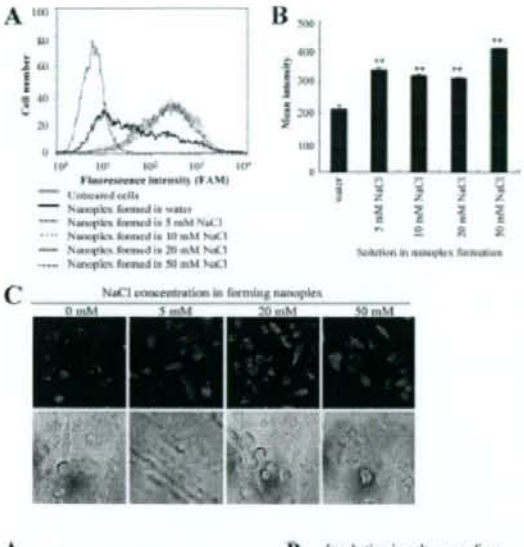

Fig. 3. Association (A, B) and Intracellular Localization (C) of NP-OH Nanoplex with PC-3 Cells 24 h after Transfection

NP-OH was mixed with 100 pmol of FAM-siRNA (A, B) or Alexa Fluor 555-labeled siRNA (C) in various concentrations of NaCl at a charge ratio (+/−) of 3/1 for 10 min, and then diluted to 100 nM with medium. The association was determined based on FAM fluorescence by flow cytometry (A, B). Each column in B represents the mean±S.D. ($n=3$). Statistical differences between different groups were analyzed with one-way analysis of variance on ranks with post-hoc Tukey–Kramer's test. $^{**}p<0.01$, compared with NP-OH nanoplex of siRNA formed in water. Alexa Fluor 555-labeled siRNA was visualized by confocal microscopy (magnification ×600) (C). Red signals show localization of Alexa Fluor 555-labeled siRNA.

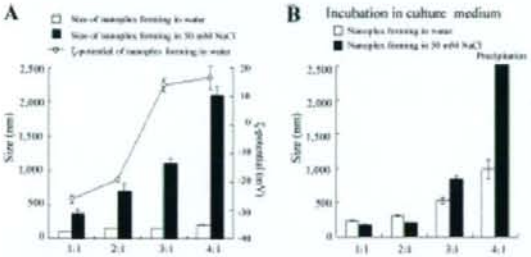

Fig. 4. Effect of Charge Ratio (+/−) on the Size and ζ-Potential of NP-OH Nanoplex (A), and Particle Size of NP-OH Nanoplexes 15 min after Incubation with Culture Medium at Room Temperature (B)

NP-OH nanoplexes were prepared by mixing siRNA with NP-OH in water or 50 mM NaCl at various ratios (+/−), and was left for 10 min. ζ-Potentials were measured after forming nanoplex in water.

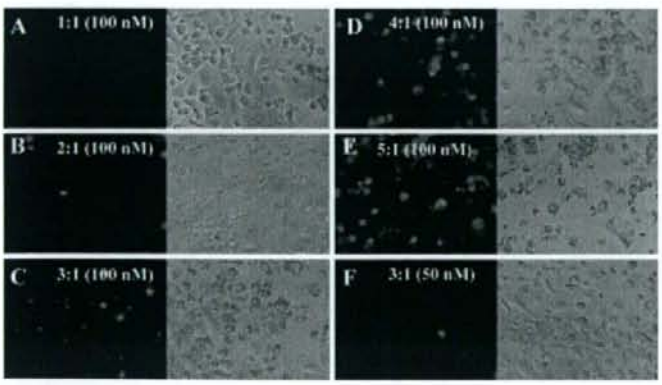

Fig. 6. Cytotoxicity of NP-OH Nanoplex Was Estimated by Staining Dead Cells with PI

NP-OH nanoplex of siRNA was prepared at various charge ratios (+/−) in 50 mM NaCl and then incubated with cells at 100 or 50 nM siRNA. Assays were performed 24 h after PC-3 cells were exposed to nanoplex.

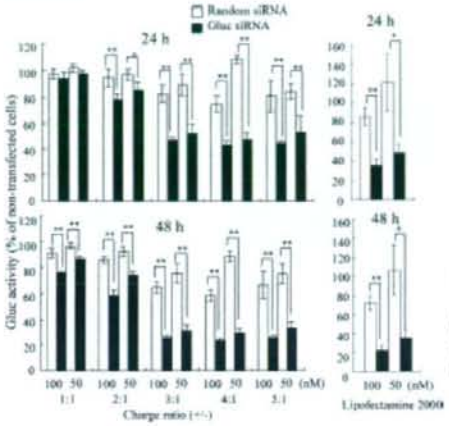

Fig. 5. The Suppression of Gluc Activity 24 and 48 h after Transfection

All NP-OH nanoplexes of Gluc or random siRNA were prepared in 50 mM NaCl and lipofectamine 2000 lipoplex was prepared according to the manufacturer's protocol. The nanoplexes and lipoplexes were incubated with PC-3-Gluc cells at 100 or 50 nM siRNA. Statistical significance was evaluated by Student's *t* test. * $p<0.05$ and ** $p<0.01$, compared with nanoplex of random siRNA. Each column represents the mean ± S.D. ($n=4$).

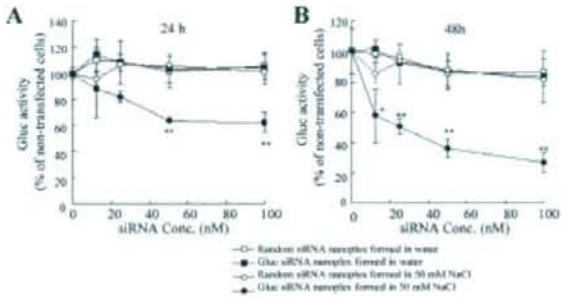

Fig. 7. Dose Dependency of siRNA-Mediated Inhibition of Gluc Activity 24 h (A) and 48 h (B) after Incubation with NP-OH Nanoplex

PC-3-Gluc cells were treated with NP-OH nanoplex of Gluc siRNA and random siRNA at a final concentration of 100, 50, 25 and 12.5 nM siRNA. Statistical differences between different groups were analyzed with one-way analysis of variance on ranks with *post-hoc* Tukey–Kramer's test. * $p<0.05$ and ** $p<0.01$, compared with nanoplex of random siRNA at each concentration. Each value represents the mean ± S.D. ($n=3$).

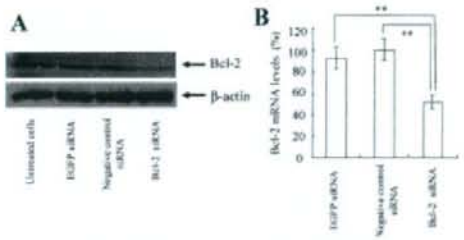

Fig. 8. Suppression of Bcl-2 Protein (A) and mRNA (B) Expression by NP-OH Nanoplex of bcl-2 siRNA

PC-3 cells were treated with NP-OH nanoplex of bcl-2, negative control or EGFP siRNA at a final concentration of 100 nM siRNA. The level of bcl-2 protein or β-actin was evaluated by Western blotting 48 h after incubation (A). Relative amount of bcl-2 mRNA in the cells was measured by an SYBR Green I-based quantitative PCR analysis (B). The y-axis indicated bcl-2 expression (%) for negative control siRNA-transfected cells. The expression level of bcl-2 mRNA was normalized for the amount of β-actin in the same sample. Each result represents the mean ± S.D. ($n=3$). ** $p<0.01$, compared with bcl-2 siRNA-transfected cells.

Finally, to investigate whether the suppression of bcl-2 expression decreased tumor cell viability, we examined cell viability 48 h after transfection of bcl-2 siRNA using a colorimetric viability assay. We used commercially available lipofectamine 2000 as the control of the transfection reagent. Cell viability significantly decreased at 50 and 100 nM siRNA in PC-3 cells 48 h after transfection by NP-OH nanoplex of bcl-2 siRNA, compared with that of the negative control or EGFP siRNA (Figs. 9A, C—F). The levels of cell viability by NP-OH nanoplex were comparable to that by lipofectamine 2000 (Fig. 9B).

DISCUSSION

In this study, to apply NP-OH for synthetic siRNA transfection, we optimized the charge ratio (+/−) of NP-OH/siRNA and nanoplex-forming solution, and evaluated the transfection efficiency into PC-3 cells. Positively charged

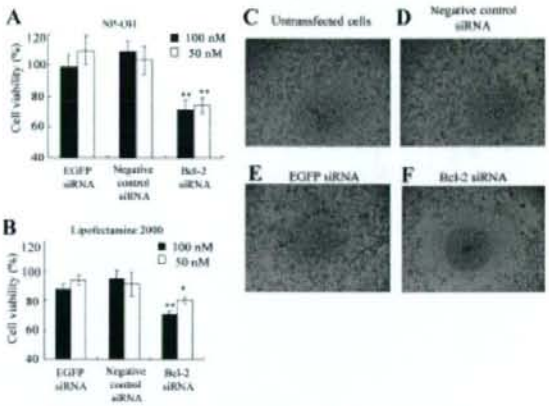

Fig. 9. Antiproliferative Activity 48 h after Transfection of NP-OH Nanoplex of bcl-2 siRNA into PC-3 Cells

PC-3 cells were treated with NP-OH nanoplex (A) or Lipofectamine 2000 lipoplex (B) of bcl-2 siRNA, random and EGFP siRNA as negative controls at a final concentration of 100 and 50 nM siRNA. Each column represents the mean ± S.D. ($n=4$). Statistical differences between different groups were analyzed with one-way analysis of variance on ranks with *post-hoc* Tukey–Kramer's test. $^{**}p<0.01$ and $^{*}p<0.05$, compared with nanoplex of the negative control siRNA. Photomicrographs of PC-3 cells transfected with NP-OH nanoplex of negative control (D), EGFP (E) and bcl-2 siRNAs (F) 48 h after transfection. Magnification ×200.

NP-OH nanoplex prepared in the presence of sodium chloride exhibited efficient siRNA transfer. Furthermore, NP-OH nanoplex of bcl-2 siRNA significantly inhibited tumor growth compared with that of negative control siRNA, and the efficacy was comparable to commercial products.

In the association of NP-OH with siRNA, a charge ratio (+/−) above 3/1 was needed for complete complex formation of NP-OH/siRNA. We previously reported that the complete nanoplex formation of NP-OH/pDNA was observed at a charge ratio (+/−) above 2/1.[19] Both pDNA and siRNA have phosphodiester backbones with negative charge and interact electrostatically with cationic liposomes or nanoparticles; however, pDNA and siRNA are different from each other in molecular weight and topography.[13] These findings suggested that a more amount of NP-OH is needed for binding to siRNA than pDNA; therefore, the optimized charge ratio (+/−) for synthetic siRNA delivery might be different from that for pDNA delivery.

To clarify the effect of the nanoplex-forming condition in water or NaCl solution on transfection efficiency, we examined the cellular association of nanoplexes. The cellular association of NP-OH nanoplex and the suppression of Gluc activity by NP-OH nanoplex were significantly increased when NP-OH nanoplex of siRNA was formed in NaCl solution compared with in water (Figs. 2A, 3A—C). This observation was similar with that of pDNA transfection by NP-OH.[19] Usually, nanoplex is prepared in water or glucose solution because nanoplexes form aggregates in the presence of ionic solutions such as saline; however, the nanoplex formed in water is exposed to ionic solution such as culture medium and subsequently increased its size. Controlling the size of the nanoplex after exposure to ionic solution is important for efficient gene transfer. The presence of NaCl during nanoplex formation can regulate the repulsion between cationic nanoplexes.[23] Moderate neutralization of the positive charge on the surface of the cationic nanoplex by a suitable concentration of NaCl can ensure sufficient repulsion of the nanoplex and prevent the aggregation of nanoplexes in ionic solution (Fig. 4B). The stabilized effect of nanoplex in ionic solution might increase cell association and transfection efficiency.

