行政院國家科學委員會專題研究計畫　期中精簡報告

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(2/3)

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執行單位：嘉南藥理科技大學藥學系

計畫主持人：郭榮華
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中　華　民　國 92 年 5 月 5 日

行政院國家科學委員會補助專題研究計畫成果報告

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※ 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(2/3) ※

※ The structural analysis and preservation of cationic

※ ※ polymer-based gene delivery systems

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計畫類別：☑個別型計畫 □整合型計畫

計畫編號：NSC 91 － 2216 － E － 041 － 001 －

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計畫主持人：郭榮華

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中 華 民 國 92 年 5 月 1 日

中文計畫摘要:

本研究的目標主要是探討保護劑對噴霧乾燥 DNA 之安定性評估。在非緊縮性保護劑(sucrose, glycine and agarose) 的作用下，噴霧乾燥製程對 DNA 很容易改變其三級結構而失去活性。以膠片電泳法檢視，SC plasmid DNA 會降解成 OC 或直線 DNA。相對之下,中 性緊縮性保護劑(polyethylene glycol 1000 and 4000) 對 DNA 提供結構保護作用。同時，過量陽離子 性緊縮性保護劑(polyethyleneimine) 亦 提供 DNA 結構維持原狀。經過六個月 4℃下保存，有緊縮性保護劑保護下，DNA 亦保持結構原狀。DNA 緊縮作用可避免噴霧乾燥引起之降解作用。

關鍵詞:DNA; 安定性; 噴霧乾燥

Abstract:

The effect of several protective agents was assessed on the stability of spray-dried plasmid DNA. The spray-drying process had adverse effects on the tertiary structure of plasmid DNA with the protective agents of sucrose, glycine and agarose. With the protection of these noncondensing agents, a band corresponding to the linear form of plasmid DNA was observed in the gel electrophoresis between the SC form and the OC form. On the contrary, spray-dried plasmid DNA maintained some degree of structural integrity under the protection of condensing

agents. For the protection by neutral condensing polymers, such as polyethylene glycol 1000 and 4000, no linear form between the SC form and the OC form of plasmid DNA was revealed in the gel electrophoresis Also, excess cationic condensing polymer, polyethyleneimine, had the ability to provide the protection of plasmid DNA from degradation as indicated by the reservation in supercoiled circular and open circular forms of plasmid DNA on the agarose gel electrophoresis Moreover, DNA topology was unchanged after 6 months of storage at 4°C by the protection of these neutral and cationic condensing agents. Accordingly, DNA condensation induced by condensing agents may provide a way to minimize damage to plasmid DNA by the process of spray drying.

Key words: DNA; stability; spray drying

計畫緣由與目的:

One of the fastest growing research areas involves plasmid DNA for gene therapy (Miller 1992). In order to obtain optimal biological effectiveness, plasmid DNA has to retain its supercoiled circular (SC) and open circular (OC) forms (Kimoto &Taketo 1996). In addition, all aspects of regulatory review must comply with the most rigorous standards to ensure the plasmid DNA products remain in a defined and characterized isoform. Therefore, the degradation of the SC and OC form is considered strictly undesirable. The degradation of plasmid DNA occurs in any aqueous solution near neutral pH by the two-step process of depurination and β -elimination, leading to cleavage of the phosphodiester backbone (Suzuki et al 1994). The introduction of structural changes will eventually convert SC form of plasmid DNA to the OC and linear forms (Middaugh et al 1998). The stability of plasmid DNA is dependent on many factors, including temperature and shear stress (Lindahl & Karlstrom 1973; Levy et al 1999).

