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Pulmonary gene delivery of hybrid vector, lipopolyplex containing N-lauroylsarcosine, via the systemic route

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Abstract

We newly prepared lipopolyplexes containing N-lauroylsarcosine (LS) as a hybrid vector for pulmonary gene delivery via the systemic route. Lipopolyplexes were composed of polyethylenimine (PEI), N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethlylammonium chloride (DOTMA), LS and plasmid DNA (pDNA). The particle size of lipopolyplex of PEI, DOTMA and pDNA (lipopolyplex 2P-2D) was not largely changed by the addition of LS, although the addition of LS decreased the high zeta potential. Lipopolyplexes containing LS with low zeta potential showed little aggregation with erythrocytes and low cytotoxicity. In HepG2 cells, lipopolyplexes containing LS showed high transgene efficiency comparable to lipopolyplex 2P-2D. After intravenous injection of the complexes into mice, lipopolyplexes containing LS showed high transgene efficiency, comparable to lipopolyplex 2P-2D. In particular, lipopolyplexes containing LS showed extremely high transgene efficiency in the lung. As a result of the analysis to identify optimum formulations, we discovered that LS contributed to the high transgene efficiency in the lung as 76.7% of the contribution index. These results suggest that lipopolyplexes containing LS are safe and useful gene delivery vectors with lung directivity.

KEY WORDS: hybrid vector; pulmonary gene delivery; luciferase; polyethylenimine; N-lauroylsarcosine

1. Introduction

Gene delivery to the lung is being investigated for a number of inherited and acquired disorders, such as cystic fibrosis [1], emphysema [2], asthma [3] and certain types of lung cancer [4, 5], and is also being considered as a means of vaccination [6]. The feasibility of intranasal and intratracheal approaches in gene delivery to the lung has been supported by many studies [7, 8]; however, airway-directed gene delivery is not simple because the lung has evolved both physical and immunologic barriers [9]. Direct gene delivery into the lung via intranasal or intratracheal routes is likely to be more challenging in patients with diseases such as cystic fibrosis characterized by a lung pathology with thick mucus production [10, 11]. Pulmonary gene delivery after systemic administration is expected as an alternative route for plasmid DNA (pDNA) complexes.

In the previous study, we demonstrated the strong potential of a lipopolyplex (ternary complex of pDNA, cationic polymer, and cationic lipid) consisting of polyethylenimine (PEI) and N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethlylammonium chloride (DOTMA) as an efficient gene delivery vector [12]. We preliminary found that a lipoplex (complex of pDNA and cationic liposomes) containing N-lauroylsarcosine (LS), which is a low-toxic bio-degradable detergent, showed high transgene efficiency in the lung after intravenous administration. LS is readily metabolized by humans to sarcosine and corresponding fatty acids [13]. Therefore, we newly prepared a hybrid vector, lipopolyplexes composed of PEI, DOTMA and LS, for pulmonary gene delivery via the systemic route. We investigated the transgene efficiency and safety of the new hybrid vector under *in vitro* and *in vivo* conditions, and analyzed the contribution of LS molecules for transgene efficiency. Lipopolyplexes

containing LS showed markedly high transgene efficiency in the lung after intravenous administration although they showed no cytotoxicity and no aggregation with erythrocytes. We discovered for the first time that LS molecules contributed to transgene efficiency of lipopolyplexes in the lung at 76.7% of the contribution index.

2. Materials and methods

2.1. Chemicals

The structure of LS is shown in Figure 1. PEI (branched form, average molecular weight of 25,000) and LS were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). DOTMA was purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Fetal bovine serum (FBS) was obtained from Biological Industries Ltd. (Kibbutz Beit Haemek, Israel). Dulbecco's modified Eagle's medium (DMEM), antibiotics (penicillin 100 U/ml and streptomycin 100 μg/ml) and other culture reagents were obtained from GIBCO BRL (Grand Island, NY, USA). Phosphate-buffered saline (PBS) was prepared by mixing isotonic phosphate buffer (pH 7.4) with an equal volume of saline. WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-2H-tetrazolium, monosodium salt) and 1-methoxy PMS (1-methoxy-5-methylphenazinium methylsulfate) were obtained from Dojindo Laboratories (Kumamoto, Japan). All other chemicals were of reagent grade and used as obtained commercially.

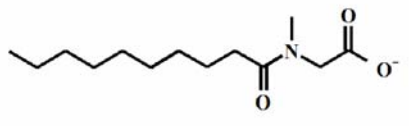

Fig. 1. Structure of LS.

