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Preparation of DNA dry powder for non-viral gene delivery by spray-freeze drying: effect of protective agents (polyethyleneimine and sugars) on the stability of DNA

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Abstract

This study investigates the feasibility of using the process of spray-freeze drying (SFD) to produce DNA dry powders for non-viral gene delivery. The effect of protective agents was assessed on the stability of DNA dry powders after SFD. The process of SFD had adverse effects on the tertiary structure of DNA with the protective agents of sucrose, trehalose and mannitol. With the protection of these sugars, a band corresponding to the linear form of DNA was observed during gel electrophoresis between the supercoiled form (SC) and the open circular (OC) form. On the contrary, excess cationic condensing polyethyleneimine (PEI), in conjunction with the above sugars, had the ability to provide protection for DNA from degradation after SFD. This is indicated by the reservation in SC and OC forms of DNA during agarose gel electrophoresis. The electrostatic forces between PEI polymer and DNA are critical for providing protection against various stresses generated by the process of SFD. Furthermore, on rehydration, the particle size and zeta potential of PEI/DNA complexes at weight ratios 3:1 of SFD dry powders were well maintained. Also, no transfection activity loss of PEI/DNA complexes at weight ratios 3:1 on NIH/3T3 cells was observed for reconstituted powders as compared with untreated control solutions. These results give a better understanding of preparing stable DNA dry powders by the process of SFD.

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Introduction

With the advent of gene therapy, great expectations have arisen from the development of DNA drugs. However, a significant challenge lies in the choice of appropriate delivery administration for acceptable bioavailability and efficacy. Due to its fragility and poor transport characteristics, DNA is typically delivered by parenteral administration (Conti et al 2000). Therefore, investigators have looked for less invasive alternatives to parenteral administration for the delivery of large DNA molecules (Freeman & Niven 1996; Liaw et al 2001). Although not as widely explored, DNA dry powders may be of paramount interest for increasing applications, such as aerosols and epidermal powder immunization, in the future (Niven 1995; Chen et al 2001).

So far, spray drying has been frequently used for preparing fine powders of peptides and proteins (Broadhead et al 1994; Maa et al 1998). The unique features of spray drying lie in its ability to involve both particle formation and drying in a single step (Master 1991). Spray-dried DNA powders with various additives have been formulated for the in-vitro and in-vivo transfection of plasmid DNA (pDNA) in the lungs (Freeman & Niven 1996; Seville et al 2002). Also, the effectiveness of protective agents has been studied to obtain stable pDNA to avoid degradation by the spray-drying process (Kuo 2003). However, an alternative technique, spray-freeze drying (SFD), has recently been reported to produce protein powders with superior aerosol performance and product yield as compared with spray-drying (Maa et al 1999; Maa & Nguyen 2001). Also, this technique has been applied in producing protein powders suitable for epidermal delivery or microencapsulation (Costantino et al 2000, 2002; Sonner et al 2002). These potential improvements in aerosolization for pulmonary delivery

routes have therefore stimulated this exploration into the feasibility of preparing DNA powders by the process of SFD.