The spray-drying process has recently been used to develop aerosol delivery for biotechnology-based pharmaceuticals (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). As a result, a fast spray-drying process may represent a subject of increasing interest in the area of the treatment of pulmonary diseases such as cystic fibrosis and lung cancer. Nonetheless, spray-dried biotechnology-based products may encounter a loss of biological activity due to certain structural alterations as a result of the processing environment. Besides thermal degradation, the instability of biopharmaceuticals during the process of spray drying has been attributed to shearing stresses in the nozzle and adsorption at the liquid/air interface of the spray solution on atomization (Niven et al 1994; Maa & Hsu 1997). In general, protective agents such as disaccharides and polyols included in the solution are

essential to protect the biological integrity of biopharmaceuticals during spray drying (Labrude et al 1989; Broadhead et al 1994). The stabilizing mechanism is primarily due to interactions, such as hydrogen bonding, between biopharmaceuticals and protective agents (Carpenter & Crowe 1989). The study of the stability profile of spray-dried plasmid DNA formulations is limited. Spray-dried DNA powders containing trehalose have been prepared, but no details of DNA topology analysis were reported (Freeman & Niven 1996).

The major purpose of this study is to evaluate the effectiveness of protective agents in order to obtain stable plasmid DNA to avoid degradation by spray-drying processing. The various noncondensing protective agents used were sucrose, glycine, and agarose. The neutral condensing protective agents studied were polyethylene glycol 1000 and 4000 whereas the cationic condensing protective agent studied was polyethyleneimine. Experimental results ofprotective agents on the stability of plasmid DNA by the process of spray drying were analyzed by agarose gel electrophoresis.

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研究方法:

Materials

The plasmid, pCMV-Luc, contains the luciferase reporter gene under control of the CMV promoter. pCMV-Luc was amplified in *E. Coli* (strain DH5α) and purified by column chromatography (QIAGEN-Mega kit, Netherlands). The purity of pCMV-Luc was established by UV spectroscopy (E260 nm/E280 nm ratio ranging from 1.87-1.89 were used). Agarose (0.7%) gel electrophoresis analysis using restriction enzymes showed that pCMV-Luc was mainly in the supercoiled form and one band corresponding to a size of 7.8 kb was visible. Reagent grade of sucrose, polyaspartic acid and glycine were obtained from Sigma Chemical Company (St. Louis, MO, USA) and used without further purification. Polyehylene glycol (PEG) 1000 and 4000 were purchased from Fluka Chemie GmbH (Switzerland). Polyethyleneimine (PEI, MW = 800 kDa) was obtained from Sigma as 50% (W/V) solution. The PEI solutions were adjusted to desired aqueous concentrations and neutralized with HCl (PH=7.0). Molecular biology grade agarose was supplied by Vegonil (Moreno Valley, CA, USA).

Spray-drying

The spray-drying process was performed using a Büchi 190 mini spray dryer (Switzerland). Atomizing air (6 bar) and feed solution passed separately to the two-fluid 0.5 mm pneumatic nozzle in co-current flow systems. The feed solution (pump setting =2) was prepared by dispersing 1 mg pCMV-Luc as well as appropriate amount of protective agents in 25 mL deionized water and sprayed at various preset inlet/outlet temperatures. The aspirator setting was kept at the scale of 12 and the air flow rate was 700 NL/hr during the process. The dried particles were separated from

the hot air stream in a cyclone and collected in a receiving vessel. The noncondensing agents used in the spray drying are sucrose (20 and 30 %), glycine (5%) + sucrose (20%), and agarose (2%); respectively. Whereas, the condensing agents used are PEG 1000 (20%), PEG 4000 (20%), and PEI-DNA (3/1 and 5/1 W/W) in 20% sucrose solution; respectively.

Agarose gel electrophoresis

After the process of spray-drying, the dried powders (1 µg pDNA/well; 10 μL+ 2 μL Type IV loading buffer /well) in TAE buffer (pH=8.0) were then loaded onto a 0.7 % agarose gel containing ethidium bromide and electrophoresed at 100 V for 1 hours. After electrophoresis, DNA was examined by UV-irradiation. For PEI cationic polymers, polyaspartic acid solution (25 mg/mL) was used to dissociate the cationic polymer- DNA complexes at ambient temperature. The resulting compound was incubated for 24 hours.

