行政院國家科學委員會專題研究計畫　成果報告

甘露醇/雙醣組成及甘露醇結晶度對陽離子性高分子基因傳送系統於冷凍乾燥中安定性之研究

計畫類別：個別型計畫
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執行期間：94 年 08 月 01 日至 95 年 07 月 31 日
執行單位：嘉南藥理科技大學藥學系

計畫主持人：宋國峻
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Abstract

The aim of this study was to evaluate the protective effect of mannitol on the aggregation behavior of PEI-DNA complexes during lyophilization. The protective effect of mannitol/disaccharide composition and mannitol crystallinity in freeze-dried formulations was also analyzed. The results allow us to assess the aggregation mechanism of cationic polymer-based gene delivery systems during freeze-drying.

Keywords: mannitol; DNA; polyethylenimine; aggregation; lyophilization

Introduction

While cationic polymer gene delivery systems have shown promise in clinical trials, relatively little attention has been paid to the development of their pharmaceutical formulations for long-term stability. Lyophilization, known as freeze-drying, is a common method used to achieve acceptable shelf life for biopharmaceuticals. Among lyoprotectants tested, disaccharides hold superior protective ability during freeze-drying of cationic polymer gene delivery systems. Mannitol, with structural similarity to disaccharides, is a commonly used excipient in freeze-dried biopharmaceutical formulations. However, the mechanism of freeze-dried stabilization of cationic polymer gene delivery systems by mannitol and is not fully studied yet. As a result, the aim of this study is to systematically evaluate the protective effect of mannitol on the aggregation behavior of cationic polymer gene delivery systems during lyophilization; the study will focus on assessing the effect of mannitol/disaccharide composition as well as mannitol crystallinity on the stability of cationic polymer gene delivery system during lyophilization.

The aggregation behavior of cationic polymer gene delivery systems after lyophilization or freeze-thawing will be investigated. The results allow us to gain insight into the mechanism of mannitol on stability of cationic polymer-based gene delivery systems during freeze-drying.

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, D-mannitol, 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.

Complex preparation and lyophilization

Typically 200 μL-aliquots of the

solutions containing both PEI-DNA complexes and excipients described in the text were prepared in this study. The pDNA (30μg) and PEI (30μg) were each diluted into 50μL of sterile double distilled H_2O (ddH_2O). 25μL of PEI was slowly added to the pDNA solution at room temperature. After 10 minutes, the resulting solution was vortexed for 30 seconds and then spun down. The solution sat for 10 minutes and the additional 25μL of PEI was added to the solution. The resulting solution sat for 10 minutes, mixed with 100 μL of excipient aqueous solutions, and transferred to 5-mL flat–bottomed borosilicate lyophilization vials. Vials were frozen by immersion in liquid nitrogen to minimize freezing damage and placed on the shelf of a freeze-drier. The temperature of the cooling collector was – 92 degrees C, the vacuum chamber pressure was set to 0.002 Torr throughout the drying process (24 hours), and the temperature of the shelf was kept at room temperature (27 degrees C). The maximal vacuum was obtained within 5 min after applying the vials. After freeze-drying, samples were sealed with stoppers under vacuum.

Light scattering analysis

The particle size of PEI-DNA complexes in suspension was determined using a Malvern Zetasizer 3000HS.

