Development of New Formulation Dry Powder for Pulmonary Delivery Using Amino Acids to Improve Stability

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Cationic polymers are being studied as non-viral gene delivery vectors. Poly[N-(2-aminoethyl)-2-aminoethyl]aspartamide] (PAsp(DET)) and their block copolymers with poly(ethylene glycol), PEG-PAsp(DET), have been reported as efficient biodegradable non-viral vectors which form a polyplex with plasmid DNA (pDNA). However, the polyplexes are not stable because PAsp(DET) and PEG-PAsp(DET) are easily subjected to hydrolysis; therefore, they need to be prepared on site. In this study, using the biodegradable polymers as non-viral vectors, PAsp(DET) and PEG-PAsp(DET), we investigated the effects of l-leucine (Leu) on the polyplex. We prepared solutions and dry powders with and without Leu. Both dry powders had large and porous particles and Leu acted as a dispersing agent. The transfection activity of the sample solutions decreased within a month. However, the decrease in the transfection activity was partially suppressed by the dry powder with Leu at 5 and 25°C at 3 months. Furthermore, transfection experiments revealed that Leu exhibited a pDNA-stabilizing effect in the solution and dry powder. Similar results were observed for pDNA integrity, where a polyplex was formed in the dry powder. The results suggest that Leu is a candidate stabilizer to protect pDNA from degradation.

Key words spray-freeze-drying (SFD); l-leucine; biodegradable polycation; gene transfection; plasmid DNA (pDNA)

Expectations for gene therapy with plasmid DNA (pDNA) are increasing in the world. Clinical trials of gene therapy against several intractable and lethal diseases have actively progressed. However, there are three major problems in the development of gene therapy: choice of gene vectors, stability of genes, and delivery of genes to target cells.

Firstly, viral gene vectors have a number of serious concerns regarding toxicity, although they exhibit high gene transfection activity and have been used in clinical studies. In contrast, non-viral gene vectors are safe, and recently, the development of non-viral gene delivery carriers has been highlighted with respect to their advantages of low host immunogenicity. Unfortunately, their efficiency of gene transfer and expression is generally too low for use clinically. Chitosan, a biodegradable polycation, is reportedly highly tolerable in the body. However, its transfection efficiency is lower than that of polyethyleneimine (PEI), a non-biodegradable polycation. Recently, polyplex micelles composed of cationic polymers and pDNAs are attracting much attention as novel vehicles for gene delivery. Polyplex micelles are expected as alternatives to viral vectors due to the finely tuned properties for specific applications by altering the structure of the polycation used for polyplex formation.

Kataoka et al. synthesized poly(aspartamide) derivatives with an ethylenediamine unit as a side chain (poly[N-[N-(2-aminoethyl)-2-aminoethyl]aspartamide]) (PAsp(DET)) and their block copolymers with poly(ethylene glycol) (PEG-PAsp(DET)) as novel biodegradable polycations for higher gene transfection efficiency. Polyplex micelles formed with pDNA, PAsp(DET), and PEG-PAsp(DET) exhibited effective endosomal escape properties based on the di-protonation of diamine side chains with decreasing pH, which improved their transfection efficiency with minimal toxicity, and they are promising candidates for local gene transfer.

Secondly, very few gene therapy studies involving the bench or clinic have taken the storage stability of gene formulations into consideration, because it has often been the case that the gene formulation is prepared on site directly before use. So far, many studies have employed a gene solution. However, the presence of water usually results in short-term storage, especially at room temperature. In order to improve the stability of the gene under preservation, a suitable formulation design is required. In this regard, dry powder formulations of gene are desirable. Such powder formulations may be reconstituted for injection, directly dispersed on organs during surgery, or inhaled by the patient at home.

Thirdly, pulmonary delivery is an attractive alternative route, especially to target and treat local infections of the lungs. There are three inhalation systems available for clinical application: nebulizers, pressurized metered-dose inhalers (pMDIs), and dry powder inhalers (DPIs). Among these systems, DPIs have attracted much attention due to their low cost, portable device, no need for propellant, and ease of handling. Spray drying (SD), lyophilization, and supercritical fluid precipitation have been employed for the preparation of gene powders. However, several stresses, including heating, freezing, spraying, and shear forces, must be considered to avoid destabilization of the gene or delivery system during powder production.