結果與討論:

The agarose gel electrophoresis of spray-dried plasmid DNA in 20% sucrose after processing at various outlet temperatures is shown in Figure 1. As compared with control plasmid DNA (Figure 1, lane 1), a sharp decrease of the SC band was observed under all processing temperatures. For all these spray-dried samples, a band corresponding to the linear form of plasmid DNA was observed in the gel between the SC form and the OC form. The results indicated that the integrity of plasmid DNA was changed by the spray-drying process, even when the outlet processing temperature was decreased as low as 56℃ (Figure 1, lane 2). The typical temperature difference between hot drying air and droplet surface during spray-drying was approximately 15℃ (Master 1991). At this temperature (around 40℃), without spray drying, plasmid DNA in aqueous solution remained in SC and OC forms (data is not shown). Accordingly, the shear stress and air-liquid interfaces of plasmid solution may result in the structural change of plasmid DNA (Maa et al 1998). Also, previous observations indicated that large molecules of DNA were easily broken by shear stress (Reese & Zimm 1990). The observation is consistent with previous studies that the induced shear stress can lower the activation energy required for plasmid DNA degradation (Adam & Zimm 1977).

To circumvent the stability issue raised by temperature and shear stress, the effects of sucrose alone as well as combing sucrose and glycine on the stability of plasmid DNA were studied (Figure 2). Similarly, the 30% sucrose (lane 2) had no significant improvement on the structural integrity of plasmid DNA after spray drying. In addition to sucrose, glycine has also been reported as a protective agent for some

protein-based products (Walsh 1998). Glycine was incorporated into the 20 % sucrose solution containing 1 mg plasmid DNA at concentrations of 5% (lane 3). Nevertheless, a linear band was still evident under these mild spray-drying conditions, suggesting that the combination of sucrose and glycine was not sufficient to protect the plasmid DNA from structural change. In order to minimize damage to DNA by shear stress, attempts were made to introduce interactions between DNA and protective agents in the solution (Sambrook & Russel 2001). For 2% agarose (lane 4), a band corresponding to the linear form of plasmid DNA was observed in the gel between the SC form and the OC form. Apparently, agarose was ineffective to protect the plasmid DNA from structural change by spray-drying process although DNA was successfully protected in agarose blocks in most molecular biology experiments.

Figure 3 shows the effect of several other protective agents on the stability of plasmid DNA after spray drying. For PEG 1000 and 4000 (lane 3, 4), no linear form of plasmid DNA was observed in the gel between the SC form and the OC form. As compared with control plasmid DNA, a slight decrease of the SC band was observed under the process of spray drying. This evidence demonstrates that spray-dried plasmid DNA mostly retained structural integrity under the protection of PEG 1000 and 4000. This protective effect of two PEGs may be attributed to the DNA condensation by neutral crowding polymers such as PEG through an excluded volume mechanism (Vasilevskaya et al 1995; Bloomfield 1996). That is, the dramatic decrease in plasmid DNA volume provoked by PEG may contribute to the minimized DNA damage by shear stress during spray drying. The protective effect of cationic polymers on the stability of plasmid DNA by the process of spray drying was also evaluated in Figure 3. Similarly, previous reports have shown that the cationic polymers may interact with the negatively charged larger piece of DNA and thus the two types of molecules may condense to compact complexes in the aqueous solutions (Boussif et al 1995; Gebhart & Kabanov 2001). Furthermore, electrostatic forces may also lead to a higher denaturation temperature of plasmid DNA and transition temperature of polymer for the cationic polymer-plasmid DNA complexes (Kuo et al 2002). The above two mechanisms suggest the addition of cationic polymers may provide the protection of plasmid DNA to withstand the thermal and shear stress during spray drying. Accordingly, the complexation of plasmid DNA with PEI was probed in aqueous solution with cationic polymer/DNA weight ratios of 5/1 and 3/1. The choice of PEI-DNA ratios (3 and 5 W/W) is based on the optimal transfection at above ratios on several cell lines. For typical PEI /DNA complexes, no band was revealed in the agarose gel electrophoresis after spray drying in aqueous solution (lane 2). To distinguish DNA from the resulting complexes in the agarose gel, the