In pDNA delivery by NP-OH transfection at a charge ratio (+/−) of 3/1, NP-OH nanoplexes of pDNA formed in both water and NaCl solution could induce luciferase expression in the cells, in which NP-OH nanoplex forming in NaCl solution increased 2-fold higher than that in water.[19] In contrast, in synthetic siRNA delivery, the nanoplex forming in NaCl solution exhibited marked RNAi activity, but that formed in water did not. The difference of transfection activity by forming NP-OH nanoplex in water between synthetic siRNA and pDNA might be due to synthetic siRNA being tightly bound to NP-OH compared with pDNA. We previously reported a marked dissociation of ODN from NP-OH nanoplex at low pH when NP-OH nanoplex of ODN was formed in NaCl solution, but slightly dissociation when the nanoplex was formed in water.[24] The presence of NaCl in forming nanoplex can weaken the association with ODN by neutralization of cationic charge on the surface of NP-OH. The high transfection efficiency of the nanoplex produced in NaCl solution may result in siRNA release from the nanoplex and the translocation of siRNA into the cytoplasm. This was supported by the distribution of siRNA being strongly observed both in the cytoplasm and nucleus using NP-OH nanoplex formed in the presence of NaCl. The intracellular localization of siRNA after transfection is crucial for its successful function as an inductor of the RNAi process; however, pDNA complexed with particles could be transferred into the nucleus for transgene expression. Thus, nanoplex formation in NaCl solution might influence the extent of siRNA dissociation from the nanoplex in cytoplasm.

Here, we used random, EGFP and negative control siRNAs as negative controls. The negative control and EGFP siRNAs had no apparent effect on cell growth (Fig. 9A), but the random siRNA showed slight suppression of luciferase activity with increasing of charge ratio (+/−) (Fig. 5). Random siRNA might induce changes in luciferase expression by a target-independent fashion (off-target effect).[25]

Many parameters including the nanoplex size and charge ratio (+/−) are known to affect transfection efficiency.[26,27] *In vitro* transfection activity increased in parallel with ζ-potential and the size of nanoplex.[27,28] In pDNA transfection, large lipoplexes over 700 nm in mean diameter prepared in NaCl solution induced efficient transfection; in contrast, lipoplexes of less than 250 nm were inefficient.[29] Positively charged lipoplex could easily associate with the negatively charged surface of the cellular membrane.[28] These findings suggested that efficient siRNA transfer of NP-OH might depend on the charge ratio (+/−) and/or size of the nanoplex.

The promise of specific RNA degradation has developed much excitement for possible use as therapy, so thus several studies have reported synthetic siRNA therapy for treating tumors. Tumor cell-specific targets such as Her-2, EGFR, Bcl-2, EphA2 and others have been selected as attractive gene targets for therapeutic intervention.[30,31] In this study, we used synthetic siRNA for bcl-2 mRNA as a therapeutic gene. In the transfection of bcl-2 siRNA, cell growth was significantly suppressed by transfection of the NP-OH nanoplex (Fig. 9A), and its activity was comparable to commercially available lipofectamine 2000 (Fig. 9B). Thus, NP-OH is appropriate for synthetic siRNA delivery to the cells.

In this study, we applied OH-Chol-based cationic nanoparticles (NP-OH) for synthetic siRNA delivery vector and optimized a charge ratio (+/−) and nanoplex-forming solution for efficient transfection. Transfection resulted in efficient siRNA transfer when positive-charged nanoplex was prepared in the presence of 50 mM NaCl. The results provided optimal conditions to form NP-OH nanoplex for synthetic siRNA delivery.

Acknowlegement We thank Mr. Motoki Hakoshima for assistance in the experimental work. This project was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by the Open Research Center Project.

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Calcium enhanced delivery of tetraarginine-PEG-lipid-coated DNA/protamine complexes

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ABSTRACT

As we have previously reported the delivery of plasmid DNA (DNA) complexed with oligoarginine-PEG artificial lipids (oligoarginine/DNA complexes), we focused on tetra- and decaarginine (Arg4, Arg10) to improve transfection efficiency by both the formation of oligoarginine-coated DNA complexed with protamine (PD), and the addition of Ca^{2+} after formation of complexes. The efficiency of DNA condensation was determined by gel electrophoresis. Cellular uptake and transfection efficiency were evaluated in human cervical carcinoma HeLa cells using flow cytometry and luciferase assay. Oligoarginine-coated PD enhanced transfection efficiency significantly more than complexes where Arg10 in both vectors exhibited higher transfection efficiency than Arg4. As assessed by gel retardation assay, high gene expression by Arg10 may be explained by Arg4 binding DNA more strongly than Arg10. The addition of Ca^{2+} to incubation medium increased transfection efficiency of Arg4-coated PD 70-fold, similar to that of Arg10-coated PD alone without an increase of cellular uptake, suggesting that Ca^{2+} induced the release of DNA from complexes in endosomes. Only Arg4 with low cytotoxicity could gain an advantage from Ca^{2+} in transfection, but Arg10 with relatively high cytotoxicity could not. The present results demonstrate that Arg4-coated PD with Ca^{2+} has great potential as an efficient non-viral vector with low toxicity.

1. Introduction

As a new gene therapy, cationic lipid-mediated gene transfer is well established and widely used (Felgner et al., 1987; Song et al., 1998; Cotten et al., 1990; Kircheis et al., 1999; Kakizawa et al., 2001). Several cell-penetrating peptides (CPPs), less than 30 amino acid residues in length, such as HIV-1 Tat fragments, have attracted much attention. CPPs contain a number of basic amino acid residues, and can deliver their associated molecules into cells (Derossi et al., 1994; Oehlke et al., 1998; Vives et al., 1997; Pooga et al., 1998; Futaki et al., 2001b; Morris et al., 2001). Oligoarginine has similar characteristic to CPPs (Mitchell et al., 2000; Wender et al., 2000; Futaki et al., 2001a,b). Oligoarginine-modified vectors with the ability to cross the plasma membrane are anticipated for the efficient delivery of plasmid DNA (DNA). Accordingly, oligoarginine incorporated into nanoparticles and liposomes was reported (Kogure et al., 2004; Zhang et al., 2006). Investigations delineating the influence of arginine length on the transfection efficiency and uptake of oligoarginine have reported that oligoarginine of eight residues in length showed higher transfection efficiency than arginine residues of 4–16 (Futaki et al., 2001a), and internalization efficiency was observed to depend on the chain length of oligoarginine peptides (Nakase et al., 2004). Octaarginine-linked stearyl residue (stearyl-Arg8) showed high transfection efficiency of DNA (Futaki et al., 2001a; Kogure et al., 2004), and efficient delivery of small interfering RNA (siRNA) (Tonges et al., 2006).

We previously reported that oligoarginine (Arg_n; n = 4, 6, 8, 10) conjugated 3,5-bis(dodecyloxy)benzamide (BDB) lipids with a poly(ethylene glycol) (PEG) spacer as a novel gene vector ((Arg)n-B). The transfection efficiency and cellular uptake of (Arg)n-B/DNA complexes ((Arg)n-B/D) increased as the number of arginine residues increased (Furuhata et al., 2006a). In contrast, with oligoarginine-modified liposome, tetraarginine-modified liposome alone showed the highest cellular uptake, and was able to deliver associated smaller protein highly among oligoarginine lipids (Furuhata et al., 2006b). We therefore hypothesized that the low transfection efficiency of Arg4-B was due to an inability to release DNA in cytoplasm. It has been reported that polycations like protamine neutralize DNA and facilitate release of DNA from cationic lipid vectors (Read et al., 2003) and therefore DNA condensed using protamine sulfate (PD), was complexed with liposomes (Li et al., 1998). Also calcium ion enhances transfection efficiency to facilitate the transfer of large molecules from endosome compartment to the cell cytoplasm (Palmer et al., 2003; Sandhu et al., 2005; Shiraishi et

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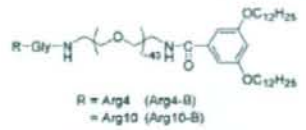

Fig. 1. Chemical structure of (Arg)n-B.

al., 2005). Here we used (Arg)n-B instead of liposomes to complex with PD and added calcium ion into the incubation medium after formation of complex to release DNA from vectors. Since (Arg)n-B forms micelles in water (Furuhata et al., 2008), by using the same method for post-PEGylation of liposomes with PEG lipid, PD could be coated by (Arg)n-B ((Arg)n-B-PD) as a novel vector. Furthermore, we recently found that at the concentration below critical micelle concentration (CMC), Arg10-B/DNA complexes (Arg10-B/D) exhibited significantly higher gene expression than that above CMC, 25 μM (Furuhata et al., 2008). Hence here we decreased from 25 μM to 5 μM for (Arg)n-B vectors.

In this study, we focused on (Arg)n-B (n = 4, 10) to improve vectors (i) by decreasing the concentration (Arg)n-B/D, (ii) by forming (Arg)n-B-coated PD, and (iii) by the addition of calcium ion into the medium. We demonstrate that the addition of Ca^{2+} to the medium dramatically increased the transfection efficiency of Arg4-B-PD with lower cytotoxicity.

2. Materials and methods

2.1. Materials

(Arg)n-B (Fig. 1) was synthesized as previously reported (Furuhata et al., 2006a). Protamine sulfate (grade III) and chloroquine were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Pica gene luciferase assay kit was purchased from Toyo Ink (Tokyo, Japan). Bicinchoninic acid (BCA) protein assay reagent was obtained from Pierce (Rockford, IL, USA). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) and Lipofectamine™ 2000 were purchased from Invitrogen Corp. (Carlsbad, CA, USA). All other chemicals used were of reagent grade.

The DNA encoding the luciferase gene under the control of the CMV promoter (pCMV-luc) was constructed as previously described (Igarashi et al., 2006). The plasmid pEGFP-C1 encoding the enhanced green fluorescent protein (EGFP) under the CMV promoter was purchased from Clontech (Palo Alto, CA, USA). Protein-free preparations of pCMV-luc and pEGFP-C1 were purified following alkaline lysis using maxiprep columns (Qiagen, Hilden, Germany). FITC-labeled 20-mer randomized oligodeoxynucleotide (FITC-ODN) was synthesized with a phosphodiester backbone (Sigma Genosys Japan, Hokkaido, Japan).

2.2. Preparation of (Arg)n-B/D and (Arg)n-B-PD complexes

Two kinds of vector/DNA complexes were prepared, (Arg)n-B/D and (Arg)n-B-PD. To prepare (Arg)n-B/D and (Arg)n-B/FITC-ODN, (Arg)n-B aqueous solution was added to an aqueous solution of DNA (pCMV-luc, pEGFP-C1 or FITC-ODN) where (Arg)n-B was fixed at a charge ratio (±) of 1.0, with gentle shaking. Here, one molecule of Arg4- and Arg10-B was considered as (+1) charge, not depending on the number of arginine residues.