In SFD, the solution is atomized by passing it through a nozzle and spraying it into cryogenic liquid nitrogen. The dispersed frozen powders are then dried by lyophilization. The effect of each SFD step – spraying, freeze–thawing and drying, on the stability of protein has been studied in detail (Webb et al 2002). It was shown that SFD induced more protein aggregates due to adsorption at air–liquid and solid–air interfaces. The resulting dry powders of SFD proteins exhibit a highly porous structure after sublimation of water vapour (Seville et al 2002; Sonner et al 2002). However, the degradation of pDNA by shearing stress in the nozzle or thermal stress during lyophilization can be critical problems for obtaining optimal gene expression (Anchordoquy et al 2001; Lengsfeld & Anchordoquy 2002). DNA, after SFD, must retain its supercoiled circular (SC) and open circular (OC) forms to obtain optimal biological effectiveness (Kimoto & Taketo 1996). Typically, the degradation of the pDNA occurs by the two-step process of depurination and β-elimination, leading to cleavage of the phosphodiester backbone, and will eventually convert the SC form to the OC and linear forms (Middaugh et al 1998). Protective agents such as disaccharides and polyols included in the solution are essential to protect the integrity and activity of biopharmaceuticals during spray-drying and lyophilization (Broadhead et al 1994; Allison & Anchordoquy 2000). One of the possibly stabilizing mechanisms is due to interactions, such as hydrogen bonding, between biopharmaceuticals and protective agents by effectively substituting for water (Carpenter & Crowe 1989). Furthermore, disaccharides are capable of maintaining particle size of lipid–DNA complexes during lyophilization by the particle isolation hypothesis (Allison et al 2000). Also, several studies have clearly demonstrated that DNA condensation induced by cationic agents, such as polymer or peptide–DNA complexes, may provide a way to minimize damage to pDNA by shear-related processes (Adami et al 1998; Kuo 2003). Among these cationic agents used, polymers are also one of the more promising delivery vehicles for non-viral gene therapy applications and could be of paramount interest for the development of commercial gene products (De Smedt et al 2000). Polyethyleneimine (PEI) polymers are the most commonly used vectors in non-viral gene delivery systems. They provided significant buffering capacity over other cationic polymers, leading to pH inhibition of lysosomal nucleases and higher gene expression (Boussif et al 1995). It has been shown that the process of jet nebulization rapidly degrades naked DNA, but that pDNA is stabilized when complexed with PEI (Densmore et al 2000). However, the effect of SFD on the stability and transfection efficiency of the PEI–pDNA complex has not been studied.

The aim of this study is to evaluate the effectiveness of protective agents as a means to avoiding pDNA degradation by SFD processing. Similar to the study carried out on proteins (Webb et al 2002), this paper investigates the effect of spraying, freeze–thawing and drying on the stability of pDNA–sugar and pDNA–PEI complexes. DNA dry powders were characterized using agarose gel electrophoresis, dynamic light scattering, zeta potential analysis and in-vitro transfection assay.

Materials and Methods

Materials

The pDNA (pSG5*lacZ*), which encodes the *lacZ* gene for β-galactosidase, was driven by an SV40 promoter to assess gene expression. The pSG5*lacZ* was amplified in *Escherichia coli* and purified by column chromatography (QIAGEN-Mega kit, Netherlands). The purity of pSG5*lacZ* was established by UV spectroscopy (E260 nm/E280 nm ratios ranging from 1.80–1.89 were used). Agarose (0.7%) gel electrophoresis analysis using restriction enzymes showed that pDNA was mainly in the SC form and one band corresponding to a size of 8 kb was visible. Sucrose, trehalose, mannitol and polyaspartic acid were obtained from Sigma Chemical Company (St Louis, MO) and used as supplied. The branched polyethyleneimine (PEI 800 K, MW = 800 kDa) was obtained from Sigma as a 50% (w/v) solution. The PEI solutions were adjusted to the desired aqueous concentrations and neutralized with HCl (to pH 7.0).

Spray-freeze drying

A schematic drawing of the apparatus for SFD is shown in Figure 1. The 1-mL feed solution (pDNA 50 $\mu g\,mL^{-1}$) was placed into a 5-mL syringe and inserted into a silicon tube. A constant pressure (30 psi) from an air pump provided the accelerating flow for the feed solution and connected to the 0.28-mm spray nozzle (Garaba Mfg Co. Ltd, Taiwan) for atomization. The feed solution was sprayed directly into a 250-mL round-bottom glass flask two-thirds full of liquid nitrogen. The whole glass flask was surrounded by liquid nitrogen and kept in an insulated chamber to prevent temperature elevation. After spraying, fresh liquid nitrogen was added to the flask to compensate for the liquid lost through evaporation. The frozen particles were then lyophilized in a freeze dryer (Model FD6-6P-D; Kingmech Co. Ltd, Taiwan), which had been precooled to $-60\,^{\circ}C$. The chamber pressure of the freeze dryer was reduced and maintained at < 80 mTorr for 48 h. The dry powders were stored in a vacuum desiccator at room temperature before analysis. All dry powders were analysed within a week.