2.2. Construction of pDNA

pCMV-luciferase was constructed by subcloning the HindIII/Xbal firefly luciferase

cDNA fragment from the pGL3-control vector (Promega, Madison, WI, USA) into the polylinker of the pcDNA3 vector (Invitrogen, Carlsbad, CA, USA). pDNA was amplified using a EndoFree® Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany). pDNA was dissolved in 5% dextrose solution and stored at -80 ^{o}C until analysis. The pDNA concentration was measured at 260 nm absorbance and adjusted to 1 mg/ml.

2.3. Preparation of polyplex and lipopolyplex

The theoretical charge ratio of pDNA/PEI complexes was calculated as the molar ratio of PEI nitrogen to pDNA phosphate. An appropriate amount of stock PEI solution (pH 7.4) was mixed with a diluted solution of pDNA (1 mg/ml) by pipetting thoroughly to prepare polyplexes (complex of pDNA and cationic polymer) at a charge ratio 2 (polyplex 2P) or 8 (polyplex 8P). To prepare lipopolyplexes, the theoretical charge ratio of DOTMA to pDNA was calculated as the molar ratio of DOTMA nitrogen to pDNA phosphate. The theoretical charge ratio of LS to pDNA was calculated as the molar ratio of LS carboxylate to pDNA phosphate. DOTMA ethanol solution was added to polyplex 2P at a charge ratio of DOTMA/pDNA 2 to preparing the lipopolyplex 2P-2D. LS ethanol solution was added to lipopolyplex 2P-2D at a charge ratio of LS/pDNA 2 and 4 to prepare lipopolyplex 2P-2D-2L and 2P-2D-4L.

2.4. Physicochemical properties and gel retardation of lipopolyplexes

The particle size and zeta potential of the polyplexes and lipopolyplexes were measured with a Zetasizer Nano ZS (Malvern Instruments, Ltd., UK). The 5 μl aliquots of complex solution containing 1 μl pDNA was mixed with 1 μl of loading

buffer (30% glycerol and 0.2% bromophenol blue) and was loaded onto a 0.6% agarose gel. Electrophoresis (i-Mupid J®, Cosmo Bio, Tokyo, Japan) was carried out at 50 V in running buffer solution (40 mM Tris/HCl, 40 mM acetic acid, and 1mM EDTA). The retardation of complexes was visualized with ethidium bromide staining.

2.5. In vitro gene expression experiments

The human hepatoma cell line HepG2 was obtained from the Cell Resource Center for Biomedical Research, Tohoku University. HepG2 cells were maintained in DMEM supplemented with 10% FBS and antibiotics under a humidified atmosphere of 5% CO_2 in air at 37 ^{o}C. The cells were plated on 12-well collagen-containing plates (Becton-Dickinson, Franklin Lakes, NL, USA) at a density 5.0×10^4 cells/well and cultivated in 1.5 ml DMEM supplemented with 10% FBS and antibiotics. In the transfection experiment for the complexes, after 24 h culture, the medium was replaced with 1.5 ml DMEM with FBS. Then 2 μg of pDNA as polyplex and lipopolyplexes were added to each well and incubated for 2 h. After transfection, the incubation medium was removed and the cells were cultured for a further 22 h at 37 ^{o}C with DMEM supplemented with 10% FBS and antibiotics until luciferase analysis. The transfected cells were washed with PBS and then lysed in 200 μl of lysis buffer (pH 7.8 and 0.1 M Tris/HCl buffer containing 0.05% Triton X-100 and 2 mM EDTA). The lysates were used for luciferase assay. The protein content of the lysates was also determined by a Bradford assay (Bio-Rad Protein Assay Dye Reagent Concentrate, Bio-Rad Laboratories, Inc., USA). In order to examine the influence of FBS pre-incubation on transgene efficiency, polyplex and lipopolyplexes were incubated with an equal volume of FBS for 10, 60, and 120 min before transfection.

