行政院國家科學委員會專題研究計畫　成果報告

陽離子性高分子基因傳送系統之載體結構分析與其安定性研究(3/3)

計畫類別：個別型計畫
計畫編號：NSC92-2216-E-041-001-
執行期間：92 年 08 月 01 日至 93 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：郭榮華
共同主持人：蕭明達，程中玉

報告類型：完整報告

處理方式：本計畫可公開查詢

中　華　民　國 93 年 9 月 24 日

行政院國家科學委員會補助專題研究計畫成果報告

※※※※※※※※※※※※※※※※※※※※※※※※

※ 陽離子性高分子基因傳送系統之載體結構分析與其安定性研究(3/3) ※

※ The structural analysis and preservation of cationic ※

※ polymer-based gene delivery systems ※

※※※※※※※※※※※※※※※※※※※※※※※※

計畫類別：☑個別型計畫　□整合型計畫

計畫編號：NSC 92 － 2216 － E － 041 － 001 －

執行期間：92 年 8 月 1 日至 93 年 7 月 31 日

計畫主持人：郭榮華

共同主持人：程中玉，蕭明達

計畫參與人員：邱鉉文

童義方

執行單位：嘉南藥理科技大學

中 華 民 國 93 年 9 月 17 日

一.中文計畫摘要:

本計劃以冷凍乾燥製程以評估陽離子性高分子-DNA 安定性。本計劃的目的如下：

(1)探討保護劑之選擇與濃度。
(2)保護劑之機轉及效能探討。
(3)冷凍乾燥對基因製劑之物化特性及細胞轉染效率之評估。

二.英文計畫摘要:

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-DNA by mannitol and its crystallization behavior has yet to be elucidated. Without lyoprotectants added, a significant increase of particle aggregation and a dramatic decrease in transfection efficiency of polyethylenimine (PEI)-DNA complexes were observed after freeze-drying or freeze-thawing. Mannitol did provide some protecting effect on the retention of particle size and transfection efficiency of PEI-DNA complexes after lyophilization or freeze-thawing. However, the crystallization of mannitol negatively affected size stability and transfection efficiency of PEI-DNA complexes during lyophilization and freezing-thawing. Co-lyophilization with disaccharides (sucrose or trehalose) resulted in avoiding particle aggregation and preserving transfection efficiency of PEI-DNA complexes during lyophilization or freeze-thawing. As compared with disaccharides, co-lyophilization of lyoprotectants with higher molecular weight (polyvinyl pyrrolidone and dextran) resulted in higher particle aggregation and lower transfection efficiency of PEI-DNA complexes after freeze drying or freeze-thawing. Disaccharides not only reduced the crystallization of mannitol of freeze-dried DNA formulations, they also protected stabilization of DNA during lyophilization or freeze-thawing. Except for lowing mannitol crystallinity, the characteristics of the excipient may influence the ability of the preservation of PEI- DNA complexes after lyophilization or freeze-thawing. This study also demonstrates that lyophilization process resulted in more aggregation than freeze-thawing.

Key words: mannitol; DNA; polyethylenimine; aggregation; lyophilization; transfection.

三.計畫緣由與目的:

The utility of polyethylenimine (PEI) as a transfection agent presents an attractive method to deliver genes for therapeutic applications [1, 2]. PEI polymers, classified as non-viral vectors, are the most commonly used vectors in cationic polymer gene delivery systems. They are believed to provide significant buffering capacity over other cationic polymers, resulting in pH inhibition of lysosomal nucleases and higher transfection activity [3, 4]. Although much is known about the delivery obstacles of cationic polymer-based gene delivery systems, relatively little attention has been paid to the development of their pharmaceutical formulations for long-term stability. Aggregation is typically pointed as the major cause of the loss of transfection rates for cationic polymer-based gene delivery systems and other colloidal carriers stored as liquid suspensions [5-7]. This is due to thermodynamic instability of the colloidal particles. Thus, this intrinsic instability places a strict limitation on the practical application of this new class of DNA-based pharmaceuticals as liquid formulations.

