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# Hydroxyethylated cationic cholesterol derivatives in liposome vectors promote gene expression in the lung

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**Abstract**

Three cationic cholesterol derivatives (CCDs), which differ in their types of amine and bear a hydroxyethyl group at the amine group, were synthesized and formulated into liposomes and nanoparticles as gene delivery vectors. *In vitro* transfection into A549 cells proved that liposomes formulated with CCDs and dioleoylphosphatidylethanolamine (DOPE) of 1/2 molar ratio were more effective than the corresponding nanoparticles with CCDs and Tween 80 at charge ratios (+/−) of 1/2, 3/1 and 5/1. Among the liposomal formulations, non-hydroxyethylated CCDs were more effective than hydroxyethylated ones *in vitro*. However, gene transfection in the lung through intratracheal injection showed opposite results to those *in vitro*, with liposomes containing hydroxyethylated CCDs being more potent than those containing non-hydroxyethylated CCDs. Transfection by liposomes with *N*,*N*-methyl hydroxyethyl aminopropane carbamoyl cholesterol iodide (MHAPC) showed the highest luciferase activity, resulting in 2- and 60-fold higher gene expression than jet-PEI and naked DNA, respectively. The distribution of MHAPC lipoplex after intratracheal injection was heterogeneous, and luciferase was expressed in epithelial cells lining the bronchi and bronchioles. All the lipoplexes led to higher TNF-α levels in the lung compared to the nanoplex and jet-PEI, but our findings suggested that modification of the cationic cholesterol with a hydroxyethyl group at the tertiary amine terminal, MHAPC, promoted gene expression in the lung without increasing the toxicity compared with other CCDs. This work firstly proved that liposomes containing hydroxyethylated CCDs could promote gene expression in the lung through intratracheal injection.


*Keywords:* Cationic liposomes; Nanoparticles; Intratracheal injection; Cationic cholesterol derivatives; Hydroxyethyl group

## 1. Introduction

Gene therapy in the lung still holds promise as an effective method for treating cystic fibrosis and lung neoplastic disease (Hoag, 2005). Gene delivery vectors are classified into viral and non-viral ones, and both of them have been well studied. Cationic lipids (Miller, 2003) and polymers (Pietersz et al., 2006), which can compact negatively charged genetic materials through electrostatic interaction and transport them into the cells, constitute a large fraction of non-viral vectors. Many cationic lipids have been reported to mediate gene delivery *in vitro* and *in vivo* (Miller, 2003), and more are being reported all the time.

After the discovery of 3β-[*N*-(*N*′,*N*′-dimethylaminoethane) carbamoyl] cholesterol (DC-Chol) (Gao and Huang, 1991), many effective cationic cholesterol derivatives (CCDs) were soon developed (Ghosh et al., 2002; Hasegawa et al., 2002; Miller, 2003; Nakanishi, 2003; Percot et al., 2004; Bajaj et al., 2007). CCDs are amphiphilic molecules that consist of a cationic headgroup attached via a linker to the cholesteryl skeleton. Clearly, the linker and the cationic headgroup are crucial for the gene transfection ability and toxicity. The inability of CCD-containing cationic liposomes to produce persistent gene expression (Scheule et al., 1997) results in a need for repeated dosing of the liposomes. Therefore, biodegradable CCDs with a linker such as carbamate ester, which can facilitate degradation *in vivo*, are strongly recommended for the design and synthesis of CCDs (Choi et al., 2001). In fact, this strategy has already been verified to be effective by the low toxicity of DC-Chol (Gao and Huang,

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1991) and cationic 3β-[L-lysinamide-carbamoyl]cholesterol derivatives (K-Chol) (Choi et al., 2001; Lee et al., 2006).

Moreover, the amine headgroups of cationic lipids definitely determine their transfection ability (Reynier et al., 2004). Among various amine headgroups, hydroxyethyl group-containing ones have exhibited higher gene transfection than the corresponding hydroxyethyl-lacking ones (Okayama et al., 1997; Venkata Srilakshmi et al., 2002; Arpicco et al., 2004). Cholesteryl-3β-carboxyaminoethylene-*N*-hydroxyethylamine (OH-Chol) is a cationic cholesterol with a hydroxyethyl group at the amine headgroup, linked to the cholesteryl skeleton by an amido bond. Liposomes containing OH-Chol and phosphatidylethanolamine (DOPE) showed high gene transfection ability (Okayama et al., 1997). Furthermore, a nanoparticle formulation with OH-Chol exhibited excellent gene transfection. Their high gene transfection activity was ascribed to the hydroxyethyl group at the cationic headgroup of OH-Chol (Hattori et al., 2007). The combination of a hydroxyethyl group at the headgroup and amine lipids, therefore, will produce effective cationic carbamate-linked lipids for gene delivery vectors.