Dynamic light scattering and zeta potential analysis

The size and zeta potential of the PEI–DNA complexes (3:1 w/w) in suspension was determined using a Zetasizer 3000 (Malvern Ltd, UK), which combines the size measurement by dynamic light scattering and the zeta potential analysis by capillary electrophoresis. Each reconstituted sample was measured in triplicate and the final DNA concentration was 10 $\mu g\,mL^{-1}$.

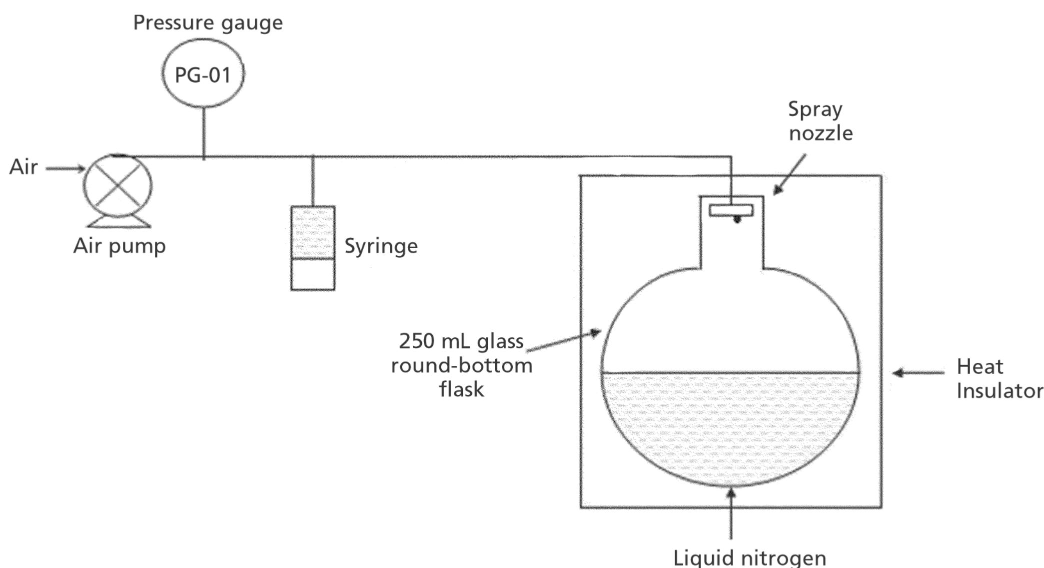

Figure 1 Schematic diagram of the apparatus used in the SFD process.

Agarose gel electrophoresis

Structural integrity of pDNA after SFD was assessed by agarose gel electrophoresis. The DNA dried powders (1 μg pDNA/well; 10 μL + 2 μL Type IV loading buffer/well) in Tris-acetate-EDTA buffer (pH 8.0) were then loaded onto a 0.7% agarose gel containing ethidium bromide and electrophoresed at 100 V for 1.5 h. After electrophoresis, DNA was examined by UV irradiation. For PEI cationic polymers, polyaspartic acid solution (20 mg mL^{-1}) was used to dissociate the cationic polymer–DNA complexes at ambient temperature (Trubetskoy et al 1999). Before electrophoresis, the resulting solution was incubated for 24 h.