Lyophilization, known as freeze-drying, is a common method used to achieve acceptable shelf life for biopharmaceuticals when the stability of aqueous solutions is inadequate. In the previous report, freeze-dried poly((2-dimethylamino)ethyl methacrylate)-DNA complexes using sucrose as a lyoprotectant, preserved their transfection rates after rehydration even when stored at 40 degrees C for 10 months [5]. Also, encouraging stabilization investigations by freeze-drying were carried out on non-viral vectors such as nanoparticles, polyethylenimine, polyethylenimine-transferrin, peptides, lipids, and polyethyleneglycol-lipds [8-14]. Full preservation of particle size and transfection efficiency for cationic polymer gene delivery systems has been reported when lyoprotectants (sugars) are included in the freeze-dried formulations [9, 14, 15].

The choice of lyoprotectant is crucial for the protection of non-viral complexes during freeze-drying. Among lyoprotectants tested, disaccharides hold superior protective ability during freeze-drying of non-viral complexes [10, 13, 16]. It was suggested that particle isolation during the freezing step is necessary for structural stabilization by disaccharides [17]. On the contrary, there are some reports of negative and controversial effects of mannitol crystallinity on stability of non-viral complexes as a freeze-dried excipient [10, 13, 16, 17]. Mannitol is a commonly used lyoprotectant in biopharmaceutical formulations during lyophilization [18, 19]. Unlike disaccharides, the reasons for the widespread use of mannitol include its tendency to crystallize results in an elegant cake structure, its high eutectic melting temperature with ice promotes efficient freeze-drying, and its chemical stability which resists hydrolysis at extreme pH [20]. The crystallization of mannitol during freeze-drying brings about various anhydrous polymorphs (α, β, and δ) and their mixtures depending on different formulations and processing conditions [21]. Mannitol crystallinity has negatively affected stability of proteins and lipid-DNA as a freeze-dried excipient [16, 22, 23]. The increase in particle size or a concurrent decrease in the transfection efficiency of non-viral complexes was monitored after lyophilization of mannitol [10, 13, 16, 17]. However, in other studies, the transfection efficiency of freeze-dried PEI-DNA was fully recovered in the presence of mannitol [14, 15]. In another approach, crystallization of mannitol during freezing may be able to spatially separate lipid-DNA complexes and attenuate particle interactions to some significant degree [17]. The mechanism of freeze-dried stabilization of cationic polymer-DNA by mannitol and its crystallization behavior has yet to be elucidated. Despite predictable difficulties associated with using mannitol as a freeze-dried excipient, it is worthwhile to explore this formulation alternative for situations where disaccharides are unsuitable.

The aim of this study was to systematically evaluate the protective effect of mannitol on the aggregation behavior of PEI-DNA complexes during lyophilization or freeze-thawing. The mannitol crystallinity in freeze-dried formulations was analyzed by a powder X-ray diffractometer. The analysis of the aggregation behavior of PEI-DNA complexes after lyophilization or freeze-thawing has been investigated by light scattering measurements and in vitro transfection assay. The results allow us to

gain some additional insight into the aggregation mechanism of cationic polymer-based gene delivery systems during freeze-drying.

References

[1] O. Boussif, F. Lezoualc'h, M.A. Zanta, M.D. Mergny, D. Scherman, B.D. Demeneix, J.P. Behr, A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine, Proc. Natl. Acad. Sci. USA 92 (1995) 7297-7301.

[2] T. Merdan, J. Kopecek, T. Kissel, Prospects for cationic polymers in gene and oligonucleotide therapy against cancer, Adv. Drug Deliv. Rev. 54 (2002) 715-758.

[3] W.T. Godbey, K.K. Wu, A.G. Mikos, Tracking the intracellular path of poly(etheylenimine) for gene delivery, Proc. Natl. Acad. Sci. USA 96 (1999) 5177-5181.

[4] W.T. Godbey, K.K. Wu, G.J. Hirasaki, A.G. Mikos, Improved packing of poly(ethylenimine)/DNA complexes increases transfection efficency, Gene Ther. 6 (1999) 1380-1388.

[5] J.Y. Cherng, H. Talsma, D.J. Crommelin, W.E. Hennink, Long term stability of poly((2-dimethylamino)ethyl methacrylate)-based gene delivery systems, Pharm. Res. 16 (1999) 1417-1423.

[6] T.J. Anchordoquy, S.D. Allison, M.C. Molina, L.G. Girouard, T.K. Carson, Physical stabilization of DNA-based therapeutics, Drug Discov. Today. 6 (2001) 463-470.

[7] E. Lai, J.H. van Zanten, Evidence of lipoplex dissociation in liquid formulations, J. Pharm. Sci. 91 (2002) 1225-1232.