To develop highly potent and biodegradable CCDs, in this study, we synthesized three types of CCDs bearing secondary, tertiary and quaternary amines, with a carbamate ester linker and a hydroxyethyl group at the amine headgroup. The synthesized CCDs were formulated into nanoparticles and liposomes, and their formulations were optimized for gene transfection of the human lung adenocarcinoma A549 cell line and into the mouse lung through intratracheal injection. The location of luciferase expression was studied by immunohistochemistry. Furthermore, the inflammatory response of the nanoplexes and lipoplexes was evaluated.

## 2. Materials and methods

### 2.1. Materials and instrumentation

DC-Chol and cholesterol chloroformate were purchased from Sigma–Aldrich (St. Louis, USA). *N,N*-Dimethyl-1,3-propanediamine; *N*-methyl-1,3-propanediamine; 1,3-propanediamine; 2-iodoethanol were purchased from Wako Pure Chemistry (Osaka, Japan). Tween 80 was obtained from NOF Co. Ltd. (Tokyo, Japan) and DOPE was from Avanti Polar Lipids Inc., (Alabaster, AL, USA). The synthesis of OH-Chol was done as previously reported (Hattori et al., 2005). RPMI-1640 culture medium was purchased from Invitrogen Corp. (Carlsbad, CA, USA). $^{1}$H NMR (270 MHz) and $^{13}$C NMR (67.8 MHz) spectra were recorded using tetramethylsilane as an internal standard with a JEOL JNM-LA270 spectrometer. Chemical ionization (CI) was carried out on JEOL JMS 600 (JEOL, Tokyo, Japan). The plasmid pCMV-luc encoding the luciferase gene under the control of the CMV promoter was constructed as previously described (Igarashi et al., 2006). A protein-free preparation of the plasmid was purified following alkaline lysis using Maxiprep columns (Qiagen, Hilden, Germany).

### 2.2. Synthesis of cationic cholesterol derivatives (Fig. 1)

#### 2.2.1. N,N,N-Dimethyl aminopropane carbamoyl cholesterol iodide (DMAPC, 1) and N,N,N-dimethyl hydroxyethyl aminopropane carbamoyl cholesterol iodide (DMHAPC, 2)

**1** was synthesized as described by Percot et al. (2004) and hydroxyethylated to **2** with some modifications. **1** and iodoethanol were refluxed in toluene (with a catalytic amount of DMF) at 105 °C for 24 h. Silica-gel chromatography with $CHCl_3$/methanol for elution gave **2** (yield 70%), a pale yellow powder.

#### 2.2.2. N-Hydroxyethyl aminopropane carbamoyl cholesterol iodide (HAPC, 3)

A solution of cholesterol chloroformate (2.7 g, 6 mmol) in 10 ml of dry dichloride methylene (DCM) was slowly added to a pre-cooled solution of 1,3-propanediamine (5 ml, 60 mmol) in 100 ml of DCM. The mixture was further stirred at RT for 1 h. After the reaction, the solvent was removed by vacuum evaporator and the residue was purified on silica gel to give aminopropane carbamoyl cholesterol (2.8 g, 95%). 2.8 g (5.75 mmol) of aminopropane carbamoyl cholesterol was dissolved in a mixture of DCM/methanol with 1 equiv. of triethylamine, and iodoethanol (1.9 mmol, 150 μl) was added and

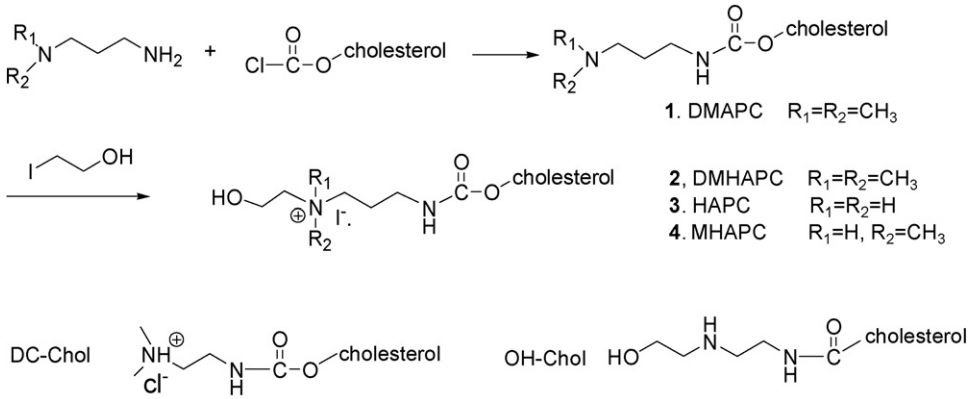


Fig. 1. Chemical structures and synthesis of cationic cholesterol derivatives.

stirred at RT for 24 h. Careful purification on silica-gel chromatography with $CHCl_3$/methanol for elution gave **3** (1.2 g, 31%). $^1$H NMR ($CDCl_3$) $\delta$: 7.62 (s, 1H, –CO–NH–C), 5.36 (t, 3H, C=CH–C), 4.41 (m, 1H, CH–O–CO–), 4.1 (m, 2H, –C–$CH_2$–O). $^{13}$C NMR ($CDCl_3$) $\delta$: 157.2 (NH–CO–O), 139.5, 122.5 (C=CH), 74.9 (=CH–O), 64.9 (–$CH_2$–OH). CI-MS *m/z*: found 658 (Calcd for $C_{33}H_{59}IN_2O_3$, 658.36).

