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Stability of chitosan–pDNA complex powder prepared by supercritical carbon dioxide process

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Abstract

The present study examined the stability of a gene in powders prepared with supercritical carbon dioxide (CO_2) from the viewpoints of the ternary structure of DNA and in vivo transfection potential. An aqueous chitosan–pCMV-Luc complex solution containing mannitol was injected into the stream of a supercritical CO_2/ethanol admixture to precipitate a gene powder. The obtained gene powders and gene solutions were placed in stability chambers at 25 or 40 °C for 4 weeks. The integrity and transfection potency of the gene were examined by electrophoresis and in vivo pulmonary transfection study in mice. The supercritical CO_2 process decreased the supercoiled DNA during the manufacturing process; however, the decrease in the remaining supercoiled and open circular DNA in the powders during storage was much slower than that in solutions. In addition, the powders had higher transfection potency than the solutions containing the same amount of DNA. The effect of chitosan on the stability of DNA in solutions was not obvious in the solutions but it improved the stability of DNA in powders during manufacturing and storage. Thus, a gene powder with a cationic vector is a promising ready-to-use formulation for inhalation therapy of pulmonary diseases.

Keywords: Plasmid DNA; Chitosan; Supercritical carbon dioxide; Dry powder; Gene delivery system; Stability

1. Introduction

The respiratory system, from the nasal cavity to alveoli, is a suitable site for gene therapy because direct access of a gene delivery system via the airway is possible. The success of gene therapeutic strategies depends on an efficient system for the delivery of nucleic acid into the target cells. Both viral and nonviral gene delivery systems have been used in clinical trials to treat maladies such as cystic fibrosis and several forms of cancer. The majority of gene delivery methods have involved primarily adenoviral or liposomal vectors (Godbey et al., 1999). Cationic polymers also have the potential

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for DNA complexation and it is recognized that they may be useful as non-viral vectors for gene delivery. Many cationic polymers such as chitosan (Richardson et al., 1999; Lee et al., 1998), polylysine (Choi et al., 1998), and poly(ethyleneimine) (Godbey et al., 1999) have been examined as DNA delivery systems, as have cationic liposomes (Niven et al., 1998) and cationic microspheres (Capan et al., 1999). Richardson et al. (1999) examined the potential of low-molecular-mass chitosan as a DNA delivery system. They observed that the highly purified chitosan fractions used were neither toxic nor hemolytic, that they had the ability to complex DNA and protect against nuclease degradation, and that low-molecular-weight chitosan can be administered intravenously without liver accumulation.

Although aqueous solution instillation appears to be the most commonly used way of gene delivery to the airway, a dosage form for inhalation would be more desirable. Pressurized metered-dose inhalers (MDIs), nebulizers, and dry powder inhalers (DPIs) are the three main delivery systems used for aerosol inhalation in humans (Timsina et al., 1994). Brown et al. prepared propellant-driven aerosols by suspending lyophilized lipofectin–DNA plasmid complex in dimethylether propellant (Brown and Chowdhury, 1997). Nebulization is one of the practical systems for the administration of non-viral gene delivery systems. Stribling et al. (1992) demonstrated that aerosol administration of a chloramphenicol acetyltransferase (CAT) expression plasmid complexed to cationic liposomes produced high-level, lung-specific CAT gene expression in mice in vivo with no apparent treatment-related damage. However, the free plasmid DNA (pDNA) was degraded rapidly during aerosolization, although complexed pDNA was largely protected (Eastman et al., 1997). Birchall et al. (2000) also reported that the process of jet nebulization adversely affected the physical stability of lipid–pDNA complexes. The physical stability and biological activity of nebulized lipid–pDNA complexes can be improved by inclusion of a condensing polycationic peptide such as protamine.

Another promising system for pulmonary gene delivery is dry powder. DPIs appear to be more promising than MDIs or nebulizers for future use because the device is small and relatively inexpensive, no propellants are used, and breath-actuation can be used successfully by many patients who have poor MDI technique (Timsina et al., 1994; Newman et al., 1994). However, information on the usefulness of dry powder as a pulmonary gene delivery system is very limited. Freeman and Niven (1996) administered 200 μg of pCMV-Luc as a spray-dried pCMV-Luc:trehalose (1:9) powder into the rat lung through the trachea to examine the effect of insufflated pCMV-Luc on the transfection of lung tissue. Unfortunately, no response to the insufflated pCMV-Luc powder could be obtained.