resulting complex solution was treated with excess polyaspartic acid to dissociate cationic polymers as shown in the previous report (Trubetskoy et al 1999). Indeed, for excess cationic polymer/DNA ratios (5/1 and 3/1), no linear form was revealed in the agarose gel electrophoresis between the SC form and the OC form after the process of spray drying, as shown in Figure 3 (lane 5,6). As previously reported, the transfection efficiency in several cell lines was much higher for SC/OC plasmid DNA than for linearized plasmid DNA (Xie & Tsong, 1993; Adami et al., 1998). Whereas, the protective effect of excess DNA/cationic polymers ratios might not be sufficient to condense the plasmid DNA from structural degradation, as demonstrated from previous studies that complexation of excess DNA with the cationic polymers resulted in a decreased thermal stability of SC DNA (Lobo et al 2002). The above results demonstrate that the condensation induced by condensing agents such as PEG and cationic polymers may provide the ability to protect plasmid DNA from structural degradation by the process of spray drying. In an effort to understand the protective mechanism by polymers, our results demonstrated that the interactions between polymer and plasmid DNA is critical to withstand the shear stress generated by the process of spray drying. Treatment of plasmid DNA with neutral crowding polymers such as PEG induced DNA condensation and this kind of interactions with DNA was sufficient to resist the DNA degradation. In addition to condensation, cationic polymers also generate electrostatic forces with the negatively charged larger piece of DNA and are effective to provide the DNA stability under spray drying.

In order to examine the long term (6 months) stability of plasmid DNA and to evaluate the possible reversibility of conformational change process, the spray-dried plasmid DNA under the protection of noncondensing agents was stored at 4℃ after 6 months and the electrophoresis results are shown in Figure 4. The intensity of SC form of spray-dried plasmid DNA in Figure 4 decreased as compared with samples without 6 months of aging (Figure 1, 2). This demonstrated that spray-dried plasmid DNA was further degraded by the aging process, even when the storage temperature remained as low as 4℃. The results were also in accordance with previous observation that the structural change of plasmid DNA was irreversible; OC and linear form cannot be converted back to SC form (Achordoquy & Koe 2000). As for PEGs (Figure 5, lane 3 and 4), no linear forms were obtained from the analysis of agarose gel electrophoresis after 6 months storage at 4℃. Whereas, for PEI-DNA complexes, no band was detected in the agarose gel after spray-drying in aqueous solution (Figure 5, lane 5). Apparently, the binding between cationic polymer and plasmid DNA was undisrupted by 6 months storage at 4℃. However, after polyaspartic acid dissociation (Figure 5, lane 2 and 6), plasmid DNA remained in SC and OC forms in aqueous solution at cationic polymer /DNA weight ratios 5/1 and 3/1.

This reflects the stabilizing effects on spray-dried plasmid DNA by the addition of cationic polymers such as PEI. These observations clearly show that the condensing agents (PEG 1000, PEG 4000, and PEI) are more effective than noncondensing agents on the long-term stability of plasmid DNA by the process of spray drying. From these stability studies, it opens up opportunities in using the process of spray drying as a means to facilitate the formulations of plasmid DNA such as arosols while preserving plasmid DNA integrity. The protective effect of PEG could be used for storage purposes. However, PEI-DNA complexes should unlikely be used as aerosols since the charge of the complex should not be suitable for transfection. Furthermore, PEI can be used for transfection in lung without the use of cationic lipids and could be of paramount interest for the development of commercial gene products.