Cell culture and transfection assay

NIH/3T3 cells (mouse fibroblasts) were cultured in Dulbecco's modified Eagle medium (DMEM, high glucose; Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, USA) and 100 U mL^{-1} penicillin/100 μg mL^{-1} streptomycin (Sigma, USA). Cells were seeded into 24-well cell culture plates at a density of 3×10^4 cells/well and grown overnight (60–75% confluence) at 37°C and 5% CO_2. The transfection activity of the suspension of PEI–DNA complexes without SFD was taken as a comparative reference. Immediately before transfection, cells were rinsed with PBS and supplemented with 1 mL fresh DMEM per well. The pDNA (2 μg) and PEI (6 μg) were each diluted into 50 μL of DMEM solution. PEI (25 μL) was slowly added to the pDNA solution and allowed to incubate at room temperature. After 10 min, the resulting solution was vortexed for 30 s and then spun down. The solution then sat for 10 min and then the additional 25 μL of PEI was added to the solution. After a further 10 min, cells were exposed to transfection mixtures for 2 h and then supplemented with 10% FBS and 1% antibiotics. Two days later, β-galactosidase gene expression was analysed by using the combined β-Gal Assay kit (Invitrogen, USA) and BCA Protein Assay Reagent Kit (Pierce, USA). The pDNA dry powders were rehydrated in free DMEM medium (2 μg pDNA/50 μL) and the above protocol for transfection was followed.

Statistical analysis

The results in Table 1 and 2 are the means ± s.d. for three replicates. Statistical differences in the particle size and zeta potential (Table 1) and the relative transfection activity (Table 2) of polyplexes of the various formulations between studies done without and with SFD were compared using Student's paired t-test. Results were considered to differ significantly when $P < 0.05$.

Table 1 Particle size and zeta potential of polyplexes in 20% sucrose, 20% trehalose and 15% mannitol solution with and without SFD.

Polyplex	Formulation	Particle size (nm)	Zeta potential (mV)
PEI–DNA (3:1 w/w)[a]			
Without SFD	20% Sucrose	150 ± 20	28 ± 2
With SFD	20% Sucrose	152 ± 28*	30 ± 4*
Without SFD	15% Mannitol	156 ± 26	30 ± 2
With SFD	15% Mannitol	159 ± 35*	34 ± 3*
Without SFD	20% Trehalose	140 ± 27	31 ± 4
With SFD	20% Trehalose	143 ± 20*	27 ± 4*

[a]Final DNA concentration was 10 μg mL^{-1}. Data are means ± s.d., n = 3. *$P > 0.05$ vs corresponding formulation without SFD.

Table 2 Relative transfection activity of polyplexes dry powder formulations after SFD.

Polyplex	Formulation	Relative transfection activity (%)
PEI–DNA[a]		
With SFD	20% Sucrose	102 ± 4*
Without SFD	20% Sucrose	100 ± 1
With SFD	20% Trehalose	94 ± 4*
Without SFD	20% Trehalose	100 ± 2
With SFD	15% Mannitol	98 ± 4*
Without SFD	15% Mannitol	100 ± 2

Data are means ± s.d., n = 3. Transfection activity of PEI–DNA (3:1 w/w) complexes in 20% sucrose, 20% trehalose and 15% mannitol solution without SFD was taken as comparative controls (*$P > 0.05$). [a]Final concentration of DNA was 2 μg/1100 μL DMEM.

Results and Discussion

To investigate the stability issue raised by SFD, we first studied the influence of sugars on the structural changes of pDNA. Agarose gel electrophoresis of pDNA powders formulated from 20% sucrose, 20% trehalose and 15% mannitol solutions after SFD is shown in Figure 2. The control pDNA exists as a mixture of both SC and OC forms (Figure 2, lane 1). As compared with control pDNA, a band corresponding to the linear form of pDNA was observed in the gel between the SC form and the OC form for all SFD pDNA with sugar (Figure 2, lanes 2–5). The results indicated that sugars alone (sucrose, trehalose, and mannitol) were not effective in protecting the pDNA from structural change by the process of SFD. Next, the separate effects of spraying and lyophilization on the stability of pDNA protected by sugars were examined (Figure 3). Spraying of pDNA under the protection of sugars resulted in the appearance of linear form of pDNA (Figure 3A, lanes 2–4), although some SC and OC forms still presented on the agarose gel. This demonstrated that the protective effect of carbohydrates was not sufficient to protect the pDNA from structural change by the induced shear stress from spraying. Figure 3B shows agarose gel electrophoresis of pDNA containing sugar solutions after lyophilization. For disac-