Supercritical fluid technology offers the possibility of producing dry powder formulations suitable for inhalation or needle-free injection (Tservistas et al., 2001). Supercritical carbon dioxide (CO_2) has been employed for the preparation of lactose (Palakodaty et al., 1998), steroids (Steckel et al., 1997), protein powder (Yeo et al., 1993), biodegradable microsphere (Bodmeier et al., 1995; Ghaderi et al., 1999), and liposomes (Frederiksen et al., 1997). Tservistas et al. (2001) described the first application of a process involving supercritical CO_2 for the production of pDNA-loaded particles. They used the technique of solution enhanced dispersion by supercritical fluids (SEDS) to coformulate the 6.9 kb plasmid pSVβ with mannitol as the excipient.

In a previous study (Okamoto et al., 2003), we prepared gene powders using supercritical CO_2 with pCMV-Luc as a reporter gene and a low-molecular-weight chitosan (Mw = 3000–30,000) as a cationic vector. The addition of chitosan suppressed the degradation of pCMV-Luc during the supercritical CO_2 process. The obtained chitosan–pCMV-Luc powders were administered to mice lungs. Administration of the chitosan–pCMV-Luc powders resulted in high levels of luciferase activity in mouse lung compared with pCMV-Luc powders without chitosan or pCMV-Luc solutions with or without chitosan. Administration of the chitosan–pCMV-Luc powder with an N/P (chitosan nitrogen per pCMV-Luc phosphate) = 5 resulted in a level of luciferase activity 27 times higher than that in the lungs of mice administered the pCMV-Luc solution. These results suggest that gene powders containing chitosan are a useful pulmonary gene delivery system.

Dry gene powders are expected to have the additional benefit of increased shelf life of the formulation. Genes are generally not stable in solution (Cherng et al., 1999; Li et al., 2000), which makes it difficult to develop ready-to-use formulations for gene therapy. A gene formulation with high stability could be a ready-to-use formulation, which would increase the

quality of life of patients and reduce the medical expenses. In the present study, we examined the stability of chitosan–pCMV-Luc powders prepared by the supercritical CO_2 process.

2. Materials and methods

2.1. Materials

The plasmid DNA pCMV-Luc, constructed by subcloning the Hind III/Xba I firefly luciferase cDNA fragment from the pGL3-control vector into the polylinker of the pcDNA3 vector, was donated by Prof. M. Hashida, Kyoto University (Sakurai et al., 2000). pCMV-Luc was amplified in the DH5 strain of *Escherichia coli*, and purified using a Qiagen Plasmid Giga Kit (Qiagen GmbH, Hilden, Germany). The concentration of pCMV-Luc was determined by measuring UV absorption at 260 nm. Plasmid DNA purity was assessed by measuring the A_{260}/A_{280} ratios.

Water-soluble chitosan (nominal Mw = 3000–30,000; Wako Pure Chemical Industries Ltd., Osaka, Japan) and mannitol (Wako Pure Chemical Industries Ltd.) were used as a nonviral vector and a dry powder carrier, respectively.

A luciferase assay system PicaGene® was purchased from Toyo Ink, Tokyo, Japan. The other reagents and solvents used were of analytical grade.

2.2. Preparation of chitosan–pCMV-Luc complex solution

The aqueous chitosan–pCMV-Luc complex solution for the stability study was prepared by admixing pCMV-Luc (120 μg) and chitosan (283 μg) in 2 mL of purified water to make an N/P ratio 5 (Table 1). The N/P ratios were calculated on the basis of chitosan nitrogen per pCMV-Luc phosphate. For the preparation of the gene powder, the same amounts of pCMV-Luc (120 μg) and chitosan (283 μg) were admixed in 0.4 mL of water containing 60 mg of mannitol and left for 30 min at room temperature before injection.