計畫成果自評:

本計畫應用噴霧乾燥以作為基因製劑之肺部給藥方式,探討保護劑或添加劑之選擇與濃度，噴霧乾燥保存機轉過程探討（發表論文: The effect of protective agents on the stability of plasmid DNA by the process of spray -drying. Journal of Pharmacy and Pharmacology: 55: 301-306, 2003）。

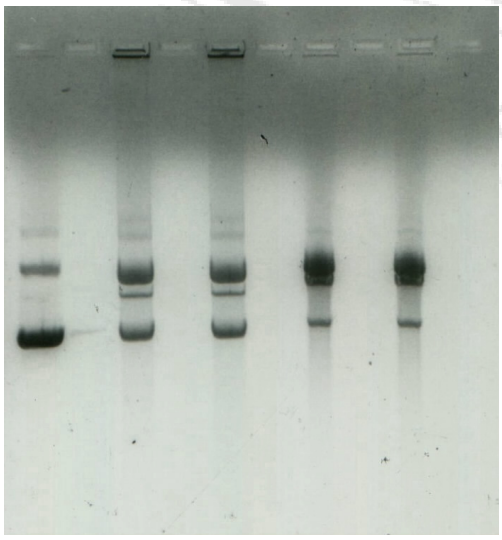

Figure 1

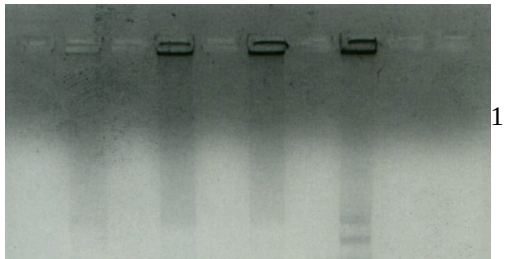

Figure 2

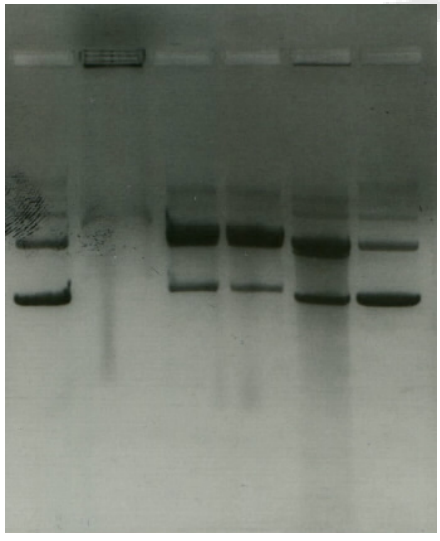

Figure 3

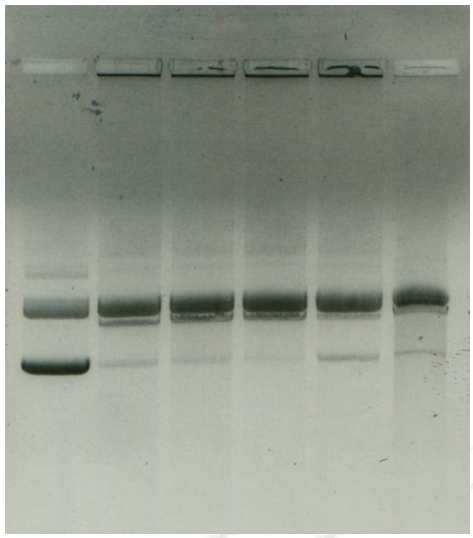

Figure 4

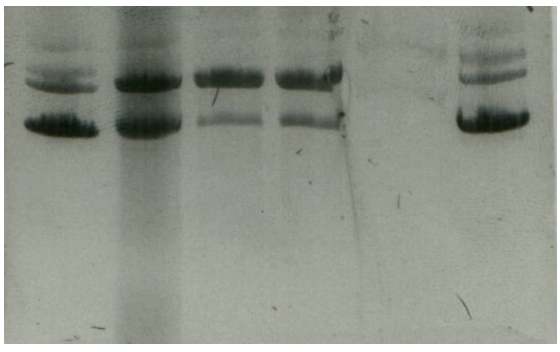

Figure 5

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