2.6. WST-1 assay

Cytotoxicity tests of polyplexes and lipopolyplexes on HepG2 cells were carried out using a WST-1 commercially available cell proliferation reagent. The assay is based on cleavage of the tetrazolium salt WST-1 by active mitochondria to produce a soluble colored formazan salt. HepG2 cells were plated on 96-well collagen-coated plates (Becton-Dickinson, Franklin Lakes, NL, USA) at a density 5.0×10^3 cells/well and cultivated in 200 µl DMEM supplemented with 10% FBS and antibiotics. Then 0.67 µl of pDNA as polyplex and lipopolyplexes were added to each well and incubated for 2 h. After transfection, the incubation medium was removed and the cells were cultured for a further 22 h at 37 ^{o}C with DMEM supplemented with 10% FBS and antibiotics. After incubation, 10 µl of WST-1 mixture solution were added in each well and incubated for 2 h at 37 ^{o}C. The absorbance in each well was measured at 450 nm wavelength with a reference wavelength of 630 nm, using a microplate reader (Multiskan Spectrum, Thermo Fisher Scientific, Inc., USA). The results are shown as a percentage of untreated cells (control). Commercially available lipofectin was also used at a charge ratio 1.75 to compare with the lipopolyplexes.

2.7. Erythrocytes aggregation assay

Male ddY mice (5–6 weeks old) were purchased from Japan SLC (Shizuoka, Japan). Erythrocyte suspension was prepared from ddY mice by centrifugation at 4 ^{o}C and 3,000 × *g* (Kubota 3700, Kubota, Tokyo, Japan) for 15 min and washed three times with PBS. The 2% erythrocytes suspension (20 µl) was incubated with an equal

volume of polyplex and lipopolyplexes containing 2 μg of pDNA for 30 min. Aggregation was observed by optical microscopy (400 × magnification).

2.8. In vivo gene expression experiments

Male ddY mice (5–6 weeks old) were purchased from Japan SLC (Shizuoka, Japan). Animal care and experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of Nagasaki University with approval from the Institutional Animal Care and Use Committee. Polyplex and lipopolyplexes containing 40 μg of pDNA were injected into the tail vein of mice (200 μl per mouse), which were sacrificed 6 h after injection. The liver, kidney, spleen, heart, and lung were dissected and homogenized with lysis buffer using a homogenizer (Omni TH-115, Yamato Scientific Co., Ltd., Tokyo, Japan). After centrifugation of the homogenates at 21,880 × *g* for 5 min, the supernatants were used for luciferase assay.

2.9. Luciferase assay

A 10 μl lysate sample was mixed with 50 μl of luciferase assay buffer (Picagene, Toyo Ink, Tokyo, Japan) and the light produced was immediately measured using a luminometer (Lumat LB 9507, EG & G Berthold, Bad Wildbad, Germany). Luciferase activity was indicated as relative light units (RLU) per mg protein for *in vitro* experiments or RLU per gram of tissue for *in vivo* experiments.

2.10. Statistical analysis

Statistical analysis was performed using Scheffe's test for multiple comparison. $p < 0.05$ was considered significant.

2.11. Data analysis for optimal formulation

The optimum formulation was estimated using a simultaneous optimization technique in which a multivariate spline interpolation (MSI) was incorporated. The contribution of hybrid vector components to transgene efficiency was also estimated according to the optimization. We carried out additional *in vivo* experiments (Table 1) and added the data for optimization. A two-factor composite second-order spherical experimental design was employed to select model formulations. The data measured for model formulations were analyzed with a computer program, dataNESIA (Yamatake Corp., Tokyo, Japan). MSI has recently been recognized as a superior method for high precision modeling of multi-dimensional data points [14]. MSI is basically a boundary element method [15]. Green functions are used to interpolate the minimum curvature of multi-dimensional data points [16]. Observational data include experimental error. To avoid problems with estimation, the multi-dimensional data surface, including experimental error, is estimated as the sum of interpolation with a Green function in a linear polynomial equation (thin-plate approximation) [16]. The smoothing parameter, which is the ratio of Green function interpolation and thin-plate approximation, is automatically estimated using a generalized cross-validation technique. The optimum formulation and the contribution of hybrid vector components to transgene efficiencies were estimated based on the standardized Euclidian distance described previously [17]. As a matter of convenience for analysis, contribution ratios were not calculated in the kidney and heart.

Table 1. Number of additional *in vivo* experiments for optimization.

complex	number of sample
2P	4
2P-0.25D	2
2P-1D	3
2P-4D	6
2P-4D-8L	6
8P-0.25D	1
8P-0.5D	1
8P-1D	1
8P-2D	5
8P-4D	1
4P	4
4P-1D	3
4P-2D	4
15P	3
1D	1
2D	1
4D	1

3. Results

3.1. Physicochemical properties of lipopolyplexes

The particle size and zeta potential of lipopolyplexes containing LS were measured with Zetasizer Nano ZS and are shown in Table 2. Data of polyplex 2P and lipopolyplex 2P-2D were reported in the previous paper [12]. The addition of cationic lipid, DOTMA, to polyplex 2P decreased the particle size and increased the zeta potential. The particle size of lipopolyplex 2P-2D was not affected by the addition of LS at 2 and 4 charge ratio, although the zeta potential decreased to 35.7 mV of lipopolyplex 2P-2D-2L and 21.5 mV of lipopolyplex 2P-2D-4L. The zeta potential of lipopolyplex 2P-2D-4L was almost equal to that of polyplex 2P.