Transfection assay

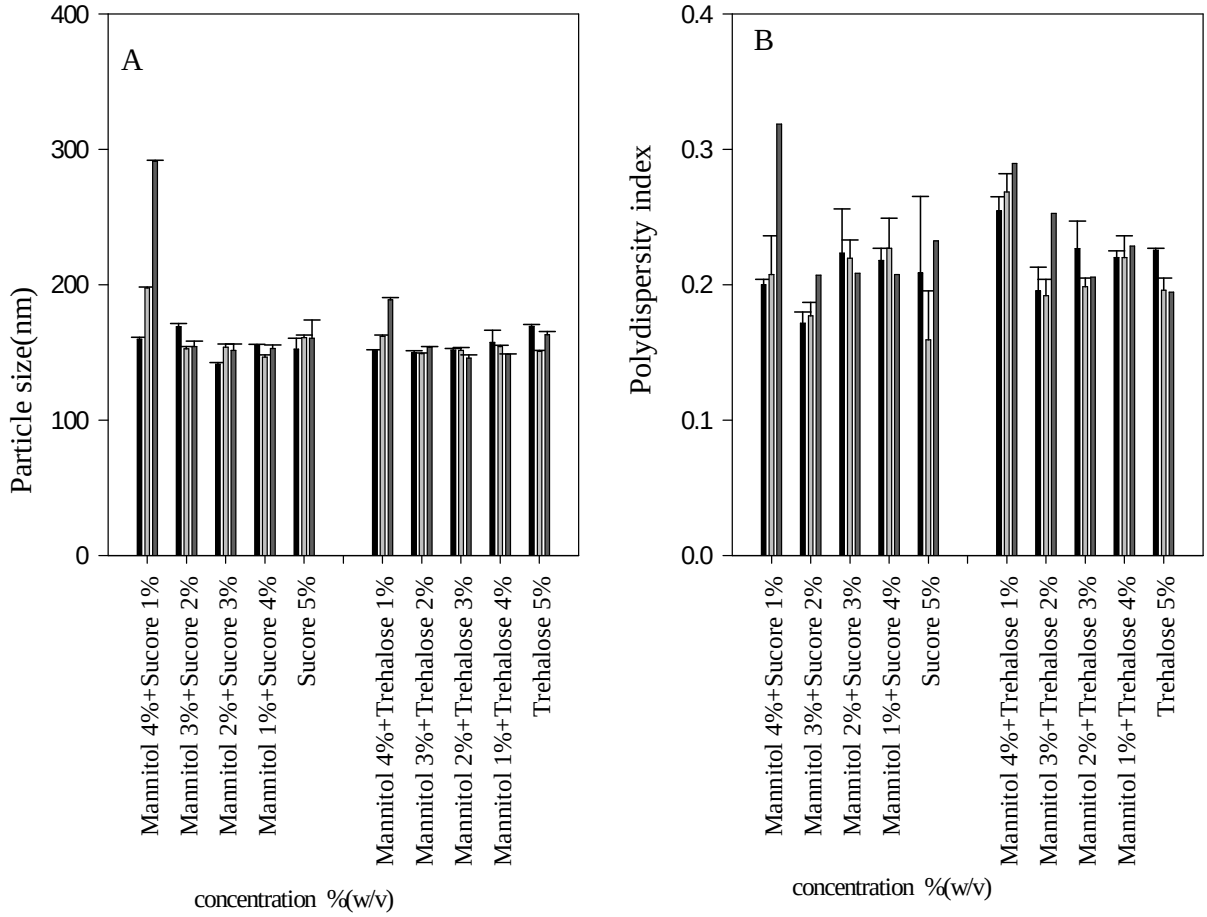
The murine macrophage-like cell line, Raw 264.7, was maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FBS and 100 U/mL penicillin / 100μg/mL streptomycin. All cell lines were incubated as a monolayer at 37 degrees C and 5% CO_2. Cells were seeded into 24-well cell culture plates at a density of 3×10^4 cells/well and grown overnight (60 to 75% confluence). The transfection activity of the suspension of PEI-DNA without additives and lyophilization was taken as a comparative reference (100%). Immediately prior to transfection, cells were rinsed with PBS and supplemented with 1 mL fresh RPMI per well. The pDNA (2μg) and PEI (2μg) were each diluted into 50μL of RPMI solution. 25μL of PEI was slowly added to the pDNA solution 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 the additional 25μL of PEI was added to the solution. After 10 more minutes, cells were exposed to transfection mixtures for 3 hours and then supplemented with 10% FBS and 1% antibiotics. Two days later, β-galactosidase gene expression was analyzed by using combined β-Gal Assay kit and BCA Protein Assay Reagent Kit. The transfection efficiency of the complexes without additives (100%) = 1320 ± 25 nmoles of ortho-nitrophenyl-β-D-galactopyranoside (ONPG)/ incubation time/ mg protein. Freeze-dried and freeze-thawing samples were firstly rehydrated with sterile ddH_2O in the amount of their original volume (200μL), and allowed to equilibrate at room temperature for 30 min. Polyplexes containing 2μg DNA were further diluted in RPMI solution (100μL) for transfection measurements.

