行政院國家科學委員會專題研究計畫　成果報告

以噴霧冷凍乾燥法製備應用於基因傳遞之多孔性 DNA 乾粉：保護劑組成及效能研究

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執行單位：嘉南藥理科技大學藥學系

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Abstract

The aim of this study was to investigate the feasibility of using the process of spray-freeze drying (SFD) to produce DNA dry powders for non-viral gene delivery. The effect of various protective agents was assessed on the stability of DNA dry powders after SFD**.**

Keywords: spray-freeze drying; DNA dry powder; polyethyleneimine; transfection activity

Introduction

With the advent of gene therapies, great expectations have arisen from the development of DNA drugs. Different investigators have looked for less invasive alternatives to parenteral administration for the delivery of large DNA molecules. Although not as widely explored, DNA dry powders may be of interest for increasing applications such as aerosols. SFD has recently been reported to produce protein powders with superior aerosol performance and product yield as compared to spray-drying. Also, this technique has been applied in producing protein powders suitable for epidermal delivery or microencapsulation. 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. The aim of this study is to evaluate the effectiveness of protective agents in order to avoid pDNA degradation by SFD processing. Similar to the study done on proteins, this paper investigates the effect of spraying, freeze-thawing, and drying, on the stability of pDNA/sugar and pDNA/PEI complex/sugar. DNA dry powders were characterized using 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 a SV40 promoter to assess gene expression. The pSG5*lacZ* was amplified in *E. Coli* and purified by column chromatography (QIAGEN-Mega kit, Netherlands). The purity of pSG5*lacZ* was established by UV spectroscopy (E260 nm/E280 nm ratio ranging from 1.80-1.89 were used) Sucrose, trehalose, mannitol, and polyaspartic acid were obtained from Sigma Chemical Company (St. Louis, MO, USA) and used as supplied. The branched polyethyleneimine (PEI 800K, MW = 800 kDa) was obtained from Sigma as a 50% (w/v) solution. The PEI solutions were adjusted to desired aqueous concentrations and neutralized with HCl (PH=7.0).

Spray-freeze drying

The 1 mL feed solution (50μg pDNA/mL) was placed into a 5 mL syringe and inserted to a silicon tube. A constant pressure (30 psi) from air pump provided the accelerating flow for the feed solution and connected to the 0.28 mm spray nozzle for atomization. The feed solution was sprayed directly into a 250 mL round-bottom glass flask with 2/3 full of liquid nitrogen. The whole glass flask was surrounded by liquid nitrogen and kept in an insulated chamber to prevent the system from 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, which had been pre-cooled to –60 ℃. The chamber

pressure of the freeze dryer was reduced and maintained at < 80 mTorr for 48 hours. The dry powders were stored in a vacuum desiccator at room temperature before analysis. All dry powders were analyzed 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 μg/mL .

Cell Culture and transfection assay

NIH/3T3 cells (mouse fibroblast) were cultured in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum and 100 U/mL penicillin / 100μg/mL streptomycin. Cells were seeded into 24-well cell culture plates at a density of 3 x 10^4 cells/well and grown overnight (60 to 75% confluence) at 37℃ and 5% CO_2. The transfection activity of the suspension of PEI/DNA complexes without SFD was taken as a comparative reference. Immediately prior to transfection, cells were rinsed with PBS and supplemented with 1 mL fresh DMEM per well. The pDNA (2μg) and PEI (6μg) were each diluted into 50μL of DMEM solution. 25μL of PEI was slowly added to pDNA solution and allowed to incubated at room temperature. After 10 minutes, the resulting solution was vortexed for 30 seconds and then spun down. The solution then sat for 10 minutes and then the additional 25μL of PEI was added to the solution. After 10 more minutes, cells were exposed to transfection mixtures for 2 hours and then supplemented with 10% FBS and 1% antibiotics. β-galactosidase gene expression was analyzed by using combined β-Gal Assay kit and BCA Protein Assay Reagent Kit. The pDNA dry powders were rehydrated in free DMEM mediumand followed the above protocol for transfection.

Results and discussion

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 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. In Table 1, no statistical difference ($P > 0.05$) in either particle size or zeta potential was observed for PEI/DNA complexes with and 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.

The transfection activity was evaluated and the results are shown in Table 2. 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. Also, the type of carbohydrate excipients plays a minor role on gene transfer of PEI/DNA complexes after SFD.

Assessment of progress

Part of the results has been written in a manuscript to be published in a journal as a research article.

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)			
Without SFD	20% Sucrose	150±20	28±2
With SFD	20% Sucrose	152±28	30±4
		(P＞0.05)	(P＞0.05)
Without SFD	15% Mannitol	156±26	30±2
With SFD	15% Mannitol	159±35	34±3
		(P＞0.05)	(P＞0.05)
Without SFD	20% Trehalose	140±27	31±4
With SFD	20% Trehalose	143±20	27±4
		(P＞0.05)	(P＞0.05)

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 (P＞0.05)
Without SFD	20% Sucrose	100± 1
With SFD	20% Trehalose	94±4 (P＞0.05)
Without SFD	20% Trehalose	100± 2
With SFD	15% Mannitol	98±4 (P＞0.05)
Without SFD	15% Mannitol	100± 2

a: transfection activities of PEI/DNA (3/1 w/w) complexes in 20% sucrose, 20% trehalose, and 15% mannitol solution without SFD was taken as comparative controls.