To prepare (Arg)n-B-PD, anionic PD at a charge ratio (±) of 0.3 was prepared by the addition of an aqueous solution of protamine to the DNA aqueous solution with rapid vortexing (2 μg protamine per 2 μg DNA). Then, the (Arg)n-B aqueous solution was added to anionic PD suspension at a charge ratio (±) of (Arg)n-B to DNA of 1.0. Each complex was left at room temperature for 10–15 min.

2.3. Electrophoretic mobility and particle size and zeta potential determination

The degree of condensation afforded by oligoarginine was investigated using agarose gel retardation. (Arg)n-B/D was loaded onto 1.5% agarose gel, and DNA bands were visualized by ethidium bromide staining. Particle size distributions and zeta potentials of vector/DNA complexes were measured 10–15 min after formation by the dynamic and electrophoresis light scattering method, respectively (ELS-Z2, Otsuka Electronics Co. Ltd., Osaka, Japan) at 25 °C, after dilution to an appropriate volume with Milli-Q water.

2.4. Gene transfection

Human cervical carcinoma HeLa cells were kindly provided by Toyobo Co., Ltd. (Osaka, Japan) and grown in DMEM supplemented with 10% FBS at 37 °C in a humidified 5% CO_2 atmosphere. HeLa cell cultures were prepared by plating cells in a 35 mm culture dish 24 h prior to each experiment. The cells were washed three times with 1 ml of serum-free DMEM. For transfection, each vector, complexed with 2 μg of DNA or FITC-labeled ODN per well, was diluted with serum-free DMEM to 1 ml, and then gently applied to the cells.

For gene expression, after vector/DNA complexes were incubated with cells for 3 h at 37 °C in serum-free DMEM, 1 ml of DMEM containing 10% FBS was added to the cells, which were incubated further for 21 h. For transfection with Lipofectamine™ 2000 (LA2000), 5 μL of LA2000 was used for 2 μg of DNA to form a DNA complex in Opti-MEM, according to the manufacturer's protocol. The incubation condition was the same as stated above. Gene transfer efficiency was measured in triplicate.

For the treatment with chloroquine, after cells were incubated for 10 min with serum-free DMEM of 1 ml with 100 μM chloroquine, gene transfection was carried out; particle vectors with DNA were incubated with cells for 4 h at 37 °C in serum-free DMEM with 100 μM chloroquine, the medium with chloroquine was replaced with 1 ml of DMEM containing 10% FBS, and then the cells were incubated for further 20 h. Twenty four hours following the gene transfection, luciferase expression was measured as described above.

For Ca^{2+} supplementation studies, vector/DNA complexes were immediately added to tubes containing an aqueous solution of 1 M $CaCl_2$ at the desired concentration and left at room temperature for 30 min before diluting to the final volume with serum-free DMEM. After incubation for 3 h in serum-free DMEM (1 ml), the cells were further incubated for 21 h at 37 °C in DMEM (2 ml) containing 5% FBS. Ca^{2+} was in medium throughout the incubations.

2.5. Luciferase assay

In the transfection experiment, incubation was terminated by washing the plates three times with cold phosphate-buffered saline (pH 7.4, PBS) as previously reported (Furuhata et al., 2006a). Cell lysis solution was added to the cell monolayers and subjected to freezing at −80 °C and thawing at 37 °C, followed by centrifugation at 15,000 rpm for 5 s. Luciferase expression in the cells was measured according to the instructions accompanying the Pica gene luciferase assay system. The protein concentration of the supernatants was determined with BCA reagent using bovine serum albumin as a standard and cps/μg protein was calculated.

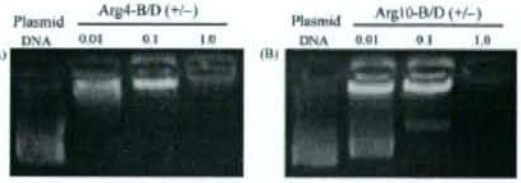

Fig. 2. Electrophoresis of Arg4-B/D (A) and Arg10-B/D (B) on agarose gels at charge ratios (±) of 0.01–1.0.

2.6. Flow cytometry

After gene transfection by incubation for 3 h at 37 °C in serum-free DMEM with or without 2 mM $CaCl_2$, the dishes were washed two times with 1 ml of PBS, and the cells were detached with 0.05% trypsin and 0.53 mM EDTA solution. The cells were centrifuged at 1500 × g, and the supernatant was discarded. The cells were resuspended with PBS containing 0.1% BSA and 1 mM EDTA, and directly introduced to a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with a 488 nm argon ion laser, as previously reported (Furuhata et al., 2006a).

2.7. Fluorescence microscopy

Cells were exposed for 3 h to the vector/DNA complexes in serum-free DMEM, and then cultured for another 21 h in DMEM containing 10% FBS. The cells were washed with PBS three times and then fixed with 4% paraformaldehyde. GFP expression was observed by fluorescence microscopy (ECLIPSE TS100-F, Nikon, Tokyo, Japan), and was imaged using the 465–495 nm excitation beam of a mercury lump. The contrast level and brightness of the images were adjusted.

2.8. Cell viability assay

Cell viabilities upon transfection using vector/DNA with Ca^{2+} were evaluated with a WST-8 assay (Dojindo, Kumamoto, Japan). Cells were seeded at a density of 3×10^4 cells/ml in growth medium containing serum per well in 96-well culture plates, and were transfected with complexes of 2 μg/ml DNA. After 24 h of incubation, the number of viable cells was determined by absorbance measured at 450 nm on an automated plate reader.

2.9. Data analysis

Significant differences in the mean values were evaluated by Student's unpaired *t*-test and one-way ANOVA followed by post-hoc analysis using Dunnett's test. A *p*-value of less than 0.05 was considered significant.

3. Results

3.1. Characteristics of vectors

To confirm the ability of (Arg)n-B to bind DNA, we prepared Arg4- and Arg10-B/D at various charge ratios and evaluated them by gel retardation assay. The results showed that Arg4-B at charge ratios (±) of more than 0.01, and Arg10-B at a charge ratio (±) of 0.1 completely bound DNA (Fig. 2). This finding suggested that Arg4-B interacted with DNA stronger than Arg10-B. In this experiment, therefore, the complexes were used at a charge ratio (±) of 1.0.

Particle sizes and zeta potential of the complexes, and Arg4- or Arg10-coated PD were about 150–230 nm and about +15–20 mV, respectively. (Arg)n-B is water-soluble, and hence, could coat PD. It was confirmed that the zeta potential of (Arg)n-B-PD changed from negatively charged PD alone at a charge ratio (±) of 0.3 (about −30 mV) to a positive charge (Fujita et al., 2008).

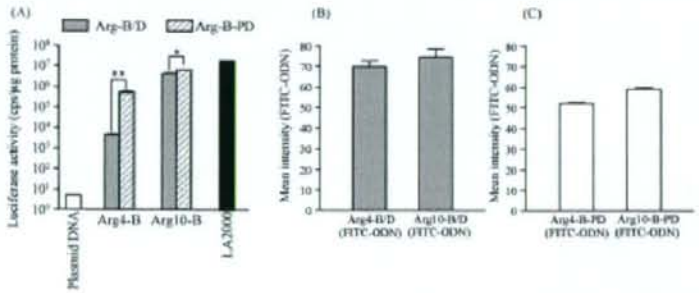

Fig. 3. Luciferase activity in HeLa cells after transfection using (Arg)n-B-PD at a charge ratio (±) of PD of 0.3 and at (Arg)n-B to DNA of 1.0 (A). Cellular uptake of (Arg)n-B/D (B) and (Arg)n-B-PD (C) using (Arg)n-B to 2 μg of FITC-ODN at a charge ratio (±) of 1.0. After incubation for 3 h in serum-free DMEM (1 ml), for luciferase activity, the cells were further incubated for 21 h at 37 °C in DMEM (2 ml) containing 10% FBS, and for FACS analysis, the cells were treated with trypsin. Each bar represents the mean ± S.D. of three experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$.

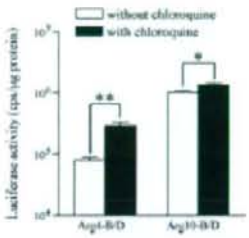

Fig. 4. Luciferase activity in HeLa cells after transfection using 5 μM (Arg)n-B/D complexes at a charge ratio (±) of 1.0 with or without chloroquine. After a 30 min incubation period with serum-free DMEM of 1 ml with 100 μM chloroquine, gene transfer was carried out. After incubation for 4 h at 37 °C in serum-free DMEM with 100 μM chloroquine, the medium with chloroquine was replaced with 1 ml of DMEM containing 10% FBS and the cells were incubated for a further 20 h.

3.2. Luciferase expression and cellular uptake of (Arg)n-B/D and (Arg)n-B-PD

Previous work has shown that below CMC (25 μM), 5 μM Arg10-B/D complexes exhibited significantly higher (1.5-fold) transfection activity than 25 μM (Furuhata et al., 2008). Hence, we used 5 μM (Arg)n-B when forming DNA complexes. Similar to the result at 25 μM (Furuhata et al., 2006a), 5 μM Arg10-B/D complexes exhibited higher luciferase activity than Arg4-B/D complexes (Fig. 3A). According to the result of gel retardation assay, low transfection efficiency by Arg4-B may be due to interaction with DNA and the inability to release.

In order to decrease the interaction of Arg4-B with DNA, we prepared negatively charged PD at a charge ratio (±) of 0.3 and then PD was coated with (Arg)n-B. Since PD exhibited the highest luciferase activity at a charge ratio (±) of 0.3 among 0.1–0.5 (Supplement Fig. 1), (Arg)n-B-PD was prepared at a charge ratio (±) of (Arg)n-B to DNA of 1.0, using PD at a charge ratio (±) of 0.3. Arg4- or Arg10-B-PD enhanced transfection efficiency significantly more than Arg4- or Arg10-B/D complexes, respectively, where Arg10-B-PD increased transfection efficiency up to commercial gene transfection reagent LA2000 ($p > 0.05$) (Fig. 3A). This finding also suggested that Arg4-B could not release DNA from complexes better than Arg10-B even when the negative charge of DNA was partially decreased by protamine.

To further examine the ability of (Arg)n-B vectors to carry genes into cells, we assayed cell internalization using FITC-ODN by flow cytometry. Unlike the large differences of transfection, similar uptake was observed by oligoarginine lengths in (Arg)n-B/D or (Arg)n-B-PD (Fig. 3B and C). These findings indicated that the

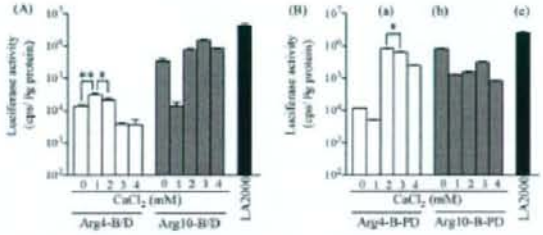

Fig. 5. Effect of Ca^{2+} concentration on luciferase activity of (Arg)n-B/D (A) and (Arg)n-B-PD (B). Vector/DNA complexes were left at room temperature for 30 min with serum-free DMEM containing an aqueous solution of $CaCl_2$, incubated with cells for 3 h, and further incubated for 21 h in DMEM containing 10% FBS. Each bar represents the mean ± S.D. of three experiments. Statistical analysis for comparing various Ca^{2+} concentrations with the control was performed using one-way ANOVA followed by post-hoc analysis using Dunnett's test, *$p < 0.05$ and **$p < 0.01$.