2.3. Preparation of chitosan–pCMV-Luc complex powder with supercritical carbon dioxide

The apparatus for preparation of the chitosan–pCMV-Luc complex powder (Fig. 1) was composed of two CO_2 pumps (SCF-Get, the maximum flow rate was 10 mL/min each), a modifier pump (PU-1580), an aqueous solution pump (PU-1580), an oven (GC353B, GL Sciences Inc., Tokyo, Japan), and a back pressure regulator (SCF-Bpg, the pressure regulation range was 1–50 MPa), which were manufactured and assembled by JASCO Co., Tokyo, Japan, except for the oven. The dry powder preparation vessel had a unique V-shaped nozzle designed by us (Fig. 1). The CO_2 flowed at a rate of 5.7 g/min and was admixed with ethanol flowing at a rate of 0.665 mL/min, which enabled water to be miscible with the non-polar CO_2, in the mixing column. The admixture flowed into the particle formation vessel (2.0 cm i.d. and 14 cm height; 35 °C and 15 MPa) through one end of the V-shaped nozzle. Water flowed into the particle formation vessel at a rate of 0.035 mL/min through the other end of the V-shaped nozzle. Since water is not soluble in pure supercritical CO_2, ethanol, which is miscible with water as well as supercritical CO_2, was added as a modifier. The CO_2/ethanol/water system in the present study made a single phase vapor region (Okamoto et al., 2003). The aqueous chitosan–pCMV-Luc complex solution

Table 1
pCMV-Luc powders prepared with supercritical carbon dioxide

Formulation	Formulation	Formulation composition (mg)			Yield (%)[a]	Mean particle diameter (μm)
		Mannitol	pCMV-Luc	Chitosan		
SL0	Solution (2 mL)	0	0.120	0		
SL5	Solution (2 mL)	0	0.120	0.283		
DP0	Powder[b]	60.0	0.120	0	71.1	12.9
DP5	Powder[b]	59.6	0.120	0.283	72.5	17.4

[a] Yield = (amount of powder recovered from the particle formation vessel)/(amount of mannitol, pCMV-Luc, and/or chitosan injected in the vessel) × 100.

[b] Nominal amounts of ingredients dissolved in 400 μL of water injected in the supercritical carbon dioxide.

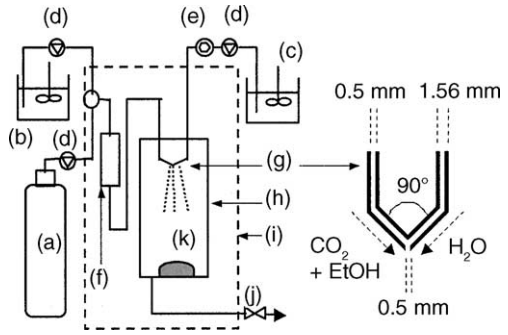

Fig. 1. Schematic diagram of the apparatus for preparation of the chitosan–pCMV-Luc complex powder with supercritical carbon dioxide: (a) CO_2 cylinder; (b) ethanol; (c) water; (d) pump; (e) manual injector for aqueous pCMV-Luc solution with or without chitosan; (f) mixing column; (g) V-shaped nozzle; (h) particle formation vessel; (i) oven; (j) back pressure regulator; and (k) product. The V-shaped nozzle has an outer diameter of 1.56 mm and an inner diameter of 0.5 mm.

(0.4 mL) was injected into the water stream through a manual injector. Half an hour after the injection, the flow of water and ethanol was stopped and the CO_2 flow was continued for additional 90 min to completely remove the water and ethanol in the vessel. The dry powder was harvested from the depressurized vessel. The formulation compositions are listed in Table 1.

2.4. Determination of pCMV-Luc integrity by electrophoresis

The powder was dissolved in an aqueous sodium dodecylsulfate (SDS) solution. The sample solution containing 0.06 μg of pCMV-Luc and 0.33% SDS was loaded on an 0.6% agarose gel containing ethidium-bromide. Electrophoresis was carried out with a current of 100 V for 2 h in TAE running buffer. A λ *Hin*dIII digest was used as a molecular mass marker.

2.5. In vivo pulmonary transfection study in mice

All the animal studies were done in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by Faculty of Pharmacy, Meijo University. Female ICR mice weighing about 20 g were anesthetized with pentobarbital (50 mg/kg, i.p.) and secured on their backs on a board during the experiments. The trachea was exposed and a 3.0 cm length of PE-60 polyethylene tubing was inserted to a depth of 1.0 cm through an incision made between the fifth or sixth tracheal rings caudal to the thyroid cartilage.