The gel retardation assay was performed to examine complex formations of the polyplex and lipopolyplexes and is shown in Fig. 2. Lipopolyplex 2P-2D, 2P-2D-2L, and 2P-2D-4L were found to tightly hold pDNA.

Table 2. Particle size and zeta potential of polyplex and lipopolyplexes

Complex	Particle size (nm)	Zeta potential (mV)
Polyplex 2P	262.7 ± 86.0 *	23.7 ± 0.6 **
Lipopolyplex 2P-2D	73.9 ± 26.8	54.8 ± 0.8
Lipopolyplex 2P-2D-2L	73.5 ± 31.9	35.7 ± 1.9 **
Lipopolyplex 2P-2D-4L	63.7 ± 19.2	21.5 ± 0.4 **

Each value represents the mean + S.D. (n = 3).

*: $p < 0.05$, **: $p < 0.01$ vs Lipopolyplex 2P-2D.

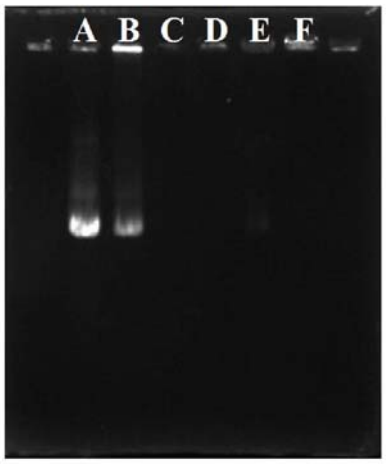

Fig. 2. Gel retardation assay was performed with electrophoresis for polyplex 2P (B), lipopolyplex 2P-2D (C), 2P-2D-2L (D), 2P-2D-4L (E), and polyplex 8P (F). Naked DNA (A) was used as a control.

3.2. In vitro transgene efficiency of lipopolyplexes

Figure 3 shows the *in vitro* transgene efficiency of lipopolyplexes containing LS in HepG2 cells and the influence of pre-incubation with FBS on efficiency. Polyplex 2P showed little transgene efficiency [12]. Polyplex 8P and lipopolyplex 2P-2D were previously reported to have high transgene efficiency [12]. Commercially available lipofectin showed high transgene efficiency (4.65×10^9 RLU/mg protein) compared to polyplex 8P and lipopolyplex 2P-2D by a similar experimental method. Transgene efficiency of lipofectin was equivalent to these of lipopolyplexes containing LS although lipofectin showed hemolysis activity and strong aggregation of erythrocytes (data not shown). The high transgene efficiency of polyplex 8P and lipopolyplex 2P-2D decreased rapidly by pre-incubation with FBS. On the other hand, lipopolyplexes containing LS showed high transgene efficiency compared to polyplex 8P and lipopolyplex 2P-2D. In particular, the high transgene efficiency of

lipopolyplex containing LS was slightly influenced by pre-incubation with FBS.

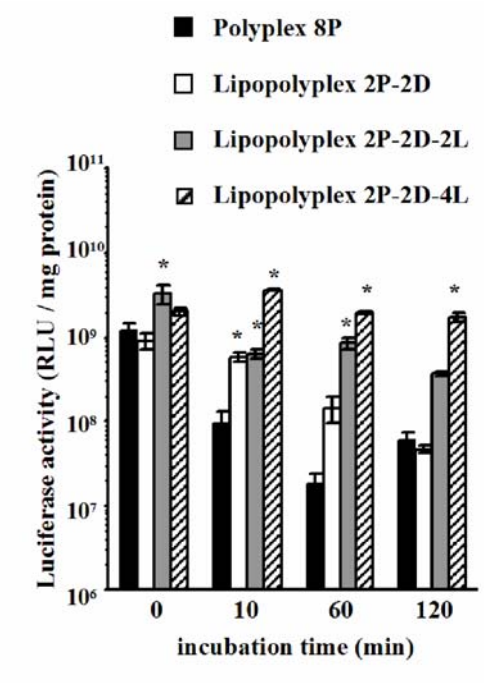

Fig. 3. Effect of pre-incubation of lipopolyplexes with FBS on their transfection efficiencies. Lipopolyplexes and polyplex 8P were incubated with an equal volume of FBS before transfection. HepG2 cells were incubated with lipopolyplexes for 2 h. At 24 h after transfection, cells were lysed for quantification of luciferase activity. The values are the means with S.D. (n= 3). *: $p < 0.05$ vs polyplex 8P.