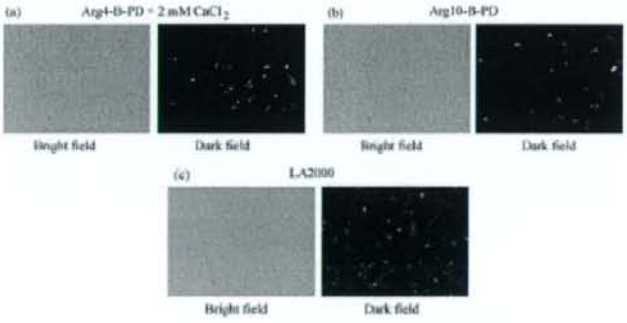

Fig. 6. GFP expression of Arg4-B-PD with Ca^{2+}, Arg10-B-PD and LA2000/DNA complexes by fluorescence microscopy (magnification × 100): (a), (b) and (c) correspond to the vector in Fig. 5(B).

uptake of vector/DNA complexes did not reflect their transfection activity.

3.3. Effect of chloroquine and Ca^{2+} on luciferase expression

Next, to clarify the discrepancy between transfection efficiency and cellular uptake, we investigated the endosomolytical properties of (Arg)n-B/D. Luciferase activities with chloroquine treatment prior to gene transfer were evaluated in the cells (Fig. 4). Chloroquine (100 μM) is known to avoid intracellular degradation by lysosomal enzymes (Carlisle et al., 2004; Hardy et al., 2006; Pelisek et al., 2006). The presence of chloroquine significantly improved the transfection efficiency of Arg4-B/D more than that of Arg10-B/D ($p < 0.01$).

Ca^{2+} is known to facilitate the transfer of large molecules from the endosome compartment to the cytoplasm (Palmer et al., 2003; Sandhu et al., 2005; Shitaishi et al., 2005). To release DNA from the complexes, we prepared DNA complexes in the presence of Ca^{2+} (Fig. 5). For (Arg)n-B/D, the transfection efficiency of Arg4 increased with Ca^{2+} concentration at 1 mM. Further increase in Ca^{2+} decreased transfection (Fig. 5A). The transfection efficiency of Arg10 increased with Ca^{2+}, except 1 mM Ca^{2+} (Fig. 5A).

For (Arg)n-B-PD, the transfection efficiency of Arg4 was significantly enhanced, ~70-fold more than that without Ca^{2+}, using 2 mM Ca^{2+} in the formulation (Fig. 5B). Any further increase in Ca^{2+} reduced transfection efficiency. The transfection efficiency of Arg10 decreased with the increase of Ca^{2+}. Arg4-B-PD with 2 mM Ca^{2+} (a) and Arg10-B-PD alone (b) showed highest transfection efficiency, similar to LA2000 (c) ($p > 0.05$). Ca^{2+} increased strongly transfection of Arg4-B-PD more than Arg4-B/D, but not that of Arg10-B-PD.

To examine the distribution of gene expression, we observed the gene expression of the plasmid pEGFP-C1 with (Arg)n-B-PD using fluorescence microscopy (Fig. 6). A slightly lower level of GFP protein was observed in cells treated with Arg4-B-PD with 2 mM Ca^{2+} and Arg10-B-PD alone than with LA2000, corresponding to the results of luciferase activity, shown in Fig. 5B (a), (b) and (c).

3.4. Effect of Ca^{2+} on cellular uptake of (Arg)n-B-PD and cytotoxicity

To investigate the decreased transfection efficiency of Arg10-B-PD with Ca^{2+}, cellular uptake was measured. We added Ca^{2+} to medium after formation of complex because it was reported that this procedure enhanced transfection (Lam and Cullis, 2000). Surprisingly, 2 mM Ca^{2+} decreased the cellular uptake of both Arg4- and Arg10-B-PD (Fig. 7). This was likely due to increase of size of (Arg)n-B-PD and decrease binding sites on the surface of cells.

It is well known that Ca^{2+} can be toxic to cells. The toxicities of (Arg)n-B-PD were assayed by determining cell viability following 24 h exposure of (Arg)n-B-PD corresponding to 0.2 μg DNA and a total lipid dose of approximately 5 μM of (Arg)n-B-PD per well. As shown in Fig. 8, Arg4-B-PD in the absence and presence of 1–4 mM Ca^{2+} exhibited little toxicity, whereas Arg10-B-PD alone and in the presence of 1 mM Ca^{2+} and LA2000/DNA complexes were highly toxic where cell viability was only ~57% with Arg10-B-PD alone and ~40% with LA2000/DNA complex. On the other hand, 77–100% cells were viable with Arg4-B-PD in the presence of up to 2 mM Ca^{2+}. This finding suggested that Arg10-B-PD with high toxicity did not receive a Ca^{2+} effect on increased transfection efficiency.

4. Discussion

This study demonstrates that we dramatically improved transfection efficiency by forming (Arg)n-B-coated PD, and that

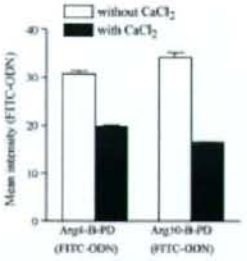

Fig. 7. Cellular uptake of Arg4-B-PD and Arg10-B-PD. (Arg)n-B at a charge ratio (±) of 1.0 was mixed with 2 μg of FITC-ODN. Cells were incubated for 3 h in serum-free DMEM with 2 mM Ca^{2+} and treated with trypsin before FACS analysis. Each bar represents the mean ± S.D. of three experiments.

of Arg4-B-PD by the addition of calcium ion into incubation medium.

The results demonstrate that (Arg)n-B could coat PD. It is well known that LPD vectors are non-viral vehicles for gene delivery composed of polycation-condensed DNA complexed with liposomes. Here we used protamine as a polycation. Protamine has been reported to enhance transfection efficiency by the ability of nuclear localization signaling (Sorgi et al., 1997; Ni et al., 1999). It was also reported that cationic PEG lipid can be inserted into preformed liposomes (Palmer et al., 2003). In this study, protamine-condensed DNA was covered with cationic oligoarginine-PEG lipids, a novel vector. PD type vector showed higher transfection efficiency than the complex type; Arg4- and Arg10-B-PD enhanced transfection efficiency 130-fold and 1.5-fold more than Arg4- and Arg10-B/D complexes, respectively. After (Arg)n-B-PD penetrated the plasma membrane, (Arg)n-B-PD might more easily release DNA from complexes than (Arg)n-B/D, likely due to the negative charge of DNA being partially decreased by protamine because (Arg)n-B interacted strongly with DNA.

Improvements of transfection did not arise from improved uptake into cells. In this regard, a number of studies have indicated that the cationic lipids contained in lipoplexes plays a direct role in stimulating uptake into cells, being due to the positive charge on lipoplexes (Miller et al., 1998). In this study at 5 μM as well as at

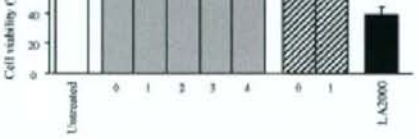

Fig. 8. Toxicity of Arg4-B-PD, Arg10-B-PD and LA2000/DNA 24 h after transfection was assessed by cell viability. Gray and hatched bars represent Arg4-B-PD and Arg10-B-PD, respectively. The numbers refer to the concentration (mM) of $CaCl_2$. Each bar represents the mean ± S.E. of four experiments. *$p < 0.05$ and **$p < 0.01$ compared with the untreated cells.

25 μM (Furuhata et al., 2006a), Arg10-B/D exhibited about 1000-fold higher transfection efficiency to Arg4-B/D in spite of similar cellular uptake (Fig. 3B).

Arg10 with both complex- and PD-type vectors exhibited higher transfection efficiency than Arg4 without an increase of cellular uptake. Similar uptake was observed with Arg4- and Arg10-B/ODN in our study. Nakase et al. (2004) reported that the cellular uptake of hexadecaarginine peptide was higher than octaarginine and tetraarginine peptide, and that of tetraarginine peptide was slight in living cells. The discrepancy with this report might be due to the different observations by using labeled oligoarginine (Nakase et al., 2004) and cargo, labeled ODN associated with oligoarginine lipid.

Presumably, since both Arg4- and Arg10-B have the same structure except for oligoarginine length, the small difference of DNA binding affinity may be caused by oligoarginine length. The poor transfection efficiency of Arg4-B/D may be due to the process of releasing DNA from the endosome compartment to the cell cytoplasm and/or the penetration of DNA into the nucleus because cellular uptake by Arg4- and Arg10-B/D was not significantly different. Ca^{2+} and chloroquine in the increase of transfection efficiency in Arg4-B was much higher than in Arg10-B. Chloroquine (100 μM) is known to avoid intracellular degradation by lysosomal enzymes (Carlisle et al., 2004; Hardy et al., 2006; Pelisek et al., 2006), which is an ion-transporting ATPase inhibitor that disrupts endosomes by preventing their acidication, enhanced CPP release from macropinosomes (Shiraishi et al., 2005). Ca^{2+} enhanced transfection of DNA/cationic lipid particulate complex by the increase of cellular uptake of DNA/lipid complex and facilitated the transfer of DNA from the endosome compartment to the cytoplasm (Palmer et al., 2003; Sandhu et al., 2005). In this study, Ca^{2+} enhanced the transfection of (Arg)n-B/D without increasing its cellular uptake. Ca^{2+} stimulated the release of DNA from endosoma/lysosomal compartments as well as the lysosomotrophic agent chloroquine. This was supported by the findings that Arg4 bound with DNA stronger than Arg10 by gel retardation assay. The difference of transfection efficiency by oligoarginine length may be caused from the different interaction with DNA.

Ca^{2+} could enhance Arg4-B-mediated gene transfer, but not Arg10-B-PD. This finding corresponded with the observation that efficient transfection of cationic liposome in the presence of Ca^{2+} is observed at lower cationic lipid-to-DNA charge ratios than in the absence of Ca^{2+} (Lam and Cullis, 2000) when lower cationic lipid was considered Arg4-B. Ca^{2+} might substitute to some extent for Arg4-B in the complex and interact directly with DNA in the complex. This finding was possibly explained that Arg10-B had more cytotoxicity than Arg4-B, which may be caused by longer Arg residues. Therefore, Arg10-B could not gain an advantage from Ca^{2+} because Ca^{2+} also induced cytotoxicity. The presence of Ca^{2+} results in a maximum increase in Arg4-B-PD transfection potency of about 70-fold and this increase was derived from the ability of Ca^{2+} to assist in destabilizing the endosomal membrane following uptake, not from an increase of uptake (Palmer et al., 2003). These findings provide new insights into the mechanism of how oligoarginine enters cells.

In summary, we improved oligoarginine vectors by the formation of (Arg)n-B-PD. Furthermore, the addition of Ca^{2+} to incubation medium increased the transfection efficiency of Arg4-B-PD, but not that of Arg10-B-PD. This difference may be due to Arg4-B binding DNA strongly and it might be hard to release the gene in cytoplasm compared with Arg10-B. Also, since Arg10-B exhibited relatively higher cytotoxicity than Arg4-B, it could not gain an advantage from Ca^{2+}. We found that the combination of Ca^{2+} and protamine could enhance Arg4-B-mediated gene transfer. Arg4-B-PD with Ca^{2+} has potential as an efficient non-viral vector with low toxicity.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2008.09.060.