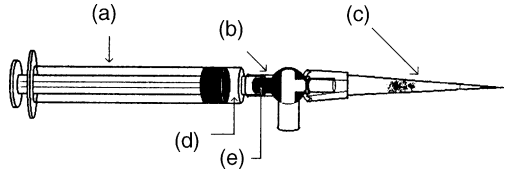

Fig. 2. Apparatus for pulmonary administration of dry powder: (a) 1 mL syringe; (b) three-way stopcock; (c) disposable tip with dry powder; (d) compressed air; and (e) administration handle.

For intratracheal administration of the solution, 50 μL of the solution SL0 or SL5 (Table 1) containing 3 μg of pCMV-Luc in a microsyringe was administered into the mouse trachea.

We administered the pCMV-Luc powders through the mouse trachea using an appropriate apparatus for mice (Fig. 2). The powder DP0 or DP5 (1.5 mg) containing 3 μg of pCMV-Luc was put in a disposable tip and dispersed in the mouse trachea by releasing air (0.25 mL) compressed in a syringe by opening a three-way stopcock connecting the disposable tip and the syringe.

Our previous study demonstrated that gene solution and powder maximized the luciferase activity in the lungs at 6 and 9 h after administration, respectively (Okamoto et al., 2003). The mice were therefore sacrificed 6 and 9 h after administration of the solution and powder, respectively, by exsanguination from the aorta in the present study. The lung was carefully removed and washed twice with ice-cold PBS and homogenized with lysis buffer. The lysis buffer consisted of 0.1 M Tris/HCl buffer (pH 7.8) containing 0.05% Triton X-100 and 2 mM EDTA. The volume of the lysis buffer added was 4 mL/g tissue. After three cycles of freezing and thawing, the homogenate was centrifuged at 15,000 rpm for 5 min at 4 °C. Twenty microliters of each supernatant were subjected to the luciferase assay with PicaGene® and the light produced was immediately measured using a luminometer (Lumat LB 9507, EG & G Berthold, Bad Wildbad, Germany) for 10 s.

2.6. Stability study protocol

Two milligrams of gene powders DP0 and DP5 were weighed in 0.6 mL microtubes and covered with Kimwipe. Four-hundred-microliter aliquots of gene

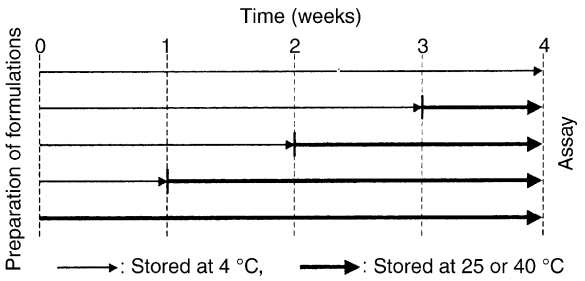

Fig. 3. Stability study schedule.

solutions SL0 and SL5 were dispensed in 0.6 mL microtubes and the caps were closed. The samples tubes were stored at 4 °C in the dark. At 0, 1, 2, and 3 weeks, thereafter, the sample tubes were transferred into chambers containing silica gel at 25 and 40 °C. The stability of the gene was examined 4 weeks after the preparation of the samples by electrophoresis (Fig. 3). Twenty milligrams of gene powders and 800 μL aliquots of gene solutions were stored at 25 and 40 °C for 4 weeks, and were then used in the in vivo pulmonary transfection study in mice to investigate the gene expression.

2.7. Effect of pulverization on the stability of a gene in powder

Seventy milligrams of the gene powder DP5 was ground with a pestle and mortar for 1 or 3 min at 27.5 °C. The stability of the gene was examined by electrophoresis.

2.8. Statistical analysis

Statistical significance of differences in luciferase activity were examined using one-way analysis of variance (ANOVA) followed by least significant difference test. The significance level was set at $p < 0.05$.

3. Results

3.1. Integrity of a gene in powders and solutions

The chitosan–pDNA complex powders DP0 and DP5 prepared by the supercritical CO_2 process with a V-shaped nozzle were of rectangular shape with the short axis less than 10 μm and long axis exceeding 10 μm (data not shown), as shown in a previous report (Okamoto et al., 2003).

The ternary structure of pCMV-Luc stored at 25 or 40 °C was examined by electrophoresis for 4 weeks (Fig. 4). The amount of supercoiled DNA in powders (DP0(0w) and DP5(0w)) was smaller than that in solutions (SL0(0w) and SL5(0w)), suggesting that the integrity of pCMV-Luc was reduced by the supercritical CO_2 process. The addition of chitosan suppressed the degradation of pCMV-Luc during the supercritical CO_2 process, as reported previously (Okamoto et al., 2003).