3.3. Cytotoxicity of lipopolyplexes

Cytotoxicity of lipopolyplexes containing LS was determined by WST-1 assay in the presence of FBS and is shown in Fig. 4. Polyplex 2P, 8P and lipopolyplex 2P-2D, 2P-2D-2L and 2P-2D-4L did not show any significant cytotoxicity. In the preliminary experiment, commercially available lipofectin showed 39.8% of cell viability. The

viability of cells treated with polyplexes and lipopolyplexes was more than 90% of control cells.

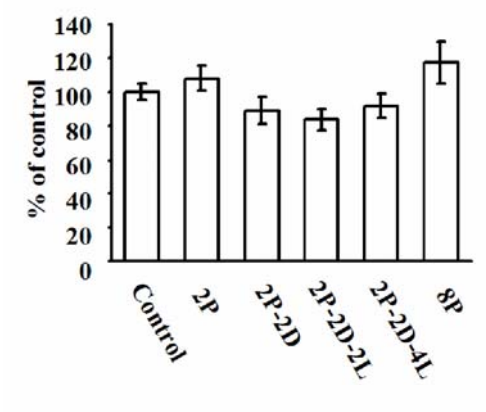

Fig. 4. Cytotoxicity of polyplexes and lipopolyplexes. Cell viability of HepG2 cells treated with complexes was measured by WST-1 assay. Cells were incubated with complexes in the presence of FBS for 2 h and cell viability was measured at 24 h after transfection. The data represent the percentage to untreated cells. Each value represents the mean with S.E. (n=8).

3.4. Erythrocyte aggregation assay

Erythrocyte aggregation assay was performed to examine the interaction of polyplexes and lipopolyplexes with mouse erythrocytes and the results are shown in Fig. 5. Polyplex 2P had slight aggregation with erythrocytes (Fig. 5A) and partial aggregation was found in lipopolyplex 2P-2D (Fig. 5B). Polyplex 8P showed strong aggregation (data not shown). In contrast, lipopolyplexes containing LS did not show any aggregation (Fig. 5C, D).

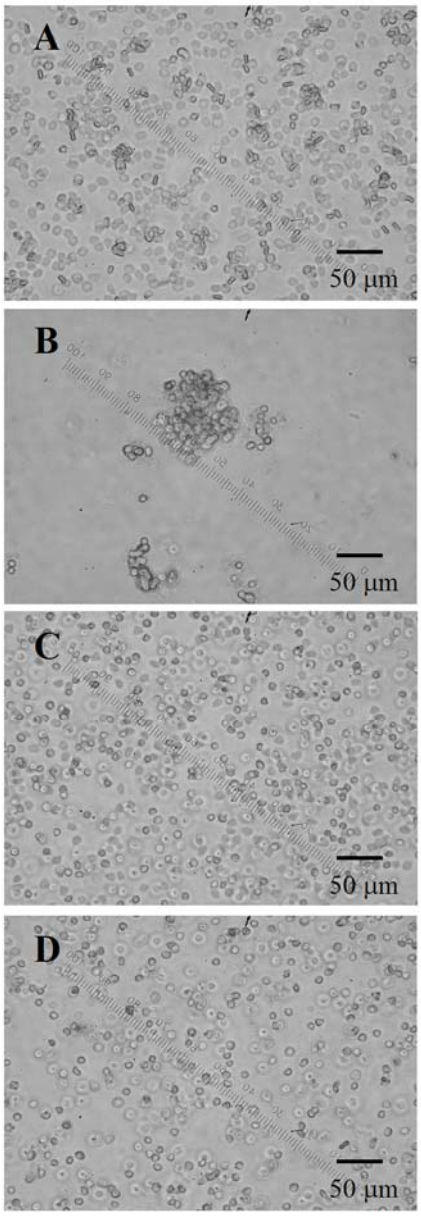

Fig. 5. Erythrocyte aggregation induced by the polyplex 2P (A) and the lipopolyplex 2P-2D (B), 2P-2D-2L (C) and 2P-2D-4L (D). The 20 µl sample of erythrocyte suspension was incubated with 20 µl of polyplex and lipopolyplexes containing 2 µg of pDNA for 30 min.