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Non-ionic Surfactant Modified Cationic Liposomes Mediated Gene Transfection *in Vitro* and in the Mouse Lung

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As reported previously, cationic liposomes formulated with dioleoylphosphatidylethanolamine (DOPE) and *N,N*-methyl hydroxyethyl aminopropane carbamoyl cholesterol (MHAPC-liposomes) achieved efficient gene transfection in the mouse lung following intratracheal injection. We have studied here the role of surfactants, mannosylerythritol lipid-A (MEL-A) and polysorbate 80 (Tween 80), in affecting gene transfection of MHAPC-lipoplexes (complex with pCMV-luc DNA) in A549 cells and in the mouse lung. MEL-A increased gene transfection of MHAPC-lipoplexes significantly *in vitro* and slightly in the mouse lung, while Tween 80 decreased it both *in vitro* and *in vivo*. As assessed by confocal laser scanning microscopy and fluorescence imaging, MEL-A might facilitate gene dissociation from MHAPC-lipoplexes with fluorescein-labeled oligodeoxynucleotide (FITC-ODN) after internalization into the cells and retained the lipoplexes in the mouse lung for prolonged time, while Tween 80 was inefficient to deliver foreign gene into target cells and in the lung. These results demonstrated that MEL-A is advantageous to Tween 80 in the modification of cationic liposomes as gene delivery vectors in the lung.

Key words non-ionic surfactant; mannosylerythritol lipid-A; Tween 80; cationic liposome

Airway gene delivery is one of the most noninvasive approaches for gene therapy of cystic fibrosis and non-small cell lung cancer.[1—3] Efficient and safe gene delivery vectors are necessary to move lung gene therapy from the laboratory to the bedside.[4,5] Cationic liposomes are currently good candidates due to their safety, high transfection ability, and they can be dosed repeatedly.[4] Many cationic cholesterol derivatives have been formulated into cationic liposomes.[6—9] We reported previously that cationic liposomes with dioleoylphosphatidylethanolamine (DOPE) and *N,N*-methyl hydroxyethyl aminopropane carbamoyl cholesterol (MHAPC, Fig. 1), a cationic cholesterol with a hydroxyethyl group in the cationic part, achieved high gene transfection efficiency in the lung following intratracheal injection.[10] To further increase the gene transfection of MHAPC-liposomes in the lung, we focused on non-ionic surfactant modification due to the reasons addressed below.

Mannosylerythritol lipid-A (MEL-A) is a newly developed biosurfactant, which consists of 4,6-di-*O*-acetyl-2,3-di-*O*-alkanoyl-β-D-mannopyranosyl-(1→4)-*O*-erythritol esterified with two fatty acids and two acetic acids (Fig. 1).[11] With the modification by MEL-A, cationic liposome exhibited high fusion ability with cells, and increased gene transfection *in vitro* by measurement of the luciferase activity.[12—15] However, the performance of MEL-A modified cationic liposomes has not been clarified for *in vivo* gene transfection. Furthermore, Tween 80 is a conventional and widely used surfactant that has been used in many cationic emulsions and cationic nanoparticles as a stabilizer to achieve high gene transfection ability.[16—21] Although MEL-A and Tween 80 have demonstrated effective roles in cationic liposomes or cationic nanoparticles and emulsions as stated above, they have never been compared in the same formulations for the promotion effect on gene transfection. In this study, we modified MHAPC-liposomes with MEL-A or Tween 80 for the enhancement of gene transfection efficiency in the mouse lung. The *in vitro* and *in vivo* gene transfection and the cellular uptake of lipoplexes were compared to evaluate the roles of surfactants in liposomes.

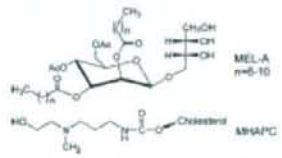

Fig. 1. Chemical Structures of MEL-A and MHAPC

MEL-A: mannosylerythritol lipid-A; MHAPC: *N,N*-methyl hydroxyethyl aminopropane carbamoyl cholesterol iodide.

MATERIALS AND METHODS

Materials MHAPC was synthesized as reported previously.[10] DOPE and Tween 80 were obtained from NOF Co., Ltd. (Tokyo, Japan), and MEL-A was purified as reported previously.[11] The plasmid p cytomegalovirus (pCMV)-luc (DNA) encoding the luciferase gene under the control of the CMV promoter was constructed as described previously.[15] Twenty-mer randomized oligodeoxynucleotide (5′-CGAGTGCACACGCCTCTCAG-3′, ODN) and fluorescein-labeled ODN with the same base sequences (FITC-ODN) were synthesized with a phosphodiester backbone (Sigma Genosys Japan, Hokkaido, Japan). ddY mice (male, 25 g body weight) were purchased from Sankyo Lab Service (Shizuoka, Japan).

Preparation and Characterization of Liposomes and Lipoplexes MHAPC was formulated into liposomes with DOPE by modified ethanol injection method,[19] namely MHAPC-liposomes (MHAPC/DOPE=1/1, m/m). For MEL-

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A and Tween 80 modified MHAPC-liposomes, 20% (molar percent in liposomes) of these surfactants were included in the lipids prior to preparation,[15] they were named as MEL-A and Tween 80, respectively. The liposomes contained 4.5 mM MHAPC concentration for all the experiments. For fluorescence labeling, 0.2% (molar ratio to total lipids) of *N*-(lissamine rhodamine B sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (rhodamine-DHPE) was included in the lipids.

The MHAPC-lipoplexes (complex with pCMV-luc DNA) for *in vitro* gene transfection at various charge ratios (+/−) of cationic lipids to DNA were formed by addition of each liposome to 2 μg of DNA (20 and 33.34 μl for the charge ratio (+/−) of 3/1 and 5/1, respectively, in which liposomes were diluted to a cationic lipid concentration of 0.9 mM). They were left at room temperature for 10 min before transfection. For *in vivo* study, the lipoplexes at charge ratios (+/−) of 3/1 and 5/1 were prepared by the addition of 40 and 66.5 μl of liposomes, respectively to 20 μg of DNA in 25 μl of MilliQ water.

The particle size and zeta potential of liposomes and lipoplexes were measured by dynamic light scanning method (ELS-Z2, Otsuka Electronics Co., Ltd., Osaka, Japan). The particle size and zeta potential of lipoplexes at a charge ratio (+/−) of 3/1 were measured both in Milli Q water (Elix® equipment, Millipore Corporate, MA, U.S.A.) and 1/10 phosphate-buffered saline (pH=7.4, 1/10 PBS).

Transfection Protocol and Luciferase Activity Measurement The human lung adenocarcinoma A549 cell line was obtained from ATCC (VA, U.S.A.). The cells were maintained in RPMI-1640 medium supplemented with 10% FBS and kanamycin (100 μg/ml) at 37 °C in a 5% CO_2 humidified incubator. Cells at a confluence level of 70% in a 35-mm culture dish were transfected with each lipoplex. For transfection, the prepared lipoplexes were diluted in 1 ml of culture medium and then incubated with the cells for 24 h. Luciferase expression in A549 cells was measured as counts per second (cps)/μg protein using the luciferase assay system (Picagene, Tokyo Ink Mfg. Co., Ltd., Tokyo, Japan) and the cps value was normalized to the protein concentration, as determined using a bicinchoninic acid protein assay (Pierce, Rockford, IL, U.S.A.).

Intratracheal injection was employed to study gene transfection in the mouse lung as reported previously.[10] Briefly, the mice were positioned in a vertical position and complex solutions were bolusly injected into the lung through opened trachea while anesthetized. *In vivo jet*-PEI (Polyplus-transfection, NY, U.S.A.) was widely used a golden standard for *in vivo* gene transfection, its complex with DNA was prepared at a charge ratio (+/−) of 5/1 according to the manufacturer's instructions. Luciferase in the lung was measured 24 h after intratracheal injection. The lung was harvested and immediately homogenized in 500 μl of cold lysis buffer (Promega Co., WI, U.S.A.). The homogenate samples were centrifugated at 15000 rpm for 5 min at 4 °C and the luciferase in the supernatant was measured as described above. The cps value was normalized to the total protein concentration as cps/mg protein.

Flow Cytometry A549 cells were prepared by plating in a 35-mm culture dish 24 h prior to each experiment. Each diluted liposome (20 μl of liposomes with a cationic lipid concentration of 0.9 mM) was mixed with 2 μg of FITC-ODN or rhodamine-labeled liposome mixed with 2 μg of ODN at a charge ratio (+/−) of 3/1, and then diluted in 1 ml of medium. Cells were incubated with the lipoplexes at 37 °C for 2 h and 24 h. After incubation, the cells were washed 2 times with PBS and detached with 0.25% trypsin and centrifuged at 1500 rpm for 3 min. The supernatant was discarded and the cell pellets were resuspended with PBS containing 0.1% BSA and 1 mM EDTA. The suspended cells were directly introduced into a FACSCalibur flow cytometer (Becton Dickinson, CA, U.S.A.). Data for 10000 fluorescent events were obtained by recording forward scatter (FSC), side scatter (SSC), and green and red fluorescence. Mean intensity values of FITC or rhodamine inside cells were calculated to compare the uptake amount of lipoplexes.

Confocal Laser Scanning Microscopy (CLSM) A549 cells were prepared by plating in a 35-mm glass dish 24 h prior to each experiment (30% confluence). Each type of rhodamine-labeled liposomes (20 μl of liposomes with a cationic lipid concentration of 0.9 mM) was mixed with 2 μg of FITC-ODN at a charge ratio (+/−) was 3/1, and then diluted in 1 ml of medium. The cells were incubated with the lipoplex at 37 °C for 2 h. After incubation, the dishes were washed 2 times with PBS and mounted in Aqua Poly/mount. Observation was performed with a Radiance 2100 confocal laser-scanning microscope (BioRad, CA, U.S.A.). FITC-ODN and rhodamine-DHPE were excited by an argon ion laser (488 nm) and a He–Ne laser (543 nm), respectively.

Fluorescence Imaging Rhodamine-DHPE (0.2%, molar ratio to total lipids) labeled MHAPC-liposomes and MEL-A and Tween 80 modified MHAPC-liposomes were complexed with 20 μg FITC-ODN. The lipoplexes were intratracheally injected into the mouse lung, after 2 h and 24 h, the lung was harvested and cryosectioned into 12 μm sections before mounting on glass slides. The sections were then embedded in Aqua Poly/mount (Polysciences Inc., Warrington, PA, U.S.A.), and the fluorescence levels of rhodamine and FITC were observed by fluorescence microscopy (Nikon Eclipse TS 100, Nikon, Tokyo, Japan). The exposure times were set as the same for each image.

Statistical Analysis The statistical significance of the data was evaluated with Student's *t*-test. A p value of 0.05 or less was considered significant.

RESULTS AND DISCUSSION

***In Vitro* Gene Transfection of MEL-A and Tween 80 Modified Lipoplexes** As reported previously,[22] the gene transfection of cationic liposomes in the presence of 10% serum was increased with elevated molar ratio of MEL-A in liposomes (from 10 to 30% of MEL-A in liposomes), and even 30% MEL-A modified liposomes did not show significant cytotoxicity at transfection concentration.[14] Therefore, we used 20% molar ratio of MEL-A or Tween 80 in this study. As MHAPC-liposomes exhibited much higher gene transfection at a charge ratio (+/−) of 3/1 than 1/2,[10] we examined charge ratios (+/−) of 3/1 and higher. As shown in Fig. 2, for all the formulations, a charge ratio (+/−) of 3/1 was much more effective than 5/1 in gene transfection. MEL-A increased gene transfection of MHAPC-liposome at both charge ratios (+/−) of 3/1 and 5/1, while Tween 80 signifi-

cantly decreased them without significant cytotoxicity (data not shown). With a notion that DNA dissociation from lipoplexes after endocytotic internalization is a key step for effective gene transfection,[20] excess cationic liposomes at a charge ratio (+/−) of 5/1 might hamper DNA dissociation, thereby decreasing gene expression as compared to 3/1.