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

[9] J.Y. Cherng, P. vd Wetering, H. Talsma, D.J. Crommelin, W.E. Hennink, Stabilization of polymer-based gene delivery systems, Int. J. Pharm. 183 (1999) 25-28.

[10] K.Y. Kwok, R.C. Adami, K.C. Hester, Y. Park, S. Thomas, K.G. Rice, Strategies for maintaining the particle size of peptide DNA condensates following freeze-drying, Int. J. Pharm. 203 (2000) 81-88.

[11] M.C. Molina, S.D. Allison, T.J. Anchordoquy, Maintenance of nonviral vector particle size during the freezing step of the lyophilization process is insufficient for preservation of activity: insight from other structural indicators, J. Pharm. Sci. 90 (2001) 1445-1455.

[12] T.K. Armstrong, L.G. Girouard, T.J. Anchordoquy, Effects of PEGylation on the preservation of cationic lipid/DNA complexes during freeze-thawing and lyophilization, J. Pharm. Sci. 91 (2002) 2549-2558.

[13] M. Sameti, G. Bohr, M.N. Ravi Kumar, C. Kneuer, U. Bakowsky, M. Nacken, H. Schmidt, C.M. Lehr, Stabilisation by freeze-drying of cationically modified silica nanoparticles for gene delivery, Int. J. Pharm. 266 (2003) 51-60.

[14] C. Brus, E. Kleemann, A. Aigner, F. Czubayko, T. Kissel, Stabilization of oligonucleotide-polyethylenimine complexes by freeze-drying: physicochemical and biological characterization, J. Control. Release 95(2004) 119-131.

[15] J.H. Kuo, R. Hwang, 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, J Pharm. Pharmacol. 56 (2004) 27-33.
[16] S.D. Allison, T.J. Anchordoquy, Mechanisms of protection of cationic lipid-DNA complexes during lyophilization, J. Pharm. Sci. 89 (2000) 682-691.
[17] S.D. Allison, M.C. Molina, T.J. Anchordoquy, Stabilization of lipid/DNA complexes during the freezing step of the lyophilization process: the particle isolation hypothesis, Biochim. Biophys. Acta. 1468 (2000) 127-138.
[18] J.F. Carpenter, B.S. Chang, W. Garzon-Rodriguez, T.W. Randolph, Rational design of stable lyophilized protein formulations: theory and practice, Pharm. Biotechnol. 13 (2002) 109-133.
[19] S.Y. Patro, E. Freund, B.S. Chang, Protein formulation and fill-finish operations, Biotechnol. Annu. Rev. 8 (2002) 55-84.
[20] C. Telang, L. Yu, R. Suryanarayanan, Effective inhibition of mannitol crystallization in frozen solutions by sodium chloride, Pharm. Res. 20 (2003) 660-667.
[21] A.I. Kim, M.J. Akers, S.L. Nail, The physical state of mannitol after freeze-drying: effects of mannitol concentration, freezing rate, and a noncrystallizing cosolute, J. Pharm. Sci. 87 (1998) 931-935.
[22] K. Izutsu, S. Yoshioka, T. Terao, Decreased protein-stabilizing effects of cryoprotectants due to crystallization, Pharm. Res. 10 (1993) 1232-1237.
[23] D. Izutsu, S. Kojima, Excipient crystallinity and its protein-structure-stabilizing effect during freeze-drying, J. Pharm. Pharmacol. 54 (2002) 1033-1039.
[24] Y.F. Maa, C. Shu, M. Ameri, C. Zuleger, J. Che, J.E. Osorio, L.G. Payne, D. Chen, Optimization of an alum-adsorbed vaccine powder formulation for epidermal powder immunization. Pharm. Res. 20 (2003) 969-977.
[25] R.E. Johnson, C.F. Kirchhoff, H.T. Gaud, Mannitol-sucrose mixtures--versatile formulations for protein lyophilization, J. Pharm. Sci. 91 (2002) 914-922.
[26] J.H. Crowe, S.B. Leslie, L.M. Crowe, Is vitrification sufficient to preserve liposomes during freeze-drying? Cryobiology 31 (1994) 355-366.
[27] J.F. Carpenter, J.H. Crowe, 1989. An infrared spectroscopic study of the interactions of carbohydrates with dried proteins, Biochemistry 28 (1989) 3916-3922.