Spray-freeze-drying (SFD) is a relatively new technology for the production of inhalable powder drugs. SFD uses a spray nozzle, liquid nitrogen, and a lyophilizer. The drug solution is sprayed directly into liquid nitrogen through a spray nozzle. The morphology and aerodynamic particle size of powders are critical factors affecting their inhalability. In general, particles with aerodynamic diameter of 1–5 µm is considered suitable for delivery by inhalation; on the other hand, a larger geometric particle size is desirable to reduce
the adhesion cohesiveness and to prevent aggregation of tiny particles.28) According to the simplest theory for spherical particles, the mass median aerodynamic diameter (MMAD) is the product of the geometric diameter and square root of the particle density,29) indicating that MMAD of low-density particles with a large geometric diameter is still in the range suitable for inhalation. SFD method is one of the practical methods for preparing low-density particles.30)

Our previous report indicated that the addition of L-leucine (Leu) to inhalable dry gene powders with PAsp(DET) and PEG-PAsp(DET) led to a decrease in the aggregation and increase in the inhalation properties.31) However, in-vitro gene transfection efficiencies against CT26 cells of the reconstituted powders containing one of PAsp(DET) and PEG-PAsp(DET) were reduced compared to the corresponding sample solutions. Recently, Kataoka and colleagues reported superior in-vitro gene transfection efficiencies of a polyplex with a 1:1 mixture of PAsp(DET) and PEG-PAsp(DET).32) In the present study, we prepared dry powders of a polyplex with a 1:1 mixture of PAsp(DET) and PEG-PAsp(DET). We examined the stability of pDNA in the sample solutions and dry powders, and found that the pDNA in the powders was more stable than that in the solutions and that Leu possessed the ability to protect pDNA from degradation.

MATERIALS AND METHODS

Materials The pDNA encoding firefly luciferase with the CAG promoter (pCAG-Luc) was provided by RIKEN Biosource Center (Tsukuba, Japan). pCAG-Luc was amplified in the DH5 strain of Escherichia coli, and purified using an EndoFree Plasmid Giga Kit (Qiagen GmbH, Hilden, Germany). The concentration and purity of pCAG-Luc were determined by measuring UV absorption at 260 nm and the A260/A280 ratio, respectively. PAsp(DET) and PEG-PAsp(DET) were supplied by Prof. K. Kataoka, The University of Tokyo.31) The polymerization levels of PAsp(DET) and PAsp(DET) segments in PEG-PAsp(DET) were calculated as 77 and 63, respectively (Fig. 1). The molecular weight of the PEG fraction was ca. 12000. Ñ(−)-Mannitol (Man; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and Leu (Sigma-Aldrich, St. Louis, MO, U.S.A.) were used as an excipient and a dispersibility enhancer for inhalable dry powder, respectively. The other reagents and solvents used were of analytical grade.

Preparation of Sample Solutions and Dry Powders We prepared two sample solutions with and without Leu (Leu(+) and Leu(−), respectively) in the present study (Table 1). PAsp(DET) and PEG-PAsp(DET) were mixed at a 1:1 m ratio in ultra-pure water (UPW), which was further mixed with a pCAG-Luc solution to prepare polyplexes. Thirty minutes later, the polyplex solution was mixed with a Man solution with or without Leu. The total mass of each formulation was adjusted to 50mg and dissolved in 2.5 mL of UPW. The molecular ratio of amine in each polymer to phosphate in pDNA (N/P) was set as 8 according to the report by Kataoka et al. that the optimum mixing ratio of PAsp(DET) and PEG-PAsp(DET) was 1:1 and N/P was 8.32)

The dry powders were prepared by the SFD process described in Fig. 2 using the sample solutions mentioned above. The solution (2.5 mL) was flowed at a rate of 5 mL/min to atomize into liquid nitrogen using a two-fluid nozzle placed approximately 15 cm above the surface of 500 mL of liquid nitrogen. The frozen particles in liquid nitrogen were collected and lyophilized with a freeze dryer.

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<th>Table 1. Formulation of the Sample Solutions</th>
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Fig. 1. Chemical Structures of Biodegradable Polycations (A) PAsp(DET) and (B) PEG-PAsp(DET).

Fig. 2. Diagram of the SFD Setup
The sample solution was atomized into liquid nitrogen using a two-fluid nozzle placed approximately 15 cm above the surface of 500 mL of liquid nitrogen. The frozen particles in liquid nitrogen were collected and lyophilized with a freeze dryer.
been evaporated, the frozen droplets were dried at a pressure of less than 2 Pa, while the shelf temperature was gradually increased from −40 to 10°C over a period of 24 h.

The formulation names for the sample solutions and dry powders were abbreviated as “SL” and “DP” in each figure and table, respectively.