The increased gene transfection of MHAPC-liposome by MEL-A was in accordance with OH-Chol-liposomes (OH-Chol=cholesteryl-3β-carboxyamindoethylene-*N*-hydroxyethylamine).[12,13] Although Tween 80 was reported to be very effective in nanoparticles and emulsions for gene transfection, it did not work for cationic liposomes.[16–21] We suppose that Tween 80 plays different roles in nanoparticles, emulsions and liposomes. In nanoparticles and emulsions, Tween 80 is an important stabilizer and emulsifier to maintain their stability, which is essential for their excellent transfection ability.[16,18] Since the liposome itself is stable system due to the lipid bilayer, the addition of Tween 80 forms a steric barrier and may prevent cellular association of the lipoplexes, thereby decreasing gene transfection.[17]

Physiochemical Properties of Liposomes and Lipoplexes The physiochemical properties of liposomes and lipoplexes had a significant effect on their *in vitro* and *in vivo* gene transfection ability, therefore, zeta potential and particle size of liposomes alone and lipoplexes at a charge ratio (+/−) of 3/1 were measured in Milli Q water and PBS (Table 1). For particle size, MHAPC-liposomes and its modified formulations had a mean particle size of about 70 nm. Modification with MEL-A and Tween 80 significantly reduced the size of their lipoplexes. MHAPC-liposomes and its modified formulations were strongly positively charged. Although MEL-A and Tween 80 didn't change the zeta potential of liposomes very much, Tween 80 significantly decreased the zeta potential of lipoplexes to 11.1 mV as compared to non-modified MHAPC-lipoplexes in 1/10 PBS. As seen from lipid-polyethylene glycol (lipid-PEG),[20] Tween 80 might also decrease zeta potential of lipoplexes by the steric layer of oxyethylene groups.

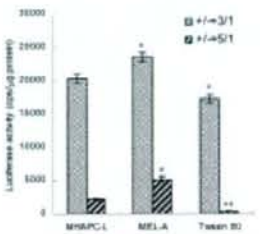

Fig. 2. Gene Transfections of MHAPC-Lipoplexes in A549 Cells at 24 h

Each result represents the mean±S.D. ($n=3$). ** $p<0.01$ and * $p<0.05$ compared with values of non-modified lipoplexes at the corresponding charge ratios.

Cellular Association of MHAPC-Lipoplexes in A549 Cells To clarify the different gene transfection levels conferred by the surfactants, we examined the cellular association of lipoplexes using FITC-ODN as the DNA source and liposomes labeled with rhodamine-DHPE. In Fig. 3A, MEL-A modification inhibited the association of MHAPC-lipoplexes at 2 h from both the FITC and rhodamine intensities; however, the associated amount of liposomes was significantly increased by MEL-A at 24 h as compared to non-modified ones. This phenomenon suggested that MEL-A could gradually increase the association of MHAPC-lipoplexes within 24 h.[15] Tween 80 modification significantly reduced the association of lipoplexes both at 2 and 24 h (Fig. 3A). Subsequently, we carried out confocal microscopy experiment to compare the effect of MEL-A and Tween 80 on MHAPC-liposome-mediated gene delivery. In Fig. 3B, red fluorescence represents the location of rhodamine-labeled lipids and green fluorescence is FITC-ODN in the cells. In the merged images, co-localization of red and green fluorescences means a stable lipoplexes (yellow dots); while the separate localization of green and red fluorescence means FITC-ODN dissociation from lipoplexes. After 2 h incubation of rhodamine-labeled MHAPC-L/FITC-ODN complexes with A549 cells, low level of FITC-ODN was seen in the cytoplasma (Fig. 3B(a)); Modification by MEL-A however showed higher level of FITC-ODN in the cytoplasma (Fig. 3B(b)), suggesting that MEL-A may promote gene dissociation from MHAPC-liposomes during the internalization step into A549 cells. MEL-A modified cationic liposomes carried out gene transfection through different process from conventional ones.[13,14] MEL-A promoted fusion between MEL-A-containing liposomes and the plasma membrane of target cells, the foreign DNA probably dissociated from the liposomes rapidly and delivered into the cytoplasma and nucleus.[13,14] Modification of MHAPC-L with Tween 80 greatly reduced the uptake of lipoplexes (Fig. 3A), and green fluorescence was barely observed in the cells (Fig. 3B(c)). The decreased cellular association of lipoplexes by Tween 80 was probably caused by the steric barrier of polyoxyethylene, the decreased zeta potential (Table 1) and the wetting surface of lipoplexes,[22] which were supposed to be disadvantageous in the cellular association of lipoplexes.

Table 1. Physiochemical Properties of Liposomes and Lipoplexes at a Charge Ratio (+/+) of 3/1

Liposome/Lipoplex		Size (nm)		Zeta potential (mV)	
		Milli Q	1/10 PBS	Milli Q	1/10 PBS
MHAPC-	Liposome	78.9	—	69.4	—
	Lipoplex	291.8±8.2	294.1±11.3	43.6±4.1	29.6±3.1
MEL-A	Liposome	71.5	—	75.9	—
	Lipoplex	162.8±4.1**	173.8±7.3**	48.8±5.4	29.4±2.7
Tween 80	Liposome	67.0	—	60.1	—
	Lipoplex	153.8±8.5**	164.3±9.1**	51.1±4.1**	11.1±2.1**

** $p<0.01$ compared with the values of non-modified MHAPC-lipoplexes. — is not measured.

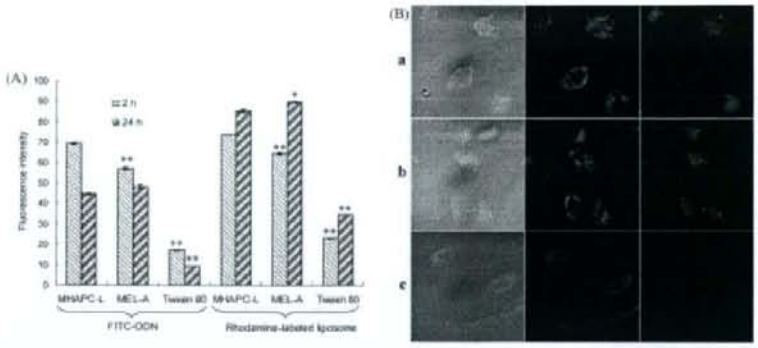

Fig. 3. Cellular Association of FITC-ODN and Rhodamine-Labeled Liposomes in Lipoplexes (+/−=3/1) Incubated with A549 Cells Measured by Flow Cytometry (A) and Confocal Laser Scanning Microscopy (B)

In A, each result represents the mean±S.D. ($n=3$). ** $p<0.01$ and * $p<0.05$ compared with values of non-modified lipoplexes for the corresponding fluorescence and time point. In B, fluorescence images of rhodamine-DHPE (middle) and FITC-ODN (right) were merged on blank images to get merged fluorescence images (left) with pseudo-color. MHAPC-L (a), MEL-A (b) and Tween 80 (c) were incubated with A549 cells for 2 h. 400×magnification for each image.

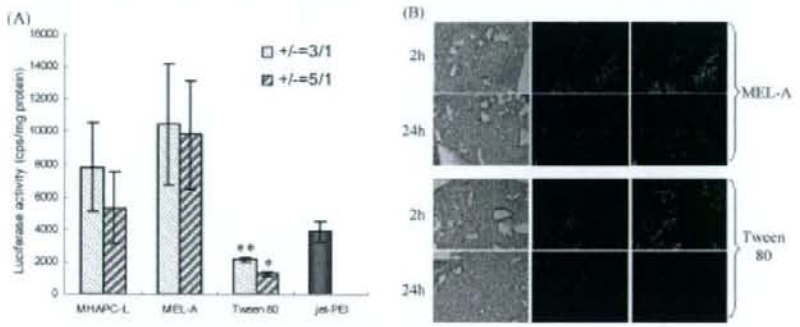

Fig. 4. Gene Transfections (A) and Retention (B) of MHAPC-Lipoplexes in the Lung

In A, *jet*-PEI was complexed with DNA at a charge ratio (+/−) of 5/1 according to manufacture's instructions. Each result represents the mean±S.D. ($n=3$). ** $p<0.01$ and * $p<0.05$ compared with values of non-modified lipoplexes at the corresponding charge ratios. In B, the retention of MEL-A or Tween 80 modified lipoplexes (complex with FITC-ODN, +/−=3/1) was evaluated at 2 and 24 h. The middle line is rhodamine and the right line is FITC. Representative images are shown for each sample. 40×magnification for each image.

***In Vivo* Gene Transfection and Retention of MEL-A and Tween 80 Modified Lipoplexes** As shown in Fig. 4A, in contrast to *in vitro* gene transfection results, a charge ratio (+/−) of 3/1 was almost equal to 5/1 in gene transfection in the lung. The effect of surfactants on gene transfection in the mouse lung had the same trend with that *in vitro* (Fig. 2). MEL-A modification increased gene transfection more than 2-fold higher than the positive control, *in vivo jet*-PEI, but Tween 80 significantly decreased the gene transfection level. The effect of MEL-A and Tween 80 on gene transfection of MHAPC-lipoplexes in the mouse lung was well reflected on the retention ability of lipoplexes (Fig. 4B). In Fig. 4B, the intensity of green fluorescence (FITC) and red fluorescence (rhodamine) indicated the amount of FITC-ODN and rhodamine-labeled liposomes in the lung sections, respectively. After intratracheal injection, the lipoplexes firstly contacted with the mucus layer covering the epithelial cells, and may be taken up by the epithelial cells if they are not quickly cleared by the mucus clearance system.[26] At 2 h after injection of lipoplexes, both MEL-A and Tween 80 modified MHAPC-L showed similar rhodamine and FITC intensities, indicating the same amount of lipoplexes was retained into the mouse lung. At 24 h, MEL-A modified lipoplexes however showed higher FITC intensity than Tween 80 modified lipoplexes, which rarely showed any FITC fluorescence. Although MEL-A and Tween 80 modified MHAPC-liposomes similarly exhibited strong interaction with DNA by circular dichroism.[25] MEL-A has adhesive ability to cells[14,15] and did not decrease the zeta potetial of MHAPC-lipoplexes as compared to Tween 80 (Table 1). These information suggested that MEL-A has higher mucoadhesion ability than Tween 80 modified formulation and could retain the lipoplexes on mucus layer

and cells of the lung for longer period than Tween 80. The prolonged retention ability of MEL-A modified MHAPC-lipoplexes might increase the association of lipoplexes with epithelial cells and gene transfection in the lung. From these results, MEL-A was superior to Tween 80 in increasing gene transfection of cationic liposomes by retaining the lipoplexes in the lung for prolonged period.

CONCLUSIONS

This study demonstrated that the gene transfection and cellular association of MHAPC-lipoplexes were differently affected by the modification of MEL-A and Tween 80 both *in vitro* and in the mouse lung. MEL-A increased gene transfection of MHAPC-lipoplexes significantly *in vitro* and slightly in the mouse lung, while Tween 80 was not effective both *in vitro* and *in vivo*. CLSM experiment demonstrated that MEL-A might induce different mechanism in the cellular uptake of lipoplexes compared with Tween 80, and facilitated foreign gene dissociation from cationic liposomes after internalization into A549 cells. MEL-A could retain the lipoplexes in the mouse lung for prolonged time while Tween 80 did not. These results provided some useful information of non-ionic surfactant modified cationic liposomes for gene transfection in the lung.