3.5. In vivo transgene efficiency of polyplexes and lipopolyplexes

In vivo transgene efficiency of lipopolyplexes containing LS were examined in ddY male mice (Fig. 6). At 6 h after intravenous injection of lipopolyplexes into mice, luciferase activities were measured in several tissues. In the previous study, we reported that polyplex 8P and lipopolyplex 2P-2D showed high transgene efficiency, as high as lipofectin, in the spleen, liver, and lung, although little transgene efficiency was observed by polyplex 2P [12]. Lipopolyplexes containing LS showed high transgene efficiency compared to lipopolyplex 2P-2D. In particular, in the lung, lipopolyplex 2P-2D-2L was found to have significantly higher expression than lipopolyplex 2P-2D.

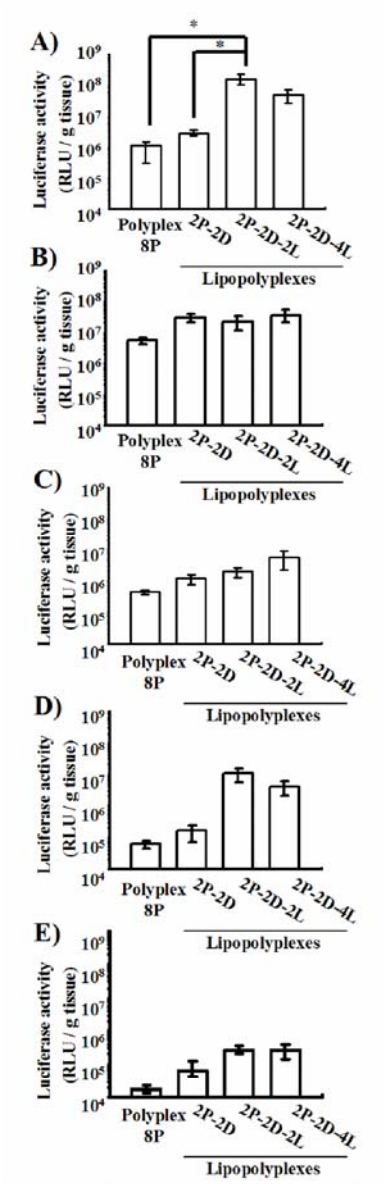

Fig. 6. *In vivo* transgene efficiencies of polyplex and lipopolyplexes in the lung (A), spleen (B), liver (C), heart (D), and kidney (E). Polyplex and lipopolyplexes were injected intravenously via the tail vein in mice (40 mg DNA per mouse). At 6 h after administration, mice were sacrificed and each organ was dissected for quantification of luciferase activity. Each value represents the mean with S.E. (n = 3- 7). *: $p < 0.05$ vs polyplex 8P.

3.6. Formulation optimization

Based on the *in vivo* results, we estimated the optimum formulation of lipopolyplexes with high transgene efficiency as a pharmaceutical formulation. In this case, the study was performed using model formulations prepared according to the two-factor spherical second-order composite experimental design. We carried out additional *in vivo* experiments and added data for optimization. The data measured for model formulations were analyzed with a computer program, dataNESIA. MSI has recently been recognized as a superior method for the high precision modeling of multi-dimensional data points [14]. The three-dimensional diagram of the contribution ratio to the transgene efficiency in the lung is shown in Fig. 7. The optimal ratios of pDNA, PEI, DOTMA, and LS as lipopolyplex were 1.0: 11.2: 2.1: 7.5 for pulmonary transgene efficiency. At that time, the contributions of PEI, DOTMA, and LS to transgene efficiency were 15.3%, 8.1%, and 76.7%, respectively. On the other hand, the optimal ratios of pDNA, PEI, DOTMA, and LS as lipopolyplex were 1.0: 12.9: 3.0: 5.8 for hepatic transgene efficiency, and 1.0: 13.4: 2.9: 5.6 for splenic transgene efficiency. The contributions of PEI, DOTMA, and LS to transgene efficiency were 50.3%, 32.0%, and 17.7% in the liver, and 63.0%, 25.1%, and 11.9% in the spleen, respectively. PEI showed the highest contribution ratio in the liver and spleen. Data of the kidney and heart were removed from the analysis because the absolute values are too small and the varieties are too large to calculate an accurate contribution index.