The gene solution without chitosan SL0 lost supercoiled DNA quickly at 25 °C and 40 °C. No bands were observed for the solution stored at 40 °C for 1 week, suggesting that intensive degradation of the gene occurred in the solution without the cationic vector. The addition of chitosan improved the stability of the gene in the solution at 40 °C; however, the bands disappeared after the 3-week storage.

Although the initial content of supercoiled DNA was small, the gene powders showed improved storage stability compared to the solutions. The supercoiled DNA was detectable after the 4-week storage at 25 and 40 °C. Even the powder without chitosan DP0 showed increased gene stability compared to the gene solutions SL0 and SL5. The gene powder DP5 was the most stable among the formulations examined in the present study. Almost no change in the integrity of the gene was observed for DP5 stored at 4 or −20 °C for 4 weeks (data not shown).

3.2. Transfection study

The transfection efficiency of the pCMV-Luc formulations stored at 4, 25, and 40 °C was examined by an in vivo transfection study in mice (Fig. 5). Almost no luciferase activity was found in the lungs of mice administered gene solutions SL0 and SL5 stored for 4 weeks at 25 and 40 °C, suggesting that pCMV-Luc was extensively decomposed. The pCMV-Luc powders DP0 and DP5 retained their transfection potential after the 4-week storage at 25 and 40 °C. In particular, the gene powder with chitosan DP5 showed a significantly higher transfection capability after the 4-week storage at 25 and 40 °C than freshly prepared SL0 and SL5. The luciferase activity in the lung produced by DP5

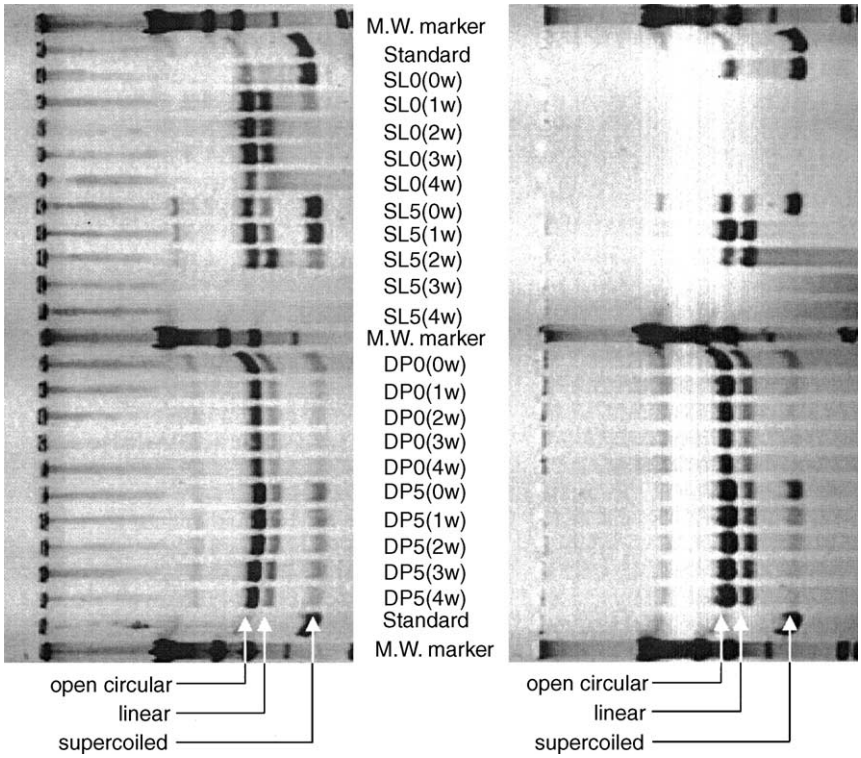

Fig. 4. The integrity of pCMV-Luc in solution without chitosan (SL0), in solution with chiotosan (SL5), in powder without chitosan (DP0), and in powder with chitosan (DP5) stored at 25 °C (left) and 40 °C (right). The numbers in the parentheses following the formulation codes represent the storage term. The standard was a freshly prepared pCMV-Luc solution.