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Surface Properties of Lipoplexes Modified with Mannosylerythritol Lipid-A and Tween 80 and Their Cellular Association

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The surface properties of cationic liposomes and lipoplexes largely determine the cellular association and gene transfection efficiency. In this study, we measured the surface properties, such as zeta potentials, surface pH and hydration levels of MHAPC- and OH-Chol-lipoplexes and their cellular association, without and with the modification of biosurfactant mannosylerythritol lipid-A (MEL-A) or Tween 80 (MHAPC=*N,N*-methyl hydroxyethyl aminopropane carbamoyl cholesterol; OH-Chol=cholesteryl-3β-carboxyamindoethylene-*N*-hydroxyethylamine). Compared to OH-Chol-lipoplexes, the higher cellular association of MHAPC-lipoplexes correlated with the significantly higher zeta potentials, lower surface pH levels and "drier" surface, as evaluated by the generalized polarization of laurdan. Both MEL-A and Tween 80 modification of MHAPC-lipoplexes did not significantly change zeta potentials and surface pH levels, while MEL-A modification of OH-Chol-lipoplexes seriously decreased them. MEL-A hydrated the liposomal surface of MHAPC-lipoplexes but dehydrated that of OH-Chol-lipoplexes, while Tween 80 hydrated those of MHAPC- and OH-Chol-lipoplexes. In all, cationic liposomes composed of lipids with secondary and tertiary amine exhibited different surface properties and cellular associations of lipoplexes, and modification with surfactants further enlarged their difference. The strong hydration ability of Tween 80 may relate to the low cellular association of lipoplexes, while the dehydration of MEL-A-modified OH-Chol-lipoplexes seemed to compensate the negative zeta potential for the cellular association of lipoplexes.

Key words cationic liposome; lipoplex; surfactant; surface pH; hydration

For effective gene transfection into mammalian cells, numerous cationic lipids have been synthesized and formulated into cationic liposomes.[1—3] In general, the diverse cationic lipids contain a hydrophobic region, a linker and a cationic headgroup, which are substantially quite variable in different cationic lipids. However, in the cationic headgroup, the basic element is amine, which is responsible for DNA complexation as a protonized form. All four amine types, from primary to quaternary amine, have been reported in diverse cationic lipids and exhibited substantially different cellular association and gene transfection capabilities.[4,5]

Understanding the key step in gene transfection, *i.e.* cellular association of lipoplexes, which is largely determined by the physiochemical properties of cationic liposomes and their lipoplexes, will provide valuable information for designing more effective cationic lipids and optimizing the liposomal formulation. Particle size and zeta potentials of lipoplexes are often reported when discussing their cellular association[6—8]; however, another physiochemical property, the surface hydration of lipoplexes, is always neglected. Although the hydration levels of lipoplexes containing different liposomal formulations have been well established,[9—11] their effect on the cellular association of lipoplexes is still lacking.

We reported previously that cationic liposomes, composed of dioleoylphosphatidylethanolamine (DOPE) and *N,N*-methyl hydroxyethyl aminopropane carbamoyl cholesterol (MHAPC) or cholesteryl-3β-carboxyamindoethylene-*N*-hydroxyethylamine (OH-Chol), demonstrated distinctive differences in their cellular association and gene transfection efficiencies *in vitro*.[12] The major differences between MHAPC and OH-Chol are the amine types and linkers shown in Fig. 1, with MHAPC having a tertiary amine linked to the cholesteryl skeleton by a carbamate ester, and OH-Chol having a secondary amine with an amido linker. To understand the different cellular association of lipoplexes composed of different cationic lipids, the effect of amine types and linkers on the surface properties, *e.g.* zeta potentials and surface hydration levels, was examined. Furthermore, mannosylerythritol lipid-A (MEL-A) is a newly developed biosurfactant[13] and modification of OH-Chol-liposomes with MEL-A markedly increased the gene transfection efficiency of plasmid DNA.[14,15] Recent findings found that MEL-A induced membrane fusion between the target cells and cationic liposomes[16,17]; however, the physiochemical mechanism of the fusion ability of MEL-A has not been fully understood. As a

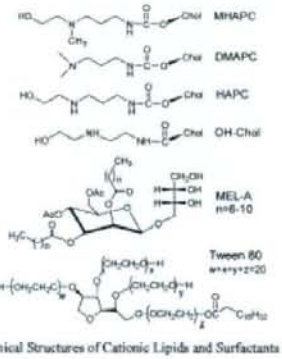

Fig. 1. Chemical Structures of Cationic Lipids and Surfactants

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control surfactant, Tween 80 was selected due to its popularity and wide application in drug delivery. Studying the surface properties of cationic liposomes and lipoplexes, without and with MEL-A or Tween 80, might help to clarify how they affect the cellular association of lipoplexes and gene transfection of MHAPC- and OH-Chol-lipoplexes.

To our knowledge, no comprehensive study has been performed to characterize the surface properties of surfactant modified liposomes and lipoplexes composed of different cationic lipids. In the present study, we measured the zeta potentials, surface pH and hydration levels of MEL-A or Tween 80-modified cationic liposomes and lipoplexes, and also discussed these findings relating to their cellular association.

Experimental

Materials MHAPC, DMAPC, HAPC and OH-Chol were synthesized as reported previously (DMAPC=*N,N,N*-dimethyl aminopropane carbamoyl cholesterol; HAPC=*N*-hydroxyethyl aminopropane carbamoyl cholesterol, Fig. 1).[3,10] DOPE and Tween 80 were obtained from NOF Co., Ltd. (Tokyo, Japan), and MEL-A was purified as reported previously.[15] 4-Heptadecyl-7-hydroxycoumarin (HC) and 6-dodecanoyl-2-dimethylaminonaphthalene (laurdan) were purchased from Lambda (Graz, Austria). *N*-(Lissamine rhodamine B sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (rhodamine-DHPE) was obtained from Invitrogen (CA, U.S.A.). The plasmid DNA pCytomegalovirus (pCMV)-luc encoding the luciferase gene under the control of the CMV promoter was constructed as described previously.[16] A protein-free preparation of the plasmid was purified following alkaline lysis using Maxiprep columns (Qiagen, Hilden, Germany).

Preparation of Liposomes and Lipoplexes MHAPC, DMAPC, HAPC and OH-Chol were formulated into liposomes with DOPE at a molar ratio of 1/1 by modified ethanol injection[10] with a concentration of 4.5 mM cationic lipid for each type of liposome, which were named MHAPC-, DMAPC-, HAPC- and OH-Chol-liposomes, respectively. For MEL-A and Tween 80-modified liposomes, 50% or 100% (molar % to DOPE, cationic lipid/DOPE/surfactant=1 : 1 : 0.5 or 1 : 1 : 1) of these surfactants was included in the lipids prior to preparation,[10] and expressed as MEL-A (0.5), MEL-A (1) and Tween 80 (0.5), respectively. To measure liposomal surface pH, 0.5% (molar % to total lipids) of HC was incorporated into lipids during preparation. To measure the hydration level of the liposomal surface, 0.2% (molar % to total lipids) of laurdan was incorporated into lipids. Liposomes were labeled with 0.2% (molar % to total lipids) of rhodamine-DHPE to measure cellular association of lipoplexes. Lipoplexes at a charge ratio (+/−, amine in cationic lipids/DNA phosphate ratio) of 3/1 were prepared by adding each liposome to an aliquot of DNA, with standing at room temperature for 5 min.

The zeta potential of liposomes and lipoplexes was measured by the dynamic light scanning method (ELS-Z2, Otsuka Electronics Co., Ltd., Osaka, Japan), both in Milli Q water (Elix® equipment, Millipore, MA, U.S.A.) and 1/10 phosphate-buffered saline (pH 7.4, 1/10 PBS).

Circular Dichroism (CD) Measurement Lipoplexes were prepared by adding 100 μl liposomes (4.5 mM cationic lipid) to 50 μl DNA (1 mg/ml), and diluted to 1 ml with PBS (pH 7.4) 5 min after the addition of DNA; the final concentration of DNA was 50 μg/ml for each sample. CD spectra were measured as the average of two scans from 220 to 320 nm at a scan rate of 50 nm/min at 25 °C on a spectropolarimeter (J-800, Jasco Co., Tokyo, Japan).

Fluorescence Measurement Twenty microliters of HC-labeled liposomes were diluted to 2 ml with PBS to a concentration of 0.045 mM of cationic lipid. Lipoplexes were prepared by adding 20 μl liposomes to 100 μl DNA (100 μg/ml), and diluted to 2 ml with PBS 5 min after adding DNA. HC fluorescence was measured by scanning the excitation wavelength between 300 and 400 nm at an emission wavelength of 450 nm (bandwidth 5 nm) at 25 °C on a fluorescence spectrometer (RF-5300PC, Shimadzu Co., Kyoto, Japan). The dissociation degree of HC, *i.e.* liposomal surface pH, can be monitored by the ratio of excitation fluorescence intensities at 380 and 330 nm (380/330).[39] A higher value of 380/330 indicates a higher liposomal surface pH.

Twenty microliters of laurdan-labeled liposomes were diluted to 2 ml with PBS to a concentration of 0.045 mM of cationic lipid. Lipoplexes were prepared by adding 20 μl liposomes to 100 μl DNA (100 μg/ml), and diluted to 2 ml with PBS 5 min after adding DNA. Laurdan fluorescence was measured by scanning the emission wavelength between 400 and 500 nm at an excitation wavelength of 340 nm (bandwidth 5 nm) at 25 °C on a Shimadzu RF-5300PC. The hydration level of the liposomal surface was monitored by the generalized polarization (GP) value, which was calculated as follows:

$$GP(Ex340)=(I_{440}-I_{490})/(I_{440}+I_{490})$$

wherein I_{440} and I_{490} are the emission intensities at wavelengths of 440 nm and 490 nm at an excitation wavelength of 340 nm.[8,23] A higher GP (Ex340) value represents a lower hydration level (dehydration) on the liposomal surface. Both the intensity ratio of 380/330 and GP (Ex340) were calculated from the absolute values of fluorescence intensity of one measurement, which was run in the same day with strictly controlled conditions. The repeated experiments showed different values, but similar trend.

Flow Cytometry Human lung adenocarcinoma A549 cells were maintained in RPMI-1640 medium supplemented with 10% FBS and kanamycin at 37 °C in a 5% CO_2 humidified incubator. The cells were prepared by plating in a 35-mm culture dish 24 h prior to each experiment. Each rhodamine-labeled liposome was mixed with 2 μg pCMV-luc at a charge ratio (+/−) of 3/1, and then diluted in 1 ml PBS (pH 7.4). After incubation with lipoplexes for 2 h, all cells were collected with FACS buffer (PBS containing 0.1% BSA and 1 mM EDTA). After centrifugation at 1500 rpm for 3 min, the supernatant was discarded and the cell pellets were resuspended with FACS buffer for 2 rounds. The suspended cells were directly introduced into a FACSCalibur flow cytometer (Becton Dickinson, CA, U.S.A.). Data for 10000 fluorescent events were obtained by recording forward scatter (FSC), side scatter (SSC) and red fluorescence. Mean intensity values of rhodamine inside cells were calculated to compare the association amount of lipoplexes.