Long-Term Stability Protocol One hundred-microliter aliquots of the sample solutions were dispensed in glass vials and secured with screw caps. Two-milligram aliquots of the dry powders were weighed in open-mouth glass vials and placed in a tight container with silica gel (Fig. 3). The sample solution and dry powders were stored at 25 and 5°C for 24 months (M). The particle size, zeta-potential, in-vitro luciferase activity, and integrity of pDNA were determined at predetermined intervals. At each interval, a fresh glass vial with the sample solution or dry powder was used.

Morphologic Analysis of Powder Particles The morphology of the dry powders was observed using a scanning electron microscope (SEM; JSM-6060, JEOL, Tokyo, Japan). The dry powders were manually dispersed on a specimen mount with double-sided tape, and coated with platinum by a sputter coater (JFC-1600, JEOL, Tokyo, Japan) before observation.

Particle Size and Zeta-Potential Measurements of Polyplexes Polyplex particle size and zeta-potential measurement was performed using Zetasizer Nano-ZS (Malvern Instruments Ltd., Worcestershire, U.K.) at 25°C. The sample solutions (2 mg/100 µL) were diluted to 2 mg/mL with UPW. The dry powders were reconstituted with UPW to a concentration of 2 mg/100 µL 30 min before the measurement. Immediately before measurement, the complexes were diluted to a final concentration of 2 mg/mL with UPW. Measurements were performed in triplicate.

Integrity Assay of pDNA The dry powders were reconstituted with UPW to a concentration of 2 mg/100 µL at room temperature. Both the sample solutions (2 mg/100 µL) and reconstituted solutions were diluted to 2 mg/400 µL with UPW. Then, 60 µL of the polyplex solution was added to each well at an amount of 3 µg as pDNA. At 4 h after exposure, the OPTI-MEM® containing the polyplex in each well was replaced with new growth medium, followed by incubation for 44 h.

After removal of the medium, lysis buffer (0.05% Triton X-100, 2 mM EDTA, 0.1 M Tris, pH 7.8) was added to each well to lyse cells. Additionally, each lysate was treated with three cycles of freezing and thawing to fully lyse cells. The lysate was centrifuged at 13000×g for 7 min at 4°C to collect the supernatant. The luciferase activity and protein concentration in each supernatant were measured by the luciferase assay system Picagene® (TOYO INK Co., Ltd., Tokyo, Japan) and Bradford protein assay, respectively.

Statistical Analysis Statistical comparisons were made with Student’s t-test. The significance level was set at p<0.05.

RESULTS AND DISCUSSION

Morphologic Analysis of Powder Particles The shape and size of the dry powder prepared by SFD were observed by SEM (Fig. 4). The shape was unique to the powders prepared by SFD and suitable for pulmonary drug delivery. In general, a large and porous particle has an aerodynamic diameter as
small as several micrometers which is suitable for inhalation and a geometric diameter of more than 10 \( \mu m \) which avoids the aggregation of powders. 33) Both the SFD powders with and without Leu had numerous pores on the surface and inside. However, there was abundant aggregations found with Leu(-)DP. On the other hand, Leu(+)-DP was dispersed to each particle, suggesting that Leu acted as a dispersing agent. It has been reported that Leu reduces the surface energy of drug particles. 34,35) Yang et al. reported that the inhalation performance of spray-dried powders was improved by an increase in Leu up to 20%. 36 In the present study, we added 5% Leu to the powder, which seemed to be sufficient to improve the dispersibility of the powder.

**Long-Term Stability Protocol: Gene Expression** In our previous report, we used mouse lung metastasis models, which were prepared by the intravenous injection of CT-26 cells to mice, to examine the *in-vivo* efficacy of a chitosan-interferon-\( \beta \) gene complex powder for inhalation. 37) In the present study, thus, we selected CT26 cells in the *in-vitro* gene transfection study, expecting a future *in-vivo* efficacy study with the same mouse lung metastasis models.

*In-vitro* luciferase gene expressions of the dry powders Leu(-)DP and Leu(+)DP were higher than the sample solutions Leu(-)SL and Leu(+)-SL after a month of storage (Fig. 5A). The gene expression of the reconstituted dry powders was comparable to the freshly prepared solutions. However, that of the sample solutions was decreased, especially when stored at room temperature. PAsp(DET) and PEG-PAsp(DET) are easily decomposed by hydrolysis. 38) So, this improvement of stability may be caused by the removal of water. In addition, the gene expression of Leu(+) samples was higher than that of Leu(-) samples. This tendency was observed for both SL and DP and was the clearest for SL at 25°C. Because the luciferase activity for SL was markedly decreased in a month, we continued further stability study with Leu(-)DP and Leu(+)DP. The luciferase gene expression of DP gradually decreased with time; however, it was maintained for 3 M (Fig. 5B). The luciferase activity for DP at 25°C was less than 1% compared with the control (Fig. 5C) at 24 M. The gene expression of dry powders was higher at a lower temperature and with Leu.