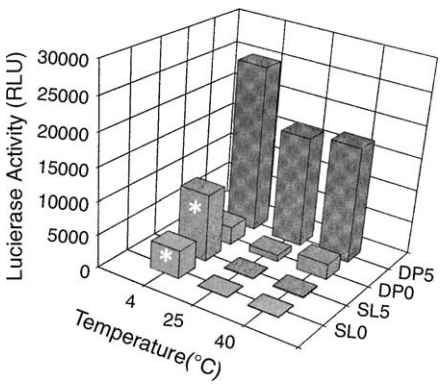

Fig. 5. The transfection efficiency of pCMV-Luc in solution without chitosan (SL0), in solution with chiotosan (SL5), in powder without chitosan (DP0), and in powder with chitosan (DP5) stored at 4, 25, and 40 °C. The formulations were stored at the indicated temperatures for 4 weeks, except for SL0 and SL5 at 4 °C (denoted by asterisk), which were freshly prepared on the day of the luciferase assay.

was significantly higher ($p < 0.05$) than those produced by SL0, SL5, and DP0 at 25 and 40 °C.

3.3. *Effect of pulverization on the stability of a gene in a powder*

To examine the tolerance of the gene powders to physical stress, the effect of pulverization on the integrity of pCMV-Luc was examined (Fig. 6). The intensity of the band of supercoiled DNA was weakened by pulverization at 27.5 °C. Grinding at 4 °C also decreased the supercoiled DNA (data not shown), suggesting that physical stress causes a gradual loss of DNA integrity.

4. Discussion

The content of supercoiled DNA in the gene powders produced by the supercritical CO_2 process was

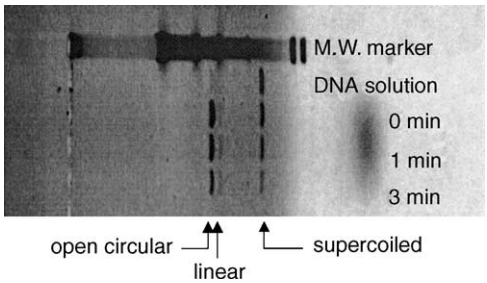

Fig. 6. The integrity of pCMV-Luc in powder with chitosan (DP5) ground with a pestle and mortar for 1 and 3 min at 27.5 °C.

decreased compared to that in the freshly prepared gene solutions, as shown in a previous study (Okamoto et al., 2003) and the present study (Fig. 4). The addition of a cationic vector, chitosan, suppressed the decomposition of DNA during the supercritical CO_2 process; however, increased amounts of open circular and linear DNAs were found in the powders immediately after the manufacturing. Although the transfection efficiency of the gene powders was much greater than that of the gene solutions, it should be determined if the increased transfection potential of the powders is retained during storage. The present study examined the stability of the gene powders from the viewpoints of the ternary structure of the DNA and in vivo transfection potential.

DNA can undergo a depurination reaction, which is often followed by β-elimination, leading to strand scission. This degradation process is associated with the conversion of supercoiled to open circular and linear forms of DNA (Cherng et al., 1999). The transfection potential of these three forms of DNA should be considered in discussing the transfection activity of gene formulations of various integrities. Niven et al. (1998) examined the expression level of CAT in mouse lung at 24 h after intravenous injection of purified open circular and supercoiled DNAs complexed with 1-[2-[9-(*z*)-octadecenoyloxy]ethyl]]-2-[8](*z*)-heptadecenyl]-3-[hydroxyethyl]imidazolinium chloride (DOTIM) and cholesterol. No significant differences were noted in expression levels between the two different forms. Voltage-pulse-mediated transfection of supercoiled and open circular DNA had nearly equal potential to transfect *E. coli*, while the linear DNA had a reduced efficiency (Kimoto and Taketo, 1996). The gene transfer efficiency of the three forms of DNA condensed with peptides possessing lysine repeats of either 18 or 36 residues was examined (Adami et al., 1998). The transfer efficiency of open circular DNA was only reduced 10% compared to that of supercoiled DNA, whereas linear DNA was nearly 90% less efficient. These findings suggest that DNA retaining its supercoiled and open circular forms is effective for transfection.