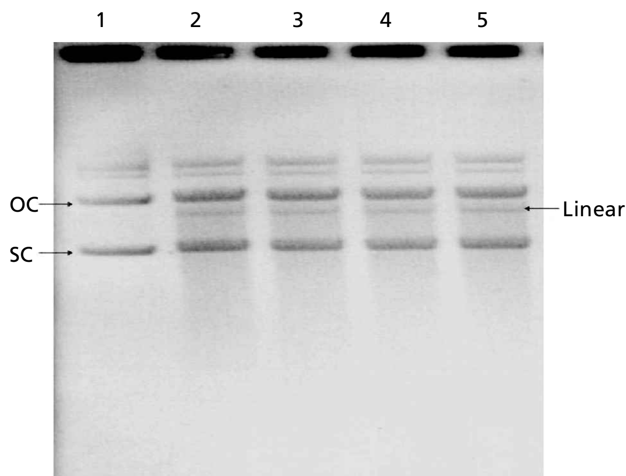

Figure 2 Agarose gel electrophophoresis of reconstituted DNA powders formulated from 20% sucrose, 20% trehalsose and 15% mannitol solutions after SFD. Lane 1, control pDNA (without SFD); lanes 2 and 3, pDNA in 20% sucrose solution after SFD; lane 4, pDNA in 20% trehalose solution after SFD; lane 5, pDNA in 15% mannitol solution after SFD. The band position for the supercoiled form (SC), open circular form (OC), and linear form of DNA is indicated with arrows.

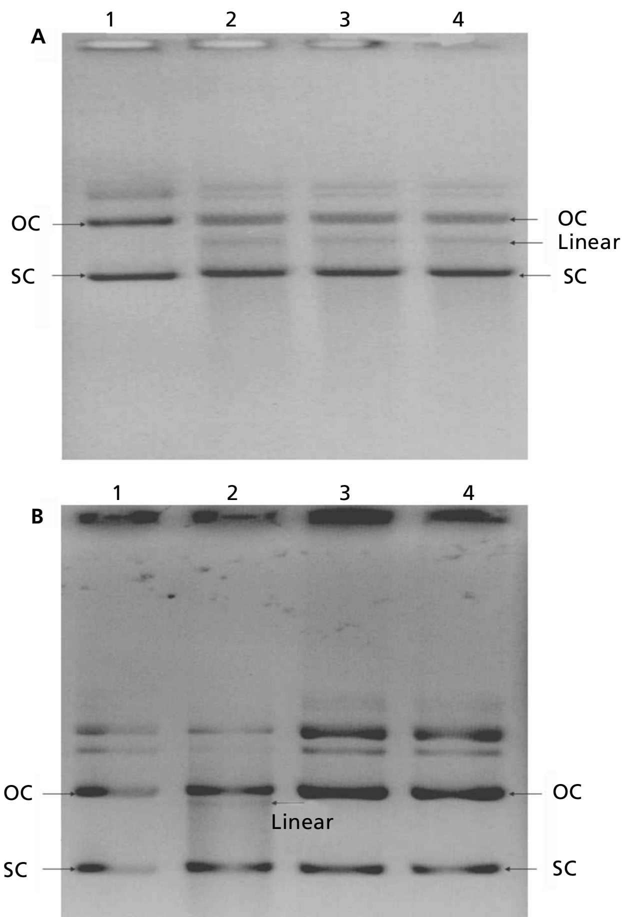

Figure 3 The separate effects of spraying and lyophilization on the stability of pDNA protected by carbohydrates. A. Spraying effects. Lane 1, control pDNA (without spraying); lane 2, pDNA in 15% mannitol solution; lane 3, pDNA in 20% trehalose solution; lane 4, pDNA in 20% sucrose solution. B. Lyophilization effects. Lane 1, control pDNA (without lyophilization); lane 2, pDNA in 15% mannitol solution; lane 3, pDNA in 20% trehalose solution; lane 4, pDNA in 20% sucrose solution. The band position for the supercoiled form (SC), open circular form (OC) and linear form of DNA is indicated with arrows.