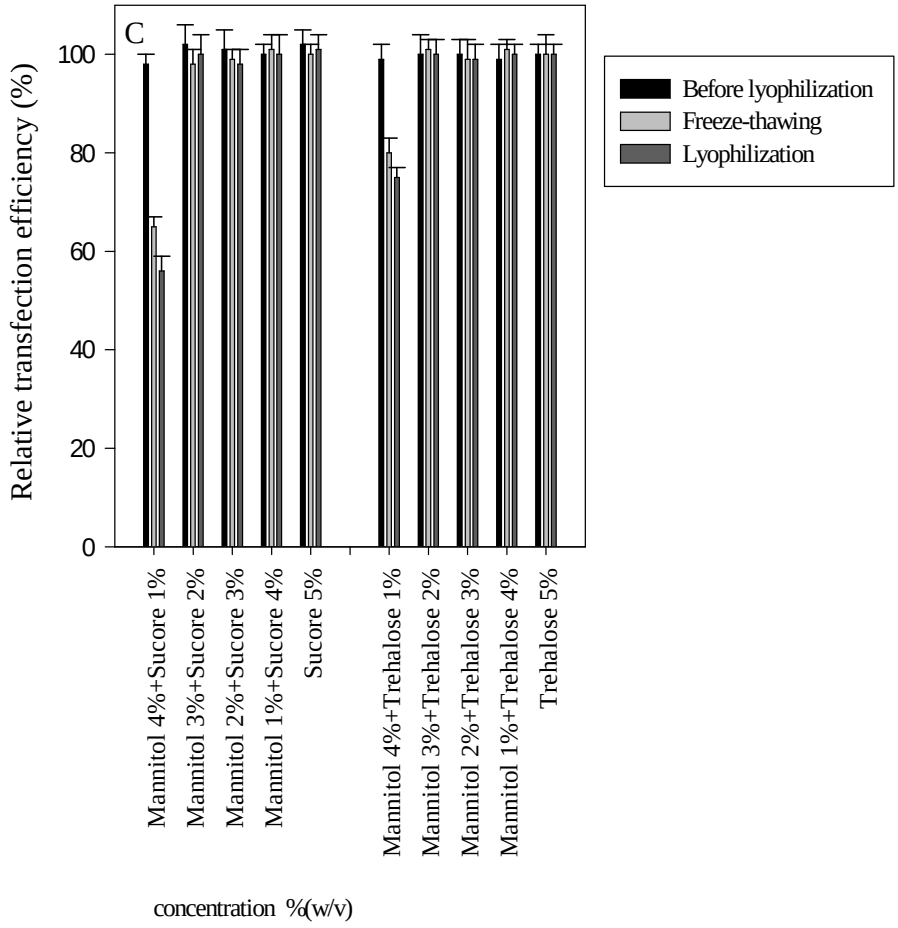
Results and discussion

Attached figures show the effects of reducing mannitol crystallinity by adding sucrose or trehalose on the retention of particle size of PEI-DNA complexes after lyophilization or freeze-thawing. When the concentration of sucrose or trehalose increased from 1 to 4 % w/v at total 5% w/v, the aggregation of PEI-DNA complexes attenuated after lyophilization or freeze-thawing. As the concentration of sucrose was 2 % w/v or above at total 5% w/v, the particle size and polydispersity index of PEI-DNA complexes after lyophilization were comparable to freshly prepared complexes. Lyophilization of PEI-DNA complexes containing 1 % w/v sucrose produced higher particle aggregation than prepared by freeze-thawing. However, lyophilization was comparable to freeze-thawing for avoiding particle aggregation when the concentration of sucrose was above 1 % w/v. Co-lyophilization with trehalose showed similar effect on the retention of particle size

of PEI-DNA complexes after lyophilization or freeze-thawing, except only 1 % w/v of trehalose was able to maintain complex size. Also, the addition of sucrose or trehalose above 1 % w/v allowed fully recovery of the transfection efficiency of PEI-DNA complexes after lyophilization or freeze-thawing. Therefore, disaccharides play the crucial role in the stabilization of DNA during lyophilization or freeze-thawing, as they have been shown to do so for proteins. Disaccharides not only reduced the crystallization of mannitol of freeze-dried DNA formulations, they also provide the protective effect on the stabilization of DNA during lyophilization or freeze-thawing.

Figure 4


A
Particle size(nm)
400
300
200
100
0
Mannitol 4%+Sucore 1%
Mannitol 3%+Sucore 2%
Mannitol 2%+Sucore 3%
Mannitol 1%+Sucore 4%
Sucore 5%
Mannitol 4%+Trehalose 1%
Mannitol 3%+Trehalose 2%
Mannitol 2%+Trehalose 3%
Mannitol 1%+Trehalose 4%
Trehalose 5%
concentration %(w/v)
B
Polydispersity index
0.4
0.3
0.2
0.1
0.0
Mannitol 4%+Sucore 1%
Mannitol 3%+Sucore 2%
Mannitol 2%+Sucore 3%
Mannitol 1%+Sucore 4%
Sucore 5%
Mannitol 4%+Trehalose 1%
Mannitol 3%+Trehalose 2%
Mannitol 2%+Trehalose 3%
Mannitol 1%+Trehalose 4%
Trehalose 5%
concentration %(w/v)


C
Relative transfection efficiency (%)
100
80
60
40
20
0
Mannitol 4%+Sucore 1%
Mannitol 3%+Sucore 2%
Mannitol 2%+Sucore 3%
Mannitol 1%+Sucore 4%
Sucore 5%
Mannitol 4%+Trehalose 1%
Mannitol 3%+Trehalose 2%
Mannitol 2%+Trehalose 3%
Mannitol 1%+Trehalose 4%
Trehalose 5%
concentration %(w/v)
Before lyophilization
Freeze-thawing
Lyophilization