It has been reported that the stability of naked DNA in solution is low (Cherng et al., 1999; Li et al., 2000). Complexation with a cationic vector generally improves the transfection potential; however, the storage stability of genes in complexes in solution is not necessarily improved. A study of the long-term stability of a gene complexed with poly((2-dimethylamino)ethyl methacrylate) in an aqueous solution at 40 °C showed that the gene lost its transfection efficiency as well as its supercoiled and open circular forms faster than naked plasmid. The faster loss of transfection potential was also observed for a freeze-dried complex (Cherng et al., 1999). The higher degradation rate of the plasmid was attributed to the accelerating effect of the tertiary amine groups of the polymer on the depurination reaction. Similarly, in the present study, no protective effect of chitosan on pCMV-Luc in aqueous solution was obvious. Supercoiled and open circular DNAs were detected for a longer period at 40 °C with chitosan (SL5); however, these two forms in SL5 disappeared faster than those in SL0 at 25 °C.

Some studies have shown that a gene is more stable in a freeze-dried powder than in solution (Cherng et al., 1999; Li et al., 2000). Genes spray-dried with cationic lipids also have long-term stability (Seville et al., 2002). The present study revealed that the gene powder prepared by the supercritical CO_2 process also had improved stability. Chitosan protected pCMV-Luc in the powder from degradation not only during the manufacturing process but also during storage. Even at 40 °C, intense bands for supercoiled and open circular DNA and high transfection potency were observed, suggesting that ready-to-use DPI formulation would be possible with gene powder.

The gene solutions used in the present study contained mannitol, while a large part of the gene powders is composed of mannitol. It has been reported that the storage stability of a gene in solution was not affected by the addition of 10% sucrose (Cherng et al., 1999). Some studies reported that the addition of sugars improved the transfection efficiency of gene powders

prepared by freeze-drying (Cherng et al., 1997). We added mannitol in the gene powders in the present study as a diluent. There is still room for examining the effect of mannitol on the improved storage stability of the gene in the powders.

The stability studies of aged gene powders so far reported examined the transfection potential by adding the reconstituted gene solution to cultured cells or by intravenously injecting the reconstituted solution into a small animal. The present study showed that the aged powders can directly deliver the gene to lung tissue by inhalation. However, the present study did not examine the inhalation performance of powders because of the limitation of the available amounts of gene powder. We consider that the high inhalation performance of DP0 and DP5 was retained during the stability study because the aged powders could be dispersed with the apparatus shown in Fig. 2 to produce high levels of luciferase in mouse lungs. In another study in our laboratory, an insulin powder was prepared with mannitol (insulin:mannitol = 5:95 in weight) by the supercritical CO_2 process. The powder had a rectangular shape with a short axis of less than 10 μm and a long axis exceeding 10 μm, the same shape as the gene powder prepared in the present study. This suggests that the morphology of the insulin and gene powders were predominantly determined by mannitol, which accounts for a large part of the powders. The insulin powder placed at 60 °C/75% relative humidity expanded with time and was hard to disperse with the apparatus shown in Fig. 2. In contrast, the insulin powder stored under dry conditions kept its shape and was dispersable with the apparatus (Todo et al., 2004). Because the gene powders stored under dry conditions for 4 weeks in the present study were successfully dispersed with the apparatus, we concluded that the mannitol-rich powders retain their shape and inhalation performance when stored under dry conditions.

We ground the gene powders for 1 min for the in vivo pulmonary transfection study in mice because the powders manufactured by the supercritical CO_2 process were too bulky to put in the tip of the apparatus for pulmonary administration and grinding was necessary to minimize the powder volume. Pulverization of the gene powder DP5 caused a decrease in supercoiled DNA at 27.5 and 4 °C, suggesting that physical stress would deteriorate the integrity of gene in powders. The effect of physical stress on the stability of the gene powder in a presentation; for example, a capsule formulation, for market should be determined.

In conclusion, the present study showed that the powder dosage form prepared using the supercritical CO_2 process and a cationic vector such as chitosan was effective for improving the gene stability during storage. The supercritical CO_2 process decreased the supercoiled DNA during the manufacturing process; however, the decrease in the remaining supercoiled and open circular DNA in the powders during storage was much slower than that in the solutions. In addition, the powders had higher transfection potency than the solutions containing the same amount of DNA. The effect of chitosan on the stability of DNA in solution was not obvious, but chitosan improved the stability of DNA in powders during manufacturing and storage. Thus, a gene powder with a cationic vector is a promising ready-to-use formulation for inhalation therapy of pulmonary diseases.

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