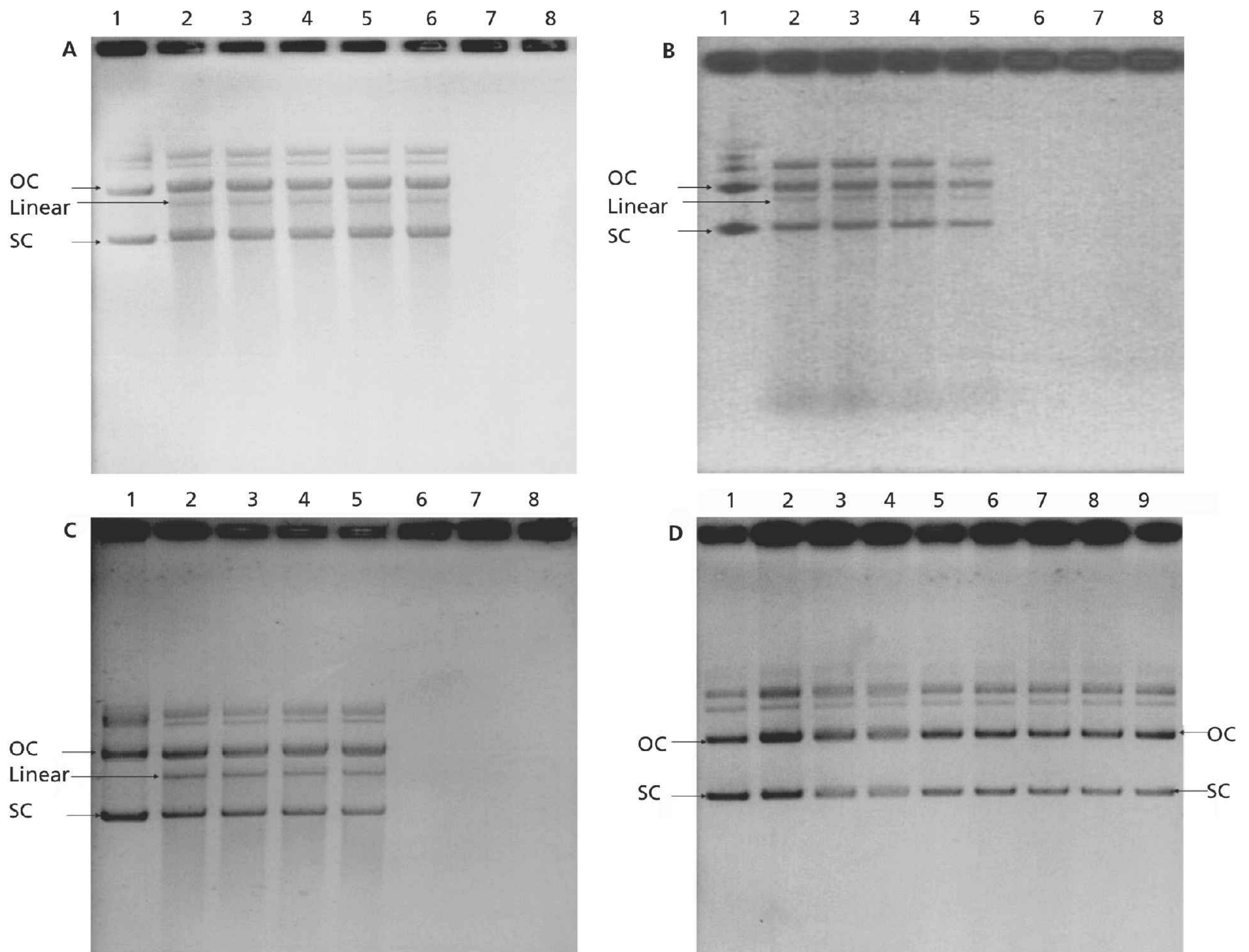

Figure 4 The protective effect of PEI on the structural integrity of DNA. SFD powders containing 15% mannitol (A), 20% trehalose (B) and 20% sucrose (C) at PEI–DNA weight ratios 1:10, 3:10, 1:2, 3:4, 1:1, 3:1, and 5:1, respectively (lanes 2–8). D. Treated with excess polyaspartic acid. Lanes 2 and 3, corresponding to lane 7 and 8 in A; lanes 4–6, corresponding to lanes 6–8 in B; lanes 7–9, corresponding to lane 6–8 in C. Lane 1 of each panel represents control pDNA without SFD. The band position for the supercoiled form (SC), open circular form (OC) and linear form of DNA is indicated with arrows.

charides tested (20% trehalose and sucrose), the same electrophoretic pattern (Figure 3B, lanes 3 and 4) as compared with control pDNA (lane 1) was observed after lyophilization. By contrast, a linear band of pDNA formulated with 15% mannitol was observed on the agarose gel. The results demonstrated that disaccharides, not mannitol, preserved the integrity of pDNA after lyophilization. Mannitol, which has the tendency to crystallize during lyophilization, did not provide the full protection of pDNA from degradation after the process of lyophilization (Allison & Anchordoquy 2000). Our results show that the spraying, which is the first step of SFD, is responsible for the degradation of pDNA formulated with sugars.

The next attempt was to introduce interactions between DNA and cationic polymers in the solution before incorporating these cationic polymer–DNA complexes into sugars. In addition to DNA condensation, cationic polymer-based gene delivery systems are also involved in the attractive bonding between cationic polymer and negative plasmid DNA. These forces may provide the ability to withstand shear stress generated by SFD. In previous studies, polymer-based gene delivery systems also preserved the transfection potential after lyophilization (Cherng et al 1997, 1999). The protective effect of PEI on the structural integrity of DNA from PEI–DNA weight ratios 1:10 to 5:1 by the process of SFD was investigated (Figure 4). For SFD powders containing 20% trehalose or 20% sucrose and PEI/DNA weight ratios 1:1, 3:1 and 5:1, no band was revealed in the agarose gel electrophoresis after SFD (Figure 4B, C, lanes 6–8). Also, for powders formulated from 15% mannitol and PEI–DNA weight ratios 3:1 and 5:1, no band was revealed in the agarose gel electrophoresis after SFD (Figure 4A, lanes 7 and 8). This indicated that pDNA was tightly bonded by the complexation of PEI polymers. Therefore, to liberate pDNA from the polyplexes, the resulting complex solution was treated with excess polyaspartic acid to dissociate pDNA from PEI in the polyplex (Trubetskoy et al 1999).

An identical electrophoretic pattern (Figure 4D, lanes 2–9), as compared with control pDNA (Figure 4D, lane 1), was observed after polyaspartic acid dissociation. Also, no linear form was revealed in the agarose gel electrophoresis between the SC form and the OC form after the process of SFD. The above results demonstrate that the electrostatic forces between the PEI polymer and pDNA are critical for providing protection against all the stresses generated by the various steps of the SFD process. SFD powders formulated from disaccharides require less PEI to prevent degradation of the structure of pDNA, as compared with formulations formulated from mannitol (reducing PEI–DNA ratio from 3:1 to 1:1). However, regardless of various carbohydrate excipients tested, sufficient cationic polymer–DNA weight ratios are essential to prevent DNA from degradation under SFD. In contrast, bands of linear DNA were observed on the agarose gel electrophoresis for SFD powders containing lower PEI–DNA weight ratios in mannitol, trehalose and sucrose (Figure 4A, lanes 2–6; Figure 4B, C, lanes 2–5).