We examined the effect of Leu by comparing the luciferase activity of the formulations with and without Leu stored at the same temperature and the same term. At 1 M, the addition of Leu significantly suppressed the decrease in luciferase activity of the solution and powder stored at 25°C. At 3 M, Leu was significantly effective at 25 and 5°C. On the other hand, the significant effect of Leu was not observed at 24 M, suggesting the effect of 5% Leu to stabilize pDNA lasted for 3 M in the present study. However, the luciferase activity of DP stored at 25°C was decreased to 36% of that of the freshly prepared solution. To improve the stabilizing effect of Leu, it may be necessary to increase the content of Leu to more than 5%.

**Long-Term Stability Protocol: Integrity of pDNA** To evaluate the integrity of pDNA in DP samples during storage, electrophoresis was carried out. The supercoiled and open-circular forms were detected in Leu(-) and Leu(+) stored at 25 and 5°C for 3 M; the bands for Leu(+) were thicker than for Leu(-), suggesting that the addition of Leu stabilized pDNA (Fig. 6A). However, the bands became thin at 24 M (Fig. 6B). This result was basically consistent with that of gene expression *in vitro*, suggesting that the *in-vitro* gene expression was governed by the integrity of pDNA, although we did not measure the uptake of the polyplexes by the cells which is another critical factor to determine the gene expression. Consequently, these results suggest that Leu acted as a stabilizer for pDNA.

**Long-Term Stability Protocol: Physicochemical Properties of Polyplex** The physicochemical properties of a polyplex such as the particle size and surface charge are important factors to determine the efficacy of gene transfection. 39) The
surface charge and particle size of polyplexes in the sample solutions and reconstituted dry powders were compared to examine the effects of Leu, the formulation, and storage term on them (Figs. 7, 8).

All of the zeta-potential values were close to neutral for the first 1 M (Figs. 7A, B). This means that a core-shell type of polyplex micelle was formed, the surface of which was covered with PEG.31) Interestingly, the powder samples showed an increasing zeta-potential when stored for 12 M or longer (Fig. 7B), suggesting that PEG-PAsp(DET) was decomposed to release the PEG moiety, resulting in the decomposition of the core-shell type of polyplex micelle.40,41) So, the positive charge in the PAsp(DET)-formed polyplex was measured at 12 and 24 M. Leu would have no effect on the decomposition of PEG-PAsp(DET), and the increase in zeta potential was not attributable to the change in luciferase activity during storage.

Although the zeta potentials of the 4 formulations at 3 M (ca. +1 mV) were statistically lower than those initial (ca. +4 mV), we recognize that they were close to zero and do not consider that this difference was critical for the property of

Fig. 6. Integrity of pDNA in Leu(−) DP and Leu(+) DP after Powder Production by SFD
(A) Stored for 3 M and (B) 24 M.

Fig. 7. Zeta Potential of the Polyplex in (A) Sample Solutions and (B) Reconstituted Dry Powders
Each value represents the mean±S.D. (n=3). Statistical differences between Leu(−)DP and Leu(+)DP were observed at 5°C/12 M, 5°C/24 M, 25°C/12 M, and 25°C/24 M.

Fig. 8. Particle Size of the Polyplex in (A) Sample Solutions and (B) Reconstituted Dry Powders
Each value represents the mean±S.D. (n=3). Statistical differences between Leu(−)DP and Leu(+)DP were observed at 5°C/0 M, 5°C/1 M, 5°C/3 M, 5°C/12 M, 25°C/0 M, 25°C/1 M, 25°C/3 M, 25°C/12 M, and 25°C/24 M.
showed that the increase in the polyplex size in the dry powders plateaued at around 450 nm after 12 M (Fig. 8B). These results suggest that Leu may stabilize PAsp(DET) as well as pDNA, although the mechanism should be studied in future.

CONCLUSION

By the addition of Leu, the integrity of genes and in-vitro gene expression was maintained with the dry powder of the polyplex composed of biodegradable polymers PAsp(DET) and PEG-PAsp(DET), although one of the polymers, PEG-PAsp(DET), seemed to be decomposed over time. The increase in the particle size and zeta potential may not be attributable to gene expression. However, the results of gene expression and integrity suggested that Leu improves the stability of pDNA and maintains the gene expression as well as the dispersibility of the powder.

Conflict of Interest The authors declare no conflict of interest.

REFERENCES

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