsule, throat, and each MSLI stage was placed onto A549 cells in six-well cell culture plates. Gene expression was determined by flow cytometry. Data represented as mean ± s.e.m.; $n = 4$. Statistical analysis by one-way analysis of variance and Duncan's multiple range test. Significance level, $p < 0.05$. Asterisks indicate significant difference from spray-dried lactose-LPD powder at each stage of MSLI.

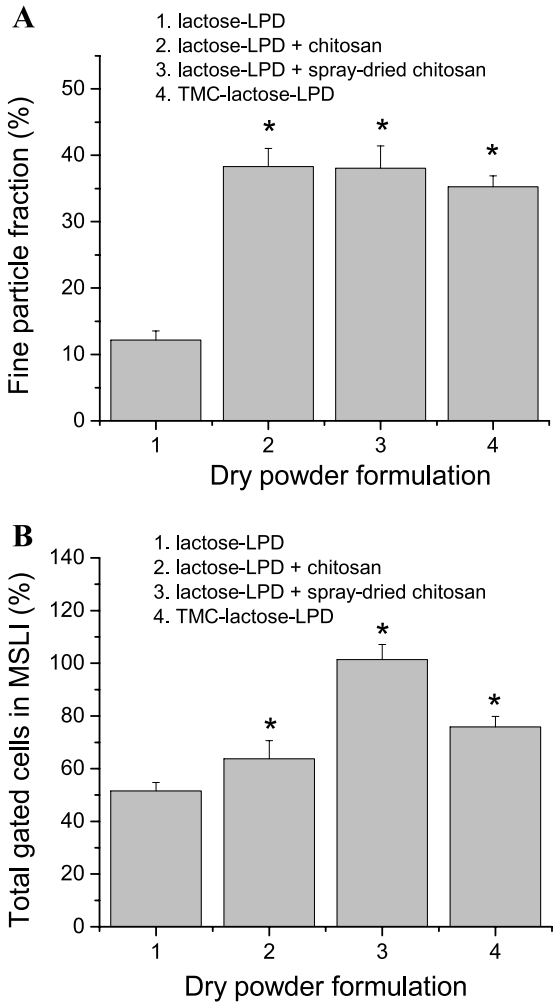

Fig. 7. (A) Fine particle fraction (FPF) of dry powder formulations. FPF was calculated by dividing the cumulative gene expression in stage 3, stage 4, and filter (particle size cut-off <6.8 μm) by the total gene expression (from capsule to device filter). (B) Cumulative gene expression of all samples collected in the *in vitro* deposition assay. Data represented as mean ± s.e.m.; $n = 4$. Statistical analysis by one-way analysis of variance and Duncan's multiple range test. Significance level, $p < 0.05$. Asterisks indicate significant difference ($P < 0.05$) from spray-dried lactose-LPD powder.

DISCUSSION

Chitosan is a biodegradable polymer with excellent biocompatibility and has been widely used in drug delivery systems due to its polycationic nature and the ability to further modify the reactive amino group to produce a range of chitosan derivatives. Chitosan and its derivatives have shown to be effective agents for improving the absorption of hydrophilic compounds and large biomacromolecules (i.e., antibiotics, insulin, peptides, and proteins). In this study, chitosan and its derivative TMC were shown to increase the gene expression of lipid/polycation condensed pDNA to human lung carcinoma cells. The mixture of spray-dried chitosan and spray-dried lactose-LPD demonstrated a significantly greater gene expression capability than that of spray-dried lactose-LPD post-mixed with unprocessed chitosan. As the spray-dried chitosan was polarized by acetic acid, this

effect may reflect enhanced solubility of spray-dried chitosan in the culture media over commercially available chitosan. It is also possible that the reduced particle size of spray-dried chitosan (< 2 μm) permits more disperse interaction with the A549 cells. It is acknowledged that chitosan relieves cellular tight junctions and enhances the transfection of biomacromolecules (26). It is not surprising that the transfection efficiency of spray-dried TMC-lactose-LPD (TMC:lactose = 1:30 w/w) is lower than that of post-mixed spray-dried chitosan with lactose-LPD (chitosan:lactose = 0.8:1 w/w) given the reduced content of chitosan in the formulation.