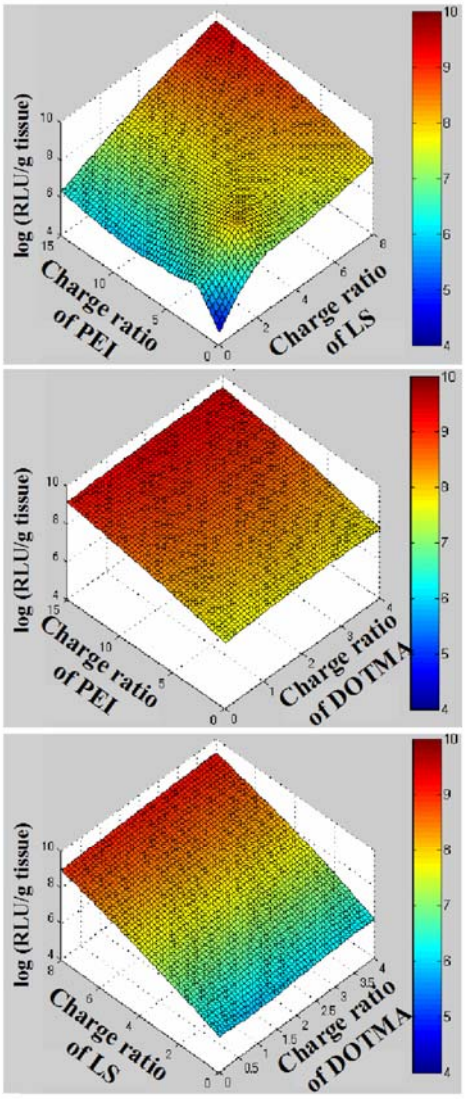

Fig. 7. Response surface of the effect of charge ratio of PEI, DOTMA, and LS on transgene efficiencies in the lung.

4. Discussion

The success of gene therapy highly depends on the development of an effective and secure delivery vector. The viral vector is highly effective and is employed for clinical trials although some severe adverse events in clinical trials have given great concern regarding its safety [18]. On the other hand, non-viral vectors have safety advantages; much less immunotoxicity, clear structure, and easy modeling [19, 20]. The formation of pDNA-vector complex is an important factor contributing to transgene efficiency. Two major formations of non-viral vector are polyplex and lipoplex, which are effective for gene delivery in several cell lines and *in vivo* experiments [21-23]. Recently, a ternary complex of cationic polymer, cationic lipid, and pDNA, named a lipopolyplex, has been developed as a second generation non-viral vector. Lipopolyplexes have shown extremely high transgene efficiency by the synergism of cationic polymers and lipid [24-27].

In the previous study, we investigated the strong potential of lipopolyplex consisting of PEI and DOTMA for efficient gene delivery [12]. We preliminary found that lipoplex containing LS had high transgene efficiency in the lung after intravenous administration. Based on these results, we newly prepared a hybrid vector, lipopolyplexes composed of PEI, DOTMA and LS, for pulmonary gene delivery via the systemic route.

We already reported that the particle size of polyplex 2P was remarkably reduced by DOTMA addition, indicating strong pDNA compaction. In the previous study, we added DOTMA to polyplexes and did not find any liposomal shape by transmission electron microscopy [12]. The particle size of complexes is an important factor to control cellular uptake because small particles should more easily enter cells via an

endocytosis pathway than larger particles. The particle size of the lipopolyplex was not greatly changed by the addition of LS, which has a negative charge. On the other hand, the high zeta potential of lipopolyplex 2P-2D gradually decreased by the addition of LS and reached about 20 mV with lipopolyplex 2P-2D-4L, similar to that of polyplex 2P. The low zeta potential of lipopolyplexes containing LS may decrease their interaction with erythrocytes, blood components and cell membranes. Significant aggregation was observed at lipopolyplex 2P-2D, which has high zeta potential. Lipopolyplexes containing LS with low zeta potential, however, were not found to aggregate erythrocytes. The slight erythrocyte aggregation of lipopolyplexes containing LS supports their low hematotoxicity and safe use *in vivo* experiments.

HepG2 cells have been often used for examining the basic *in vitro* transgene efficiency of non-viral vectors [26, 27]. Therefore, we compare the *in vitro* transgene efficiency of polyplexes and lipopolyplexes in the HepG2 cells. Lipopolyplexes containing LS showed high transgene efficiency in HepG2 cells. Polyplex 8P and lipopolyplex 2P-2D, which have high zeta potential, showed high transgene efficiency and polyplex 2P, which has low zeta potential, showed little transgene efficiency. We found that lipopolyplexes containing LS showed high transgene efficiency compared to polyplex 8P and lipopolyplex 2P-2D, regardless of their low zeta potential. In particular, lipopolyplex 2P-2D-2L showed significantly higher transgene efficiency than polyplex 8P. The lipopolyplex 2P-2D-2L also showed equivalent transgene efficiency with lower cytotoxicity comparing to commercial available non-viral vector, lipofectin. These results might be concerned with the membrane fusion characteristics of LS molecules. The high transgene efficiency of lipopolyplexes containing LS was confirmed in the preliminary experiment of the lung cell line A549 (data not shown).