The particle size and zeta potential of PEI–DNA complexes, at weight ratios 3:1 for dry powders in the hydrated state, were tested to further confirm the physical stability of pDNA dry powder formulations. The choice of PEI–DNA ratio (3:1 w/w) is based on the optimal gene expression in serum-free conditions and protection ability of PEI. As discussed elsewhere, the transfection efficiency on rehydration of lipid–DNA complexes after lyophilization is dependent on the consistency of both particle size and zeta potential (Molina et al 2001). In Table 1, no statistical difference ($P > 0.05$) in either particle size or zeta potential was observed for PEI–DNA complexes with or without SFD. These data suggest that particle size and zeta potential of polyplexes are well maintained in 20% sucrose, 20% trehalose and 15% mannitol solution after SFD. However, these findings are contrary to those of previous studies wherein the zeta potential of PEI–DNA complexes was significantly lowered at similar sucrose concentration during freezing (Molina et al 2001). The difference may be due to the higher molecular weight of PEI (800 K) used in our study, as compared with the lower molecular weight of PEI (70 K) used in the previous report. DNA was more stable under the protection of cationic polymer with higher molecular weight after SFD.

Knowing that the physical stability of pDNA dry powder formulations are well protected after SFD, the transfection activity was then evaluated to comply with the required standard in efficacy of pDNA products; and the results are shown in Table 2. Since sucrose may affect the transfection activity of cationic agent–DNA complexes on cell lines, the same concentration of control solutions was adjusted to avoid this enhanced effect (Ciftci & Levy 2001). PEI–DNA complexes containing sucrose, trehalose and mannitol retained the relative transfection activity on NIH/3T3 cells after SFD, as compared with similar PEI–DNA formulations without SFD. These findings suggest that the SFD process can be applied to preserve the transfection activity if the physical stability of pDNA is maintained. Also, the type of carbohydrate excipients plays a minor role on gene transfer of PEI–DNA complexes after SFD. The mechanism of protecting pDNA during the sequential steps of SFD is complex. We have demonstrated that excess cationic condensing polymers (PEI) in conjunction with sugars have the ability to provide protection for pDNA from degradation after SFD. DNA dry powders prepared by SFD may open a new area of applications for gene delivery routes. For example, low-density porous powders with particle diameters of approximately 20 μm might facilitate more efficient pulmonary delivery to lungs as shown in a previous study (Edwards et al 1997; Maa & Nguyen 2001). PEI–DNA complexes in SFD dry powders may enhance cellular uptake (Oh et al 2002). For another example, SFD dry powders with CpG DNA from bacteria may be formulated for epidermal powder immunization and lead to a Th-1 type of response as indicated by a previous study (Chen et al 2001). Also, potent activation of innate responses may be further induced by intracellular entry and nuclear translocation of PEI–DNA complexes as indicated in cationic lipid–DNA complexes (Freimark et al 1998; Dow et al 1999).

Conclusions

In summary, the results of this study present the possibilities for the preparation of DNA dry powders via the process of SFD. The process of SFD had adverse effects on the tertiary structure of pDNA with the protective agents of sucrose, trehalose and mannitol. With the protection of these sugars, a band corresponding to the linear form of pDNA was observed between the SC form and the OC form in the gel electrophoresis. On the contrary, excess cationic condensing polymers (PEI 800 K) in conjunction with the above sugars had the ability to provide protection for pDNA from degradation, as indicated by the reservation in SC and OC forms of pDNA on agarose gel electrophoresis. Furthermore, the particle size and zeta potential of PEI–DNA (3:1 w/w) complexes on rehydration of dry powders were well maintained after SFD. Throughout the SFD process, transfection activity of dry powders of DNA–PEI (3:1 w/w) complexes was comparable with untreated controls. Taken together, opportunities may open for using the process of SFD as a method to facilitate the formulation of pDNA in aerosols and powder immunization.

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