It should be noted that the degree of substitution in the chitosan polymer chain has an important impact on the biocompatibility of TMC, with cell (e.g., COS-7 and MCF-7) viability decreasing with increasing degree of trimethylation (27). We have tested the cytotoxicity of TMC on A549 cells using the well-characterized MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (data not shown). Three TMC derivatives were tested, having degrees of trimethylation of 30 (TMC30), 60 (TMC60), and 90% (TMC90), respectively. Following 24 h of incubation, 50 μg/mL of TMC90 causes 70% cell death, whilst the same concentration of TMC60 mediates 34% cell death. A549 cells incubated with TMC30, however, generally remained viable (88.4% cell viability) up to a concentration of 1000 μg/mL. Chitosan derivatives with a limited degree of substitution are therefore more suitable to be used as dispersibility enhancers in spray-dried formulations for inhalation. Thanou *et al.* (1999) reported that TMC is nontoxic to ciliated embryo trachea when the degree of trimethylation is under 60% (28). Therefore, although further *in vivo* assessment would be required, including inflammatory response, the available data suggest that TMC30 could be biocompatible when used as a dispersibility enhancer in spray-dried formulations for inhalation.

A well-characterized mechanism for enhancing the dispersibility and deposition of dry powders for pulmonary administration involves the addition of relatively large carrier particles that are subsequently separated from the therapeutic "fines" during inhalation. This mechanism is employed in this paper by post-mixing spray-dried lactose-LPD particles with commercial-grade chitosan, leading to an increased emitted dose and enhanced pulmonary deposition. A second mechanism reported in this paper involves the addition of "co-fines," e.g., spray-dried chitosan, that adsorb onto the active particles, e.g., spray-dried lactose-LPD, to form weak conglomerates with improved capsule emission and airway dispersion characteristics. The third mechanism reported in this paper involves the addition of a co-excipient before spray-drying to impart modified surface properties to the primary excipient particles. Various compounds, such as leucine, phenylalanine, and cyclodextrin (2–4), have shown to increase the dispersibility of the dry powders when employed in this fashion. These compounds enhance the flowability of the spray-dried powders through alteration of surface properties such as morphology, surface charge, surface tension, surface cohesion and adhesion, surface energy, or hydrophilicity.

In this study, we show that chitosan and its derivatives affect both powder dispersibility and cell transfection. Whilst the increase in cellular transfection represents an important advantage of these systems, the predominant effect is through dispersibility enhancement, as evidenced by non-spray-dried chitosan improving gene expression at different levels of the simulated lung (Fig. 6) without enhancing gene expression when compared with unmodified lactose-LPD in a basic cell culture environment (Fig. 5). We have also repeated this work using salbutamol as a model low-molecular-weight drug (data not shown) and observed an enhancement of powder dispersibility similar to that shown in this paper. In addition, previous publications using amino acids (3) and absorption enhancers (4) have shown that dispersibility-enhancing excipients that modify the dry powder surface characteristics and aggregation behavior are able to increase nonviral gene transfer to the lower regions of the lung without any effect on cell transfection.

As absorption enhancers, chitosan and its derivatives have been widely studied for therapeutic drug delivery via various administration routes (e.g., oral, nasal); however, their function as dispersibility enhancers in dry powder formulations for pulmonary deposition has not been reported previously. Our results demonstrate that both chitosan and its soluble derivative TMC can significantly increase the emitted dose (> 90%) and the *in vitro* pulmonary deposition (FPF > 35%) of dry powder dispersions. Given their additional value in enhancing cellular uptake of materials and their impressive biocompatibility, these materials demonstrate potential for inclusion in dry powder formulations for the pulmonary deposition of pharmaceutically active agents.

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