The transgene efficiency of cationic vectors was reported to be decreased by fetal

calf serum [28, 29]. We then evaluated an effect of pre-incubation with FBS on transgene efficiency of polyplex and lipopolyplexes. We found that pre-incubation of polyplex 8P and lipopolyplex 2P-2D with FBS prior to transfection resulted in a time-dependent decrease of transgene efficiency, although transgene efficiency of lipopolyplexes containing LS were less affected by pre-incubation with FBS. The serum-resistant activity of lipopolyplexes containing LS would be useful in their clinical use. These results may lower the interaction of lipopolyplexes containing LS with blood components because of their low zeta potential. Polyplexes and lipopolyplexes showed lower cytotoxicity than lipofectin in the presence of FBS.

Based on these results, we tested the efficiency of polyplex and lipopolyplexes for *in vivo* gene delivery. After intravenous injection of the complexes into mice, luciferace activities in several tissues were determined by luminescent analysis. In spite of their low zeta potential and low toxicity, lipopolyplexes containing LS showed high transgene efficiency compared to polyplex 8P and lipopolyplex 2P-2D. In particular, in the lung, lipopolyplex 2P-2D-2L showed significantly higher transgene efficiency than polyplex 8P and lipopolyplex 2P-2D.

The gene transfection with non-viral vectors have many processes such as condensing pDNA by electrostatic interaction, binding to cell surface and taken up by endocytotic pathway, and releasing pDNA into the cytoplasm [30]. Lipopolyplexes containing LS showed the highest transgene efficiency in the lung and had high transgene efficiency in the liver and the spleen. The lipopolyplexes containing LS may have several uptake mechanisms according to organs. However, the mechanism for the lung directivity of lipopolyplexes containing LS was not clear. The LS structure was similar to that of lauric acid. As the results of the lipopolyplexes containing lauric acid, however, they showed much lower transgene efficiencies than lipopolyplex

2P-2D-2L in the preliminary *in vivo* experiment ($4.52 \times 10^6 \pm 2.75 \times 10^6$ RLU/g tissue in the lung). So, we suggest the specific uptake of lipopolyplexes containing LS by pulmonary cells via recognition of LS structure. On the other hand, the low cytotoxicity and erythrocyte aggregation of lipopolyplex containing LS must be due to low zeta potential on the particle surface by addition of LS. The complexes with low zeta potential decrease the electrostatic interaction with the cellular membrane.

In order to confirm the contribution of LS to the lung directivity, we carried out additional *in vivo* experiments (Table 1) and analyze all data for designing the optimum formulation. The optimum formulation was estimated using a simultaneous optimization technique in which MSI was incorporated. As a result of this analysis, we found that PEI and DOTMA contributed to the high transgene efficiency of the complex composed of LS, DOTMA, and PEI in the liver and spleen. In contrast, in the lung, the high transgene efficiency of this complex depended on the contribution of LS, which was 76.7% of the contribution index. These results suggest that lipopolyplexes containing LS are a useful gene delivery vector with lung directivity.

Complexes with a strong positive charge showed high transgene efficiency because of their strong interaction with cell membranes; however, the strong positive charge on the complexes aggregates with erythrocytes [31] and is eliminated rapidly from the blood. The large aggregates of lipoplexes with erythrocytes may cause clinical problems because of microinfarction, causing tissue ischemia and possible myocardial damage [32]; however, we discovered that lipopolyplexes containing LS, which showed a low positive charge, had high transgene efficiency and low toxicity under *in vitro* and *in vivo* conditions. In particular, the addition of LS to lipopolyplex largely increased transgene efficiency in the lung. Further study is necessary to examine the mechanism of their high transgene efficiency.

5. Conclusion

We newly prepared lipopolyplexes containing LS as a hybrid vector for pulmonary gene delivery via the systemic route. The lipopolyplexes containing LS had high transgene efficiency in HepG2 cells without any cytotoxicity. They also showed no aggregation with erythrocytes and markedly high transgene efficiency in the lung after their intravenous administration in mice. We discovered that LS molecules contributed to the high transgene efficiency in the lung as 76.7% of the contribution index.

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