四.結果與討論:

To circumvent the aggregation issue raised by various thermal stresses, the influence of lyophilization and freeze-thawing on the particle size changes of PEI-DNA complexes without a lyoprotectant was first studied. As shown in Figure1, freeze-thawing of PEI-DNA complexes without a lyoprotectant resulted in a remarkable increase in mean particle size (500 nm, Figure 1A) and polydispersity index (0.451, Figure 1B) compared with freshly prepared complexes (mean particle size = 145 nm; polydispersity index = 0.201; control). This indicates that a mild aggregation of PEI-DNA complexes was observed after freeze-thawing even without sufficient time for particle collision. Also, freeze-thawing of PEI-DNA complexes without additives resulted in a significant

loss in transfection efficiency as compared with freshly prepared complexes (control, Figure 1C). Furthermore, lyophilization of PEI-DNA complexes without a lyoprotectant resulted in formation of larger aggregates and a further decrease in transfection efficiency than complexes after freeze-thawing (Figure 1). Except for the freezing step, the drying process of lyophilization also contributes to this drastic size increase and transfection decrease of PEI-DNA complexes without a lyoprotectant. The results are consistent with other studies indicating that transfection efficiencies of cationic polymer gene delivery systems lyophilized without additives are dramatically reduced after rehydration [8, 9].

Having evaluated the amount of aggregation during lyophilization and freeze-thawing, PEI-DNA complexes in the presence of mannitol as a lyoprotectant were tested to assess its protection properties. As illustrated in Figure 2A and 2B, either after freeze-thawing or lyophilization, the mean particle size and polydispersity index of PEI-DNA complexes containing mannitol from 1 to 10 % w/v were significant higher than freshly prepared complexes. Mannitol did provide some protecting effect on the retention of particle size of PEI-DNA complexes after lyophilization or freeze-thawing; as compared with complexes without a lyoprotectant (Figure 1). Also, lyophilization process resulted in more aggregation than freeze-thawing as they have been shown in Figure 1. The protective effect on the retention of particle size by mannitol leveled off at concentrations of more than 5% during lyophilization and freeze-thawing. In the addition of mannitol from 1 to 10 % w/v, PEI-DNA complexes after lyophilization and freeze-thawing lost approximately 65% to 75% of their ability to transfect cells (Figure 2C). Despite mannitol has the potential to form hydrogen bonding with DNA and replace water, our findings indicate that mannitol crystallization negatively affected size stability and transfection efficiency of PEI-DNA complexes during lyophilization and freeze-thawing, consistent with recent studies of PEI-DNA or other carrier systems [10, 14, 17]. Further concentrated components in the unfrozen fraction induced by phase separation of mannitol from PEI-DNA complexes is responsible for the extensive aggregation during lyophilization and freeze-thawing, as also observed in previous study of lipid-DNA complexes [16].

To evaluate the effect between the mannitol crystallinity and aggregation behavior of PEI-DNA complexes, combinations of disaccharides and mannitol were next studied for reducing the degree of crystallinity of mannitol during lyophilization. It is widely acknowledged that disaccharides hold superior protective ability during freeze-drying of polymer-DNA complexes [5, 8, 9]. However, the disadvantages of disaccharides as stabilizers are their chemical instability at low or high pH and longer lyophilization cycle to prevent the cake from collapsing. Combinations of disaccharides and mannitol have been demonstrated as additives for the freeze-drying and spray freeze-drying of proteins [24, 25]. It was proposed that water substitution and excipient vitrification are necessary for structural stabilization [26, 27]. The resulting cakes or powders enclose both an amorphous disaccharide phase for protein stability and crystalline mannitol to avoid breakdown or improve powder properties [24, 25]. Also, the relative concentration threshold above which crystalline

mannitol is observed is 30 % w/w in a mannitol-disaccharides system after freeze-drying [21]. As shown in Figure 3a, the diffraction peaks of the freeze-dried 5% w/v mannitol powders containing PEI-DNA complexes indicate the beta and delta polymorph were mainly formed, and consistence with the reference diffractogram of the previous study [21]. With the addition of sucrose or trehalose from 1 to 3 % w/v at total 5% w/v, mannitol crystallinity was reduced as illustrated by the decreased intensity at the corresponding peaks in the XRD pattern of the freeze-dried powders containing PEI-DNA complexes (data not shown). When the concentration of sucrose or trehalose to mannitol was 4/1 w/w, XRD patterns led to amorphous structures as illustrated by the broad halo and lack of resolved reflections (Figure 3b and 3c). Figure 4A and 4B shows the effect 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. At the concentration of sucrose = 1 % w/v at total 5% w/v, the aggregation of PEI-DNA complexes was less dramatic than full mannitol formulations after lyophilization or freeze-thawing (Figure 2A and 2B). 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 (Figure 4C). Therefore, disaccharides play the crucial role on the stabilization of DNA during lyophilization or freeze-thawing, as they have been shown to do so for proteins [25]. 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. This may result from both their bulking ability and through direct hydrogen bonding to the surface of DNA complexes, as indicated in other studies [16, 17].