Results and Discussion

Zeta Potentials of Liposomes and Lipoplexes As efficient gene transfection vectors, cationic liposomes and lipoplexes realize their applications through electrostatic interaction with the plasma membrane.[7] With the notion that a positively charged particle facilitates interaction with the cell membrane, and *vice versa*, the zeta potentials of liposomes and lipoplexes were measured to elucidate their cellular association.

As shown in Fig. 2, all the liposomes formulations of MHAPC and OH-Chol exhibited a high positive zeta potential ranging from 50 to 75 mV. MHAPC-lipoplexes exhibited much higher zeta potentials than OH-Chol-lipoplexes both in Milli Q and PBS. Interestingly, MEL-A did not change the zeta potential of MHAPC-lipoplexes, while it markedly decreased that of OH-Chol-lipoplexes to a negative value of −14.6 and −21.7 mV in Milli Q and PBS, respectively. Tween 80 modification slightly decreased the positive zeta potential of MHAPC-lipoplexes, while it decreased the zeta potential of OH-Chol-lipoplexes to a negative value, but to a lesser extent than MEL-A.

The zeta potentials of lipoplexes can be affected by many factors, such as lipid compositions, the size of lipoplexes, the measurement conditions, *etc.* The non-modified MHAPC- and OH-Chol-lipoplexes were about 300 and 400 nm, respectively, but those with MEL-A and Tween 80 modification became smaller and similar (200—250 nm) (data not shown). Since the lipoplex sizes were similar and the measurement conditions were the same, the differences in zeta potential between MHAPC- and OH-Chol-lipoplexes may be ascribed to their structural differences (Fig. 1). MHAPC has a tertiary amine in the headgroup, while OH-Chol possesses a secondary amine. The differences in zeta potential may arise from different protonation levels of the amines. Furthermore, MEL-A modification also generated totally different results on the zeta potentials of MHAPC- and OH-Chol-lipoplexes, suggesting that MEL-A might interact with OH-Chol and affect its protonation level in lipoplexes.

CD Spectra Since MEL-A and Tween 80 affected the

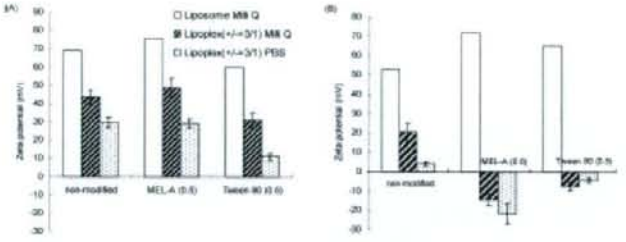

Fig. 2. Zeta Potentials of MHAPC- (A) and OH-Chol- (B) Liposomes and Lipoplexes in Milli Q and PBS
Each lipoplex result represents the mean±S.D. ($n=3$).

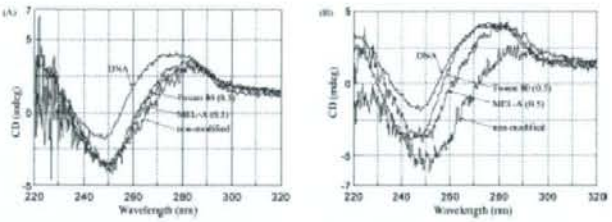

Fig. 3. CD Spectra of DNA in MHAPC- (A) and OH-Chol- (B) Lipoplexes in PBS at a Charge Ratio (+/−) of 3/1

zeta potentials of MHAPC- and OH-Chol-lipoplexes differently, we suppose that the interaction between DNA and cationic liposomes would be substantially different. In Fig. 3A, all MHAPC formulations showed enhanced negative ellipticity, a flattening of the positive bands and an overall red shift of the bands, that is, the typical features of the C form of DNA.[10,22] This suggested that the MHAPC-liposomes had strong complexation with DNA and surfactants did not alter the interaction. As shown in Fig. 3B, non-modified OH-Chol-lipoplexes also exhibited a complete C form of DNA, while modification by MEL-A and Tween 80 only generated modest negative ellipticity. These data suggested that MEL-A- and Tween 80-modified OH-Chol-liposomes were not effective in DNA complexation, correlating to the negative zeta potentials of the lipoplexes as shown in Fig. 2B.

Liposomal Surface pH as Monitored by Dissociation Degree of HC 4-Heptadecyl-7-hydroxycoumarin (HC) is a weak acid fluorophore. At a pH $>pK_a$, the maximum excitation is shifted to the wavelength of about 380 nm. Fluorescence intensity at the excitation wavelength of 330 nm is the pH-independent isosbestic point, which reflects the actual level of HC in liposomes; therefore, the dissociation degree of HC in liposomes can be monitored by the ratio of fluorescence intensities when excitated at 380 and 330 nm (380/330). A high value of 380/330 indicates a more dissociated form of HC, *i.e.* a higher liposomal surface pH.[20] The different cationic lipids in liposome formulations adopted different protonation levels due to high pH values on the liposomal surface.[20]

As shown in Fig. 4, the liposomes and lipoplexes showed the same trend of change of liposomal surface pH after surfactant modifications. All lipoplexes showed decreased pH compared to liposomes; in particular, OH-Chol formulations showed a remarkable decrease of pH. This suggested that DNA complexation may further increase proton concentration on the liposomal surface and the protonation of cationic lipids. MHAPC-lipoplexes demonstrated much lower pH values than OH-Chol-lipoplexes (values of 380/330 are <1 and >1 for MHAPC- and OH-Chol-lipoplexes, respectively), indicating rich protons on the surface of MHAPC-lipoplexes while deficient on OH-Chol-lipoplexes. The different proton levels on the liposomal surface would substantially affect the protonation levels of cationic lipids. In the proton-rich environment, MHAPC with the tertiary amine should have a higher protonation level than OH-Chol, corresponding to the higher zeta potentials of MHAPC-lipoplexes (Fig. 2A). The relatively low proton environment of OH-Chol-lipoplexes made the secondary amine in OH-Chol a major free amine form with lower zeta potential (Fig. 2B).

MEL-A modification of MHAPC-lipoplexes did not greatly influence their surface pH (Fig. 4A), while it evidently decreased that of OH-Chol-lipoplexes with an increased amount of MEL-A (Fig. 4B). This suggested that MEL-A did not change the protonation of the tertiary amine of MHAPC; but significantly affected the protonation of the secondary amine in OH-Chol. These results corresponded well with the striking negative zeta potential of MEL-A modified OH-Chol-lipoplexes (Fig. 2B). The slight effect of Tween 80 on the surface pH of both MHAPC- and OH-Chol-lipoplexes reflected the structural differences between MEL-A and Tween 80. As shown in Fig. 1, MEL-A bears hydroxyl groups in the hydrophilic part and Tween 80 has polyoxyethylene groups. We suppose that the hydroxyl groups in MEL-A may have hydrogen bond interactions with the secondary

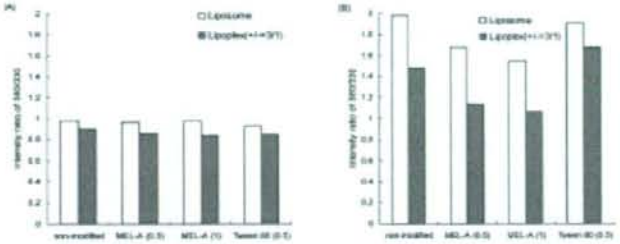

Fig. 4. The Change of Liposomal Surface pH (Dissociation Degree of HC) in MHAPC- (A) and OH-Chol- (B) Liposomes and Lipoplexes as Monitored by the Intensity Ratio of 380/330 nm in PBS

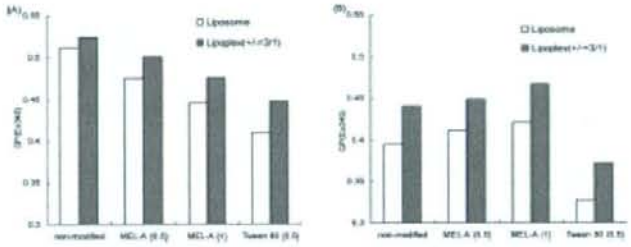

Fig. 5. The Change of Surface Hydration of MHAPC- (A) and OH-Chol- (B) Liposomes and Lipoplexes as Monitored by Laurdan Generalized Polarization (GP) in PBS

amine of OH-Chol, but the polyoxyethylene in Tween 80 does not, thereby affecting the liposomal surface pH of OH-Chol-lipoplexes. For MHAPC, due to the propane in the cationic part, the amine proton may form hydrogen-bond with the amine in the carbamate linker (inner molecular), thereby forming a stable hexacyclic structure. For OH-Chol, the proton of amine may be difficult to form an unstable pentacyclic when involved with hydrogen-bond. Therefore, the neighboring hydroxyl group in MEL-A may prefer to form hydrogen-bond with the amine of OH-Chol. The hydrogen bond interaction between MEL-A and OH-Chol may induce proton dissociation from protonized amine in OH-Chol, thereby decreasing the liposomal surface pH of MEL-A-modified OH-Chol formulations (Fig. 4B) and decreasing the protonation level of secondary amine of OH-Chol, which caused the negative zeta potentials of MEL-A-modified OH-Chol-lipoplexes (Fig. 2B).

The Hydration Level of Liposomes and Lipoplexes as Monitored by Laurdan GP Value It is well-known that polyethyleneglycolylated liposomes and lipoplexes substantially prohibit their association with the cell membrane due to the PEG watery layer.[23–25] This information suggested that the liposomal hydration level is another important factor affecting its association with the cell membrane. Here we examined the effect of MEL-A and Tween 80 on hydration levels of liposomes and lipoplexes. Laurdan was firstly used to follow changes in the hydration level of liposomes by Parasassi *et al.*[21] In solvents with high polarity, laurdan shifts its emission spectrum to higher wavelengths due to dipolar relaxation processes.[21] When associated with liposomes, laurdan emission spectra depend strongly on the different hydration levels of lipid bilayers.

As shown in Fig. 5, liposomes and lipoplexes showed the same trend of change in hydration levels after surfactant modification. All lipoplexes demonstrated higher GP values, *i.e.* lower hydration levels, than the corresponding liposomes formulations. This suggested that the liposomal surface was further dehydrated after complexation with DNA.[9,26,27] By comparing the GP values of MHAPC- and OH-Chol-lipoplexes, MHAPC-lipoplexes showed relatively higher GP values than their counterparts, suggesting that the surfaces of MHAPC-lipoplexes were "drier" than those of OH-Chol-lipoplexes. The carbamate linker in MHAPC might contribute to the "dry" surface of MHAPC-lipoplexes. As shown in Fig. 6, cationic liposomes and their lipoplexes containing lipids with carbamate linker (DMAPC, MHAPC and HAPC) showed higher GP values than those of OH-Chol, which has an amido linker. For MHAPC-lipoplexes, both MEL-A and Tween 80 modification hydrated the liposomal surface by decreasing the GP values (Fig. 5A), with Tween 80 more effective than MEL-A. For OH-Chol-lipoplexes, in contrast, MEL-A dehydrated the liposomal surface, while Tween 80 maintained its effective wetting capability (Fig. 5B). The structural difference between MEL-A and Tween 80 may help to clarify the effective wetting ability of Tween 80. In one molecule of Tween 80, twenty oxyethylene residues were present in the hydrophilic region, while MEL-A possessed only three hydroxyl groups. The water molecules bound