In addition to disaccharides, co-lyophilization of polyvinyl pyrrolidone and dextran with mannitol was also performed to further study the effect of reducing mannitol crystallinity for the stabilization of PEI-DNA complexes after lyophilization or freeze-thawing. The selection of polyvinyl pyrrolidone and dextran as protective additives is based on their ability to inhibit mannitol crystallization from frozen solutions [20, 24]. Similarly to disaccharides, the XRD spectra showed fully amorphous structure and no signs of crystalline mannitol when the concentration of polyvinyl pyrrolidone or dextran to mannitol was 4/1 w/w at total 5% w/v (Figure 5). As illustrated in Figure 6, by reducing mannitol crystallinity, a moderate increase in

particle aggregation was still observed with adding polyvinyl pyrrolidone and dextran after freeze drying or freeze-thawing. Again, particle size was better preserved after freeze-thawing as compared with lyophilization. Moreover, compared with disaccharides, transfection efficiencies were not completely preserved after lyophilization or freeze-thawing. Also, the recovery of particle size did not correlate well with transfection efficiencies after lyophilization or freeze-thawing. Therefore, except for mannitol crystallinity, the characteristics of the excipient may influence the ability of the preservation of PEI- DNA complexes after lyophilization or freeze-thawing. These results are consistent with recent studies that show different cryoformulation medium may affect stability of lipid-DNA complexes [16, 17]. The lyoprotectants with higher molecular weight (polyvinyl pyrrolidone and dextran) are not capable of fully preventing particle aggregation as compared with sucrose and trehalose. It appears that interactions between lyoprotectants with higher molecular weight and DNA complexes might not be enough to fully stabilize DNA after lyophilization or freeze-thawing. Due to their steric restrictions, the lyoprotectants with higher molecular weight do not effectively bond to DNA. Our results are consistent with the hypothesis of water replacement in preserving transfection efficiencies of lipid-DNA complexes during lyophilization [17].

Figure 1 The influence of lyophilization and freeze-thawing on the particle size (A), polydispersity index (B), and transfection efficiencies (C) of PEI-DNA complexes without a lyoprotectant. Control samples represent freshly prepared complexes prior to lyophilization or freeze-thawing. Data are given as means ± standard deviation of single measurements on triplicate samples.

Figure 2 Effect of mannitol concentration on the retention of particle size (A), polydispersity index (B), and transfection efficiencies (C) of PEI-DNA complexes during lyophilization and freeze-thawing. Data are given as means ± standard deviation of single measurements on triplicate samples.

Figure 3 Powder X-ray diffraction patterns of freeze-dried PEI-DNA complexes. The lyoprotectant formulations for each experiment are illustrated as following: (a) mannitol: 5% w/v; (b) mannitol: 1% w/v; trehalose: 4% w/v; (c) mannitol: 1% w/v; sucrose: 4% w/v.

Figure 4 Effect of reducing mannitol crystallinity by adding sucrose or trehalose on the retention of particle size (A), polydispersity index (B), and transfection efficiencies (C) of PEI-DNA complexes during lyophilization and freeze-thawing. Data are given as means ± standard deviation of single measurements on triplicate samples.

Figure 5 Powder X-ray diffraction patterns of freeze-dried PEI-DNA complexes. The lyoprotectant formulations for each experiment are illustrated as following: (a) mannitol: 5% w/v; (b) mannitol: 1% w/v; polyvinyl pyrrolidone: 4% w/v; (c) mannitol: 1% w/v; dextran: 4% w/v.

Figure 6 Effect of reducing mannitol crystallinity by adding polyvinyl pyrrolidone or dextran on the retention of particle size (A), polydispersity index (B), and transfection efficiencies (C) of PEI-DNA complexes during lyophilization and freeze-thawing. Data are given as means ± standard deviation of single measurements on triplicate samples.