### 2.2.3. *N,N-Methyl hydroxyethyl aminopropane carbamoyl cholesterol iodide (MHAPC, **4**)*

Similarly to **3**, **4** (3.6 g, 94%) was synthesized from *N*-methyl-propanediamine (3.7 ml, 30 mmol) and cholesterol chloroformate (2.7 g, 6 mmol) and subsequently hydroxyethylated with iodoethanol (6.84 mmol, 540 μl). $^1$H NMR ($CDCl_3$) $\delta$: 7.91 (s, 1H, –CO–NH–C), 5.36 (t, 3H, C=CH–C), 4.41 (m, 1H, CH–O–CO–), 4.1 (m, 2H, –C–$CH_2$–O), 2.92 (s, 3H, $CH_3$–$N^+$). $^{13}$C NMR ($CDCl_3$) $\delta$: 158.1 (NH–CO–O), 139.2, 122.8 (C=CH), 74.8 (=CH–O), 63.2 (–$CH_2$–OH). CI-MS *m/z*: found 673 (Calcd for $C_{34}H_{61}IN_2O_3$, 672.37).

## 2.3. Preparation of liposomes/lipoplexes and nanoparticles/nanoplexes

The synthesized CCDs, namely DMAPC, DMHAPC, HAPC and MHAPC, together with DC-Chol and OH-Chol (Fig. 1), were formulated into liposomes with DOPE and into nanoparticles with 5% Tween 80 by a modified ethanol injection method (Hattori et al., 2005). The molar ratio of CCDs to DOPE in the liposomes was varied from 2/1 to 1/1 to 1/2. Each type of liposomes and nanoparticles contained 0.9 mM CCD lipid concentration for *in vitro* transfection and 4.5 mM for *in vivo* experiments.

The CCD liposome/DNA complex (CCD lipoplex) and CCD nanoparticle/DNA complex (CCD nanoplex) for *in vitro* transfection at various charge ratios (+/−) of CCD to DNA were prepared by addition of each liposome or nanoparticle preparations (1.67, 10, 16.7 μl for the charge ratio (+/−) of 1/2, 3/1 and 5/1) to 1 μg of DNA in 5 μl of MilliQ water with 10 rounds of pipetting. After the preparations were left at room temperature for 15 min, the size of each lipoplex/nanoplex in water was measured after incubation with RPMI-1640 medium for a further 15 min. The mean particle size was measured by the dynamic light scanning method (ELS-Z2, Otsuka Electronics Co. Ltd., Osaka, Japan). For the *in vivo* study, the lipoplexes and nanoplexes at charge ratio (+/−) of 3/1 were prepared by the addition of 40 μl of liposomes or nanoparticles, respectively to 20 μg of DNA in 25 μl of MilliQ water. The total injection volume was fixed at 65 μl per mouse.

## 2.4. Cell culture

The human lung adenocarcinoma A549 cell line was kindly provided by OncoTherapy Science, Inc. (Kanagawa, Japan). 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.

## 2.5. Gene transfection in A549 cell line and in the lung

For transfection into A549 cells, the lipoplexes or nanoplexes were diluted in 500 μl of 10% FBS supplemented RPMI-1640 and then incubated with the cells in 12-well plates for 24 h. As a positive control, the Lipofectamine 2000 (LA2000, Invitrogen Corp.)/DNA complex was prepared according to the manufacturer's protocol.

To study the gene expression in the mouse lung, intratracheal injection through the exposed trachea was used as an injection method. Briefly, a ddY mouse (male, 5 weeks of age, Sankyo Lab., Shizuoka, Japan) was anesthetized with phenobarbital sodium (50 μg/g body weight) by intraperitoneal injection (i.p.). Then the mouse was positioned in a vertical position and the trachea was exposed by blunt dissection of the neck. Sixty-five microliters of complex suspension per mouse was bolus injected into the trachea using a 29G injection syringe. The jet-PEI (polyplus-transfection, NY, USA)/DNA complex was prepared at a (+/−) ratio of 5/1 according to the manufacturer's instructions.

## 2.6. Luciferase assay and TNF-α in the lung tissue

Luciferase expression in A549 cells was measured as counts per second (cps)/μg total protein using the luciferase assay system (Picagene, Tokyo Ink Mfg. Co. Ltd., Tokyo, Japan) and BCA reagent (Pierce, IL, USA) as previously described (Maitani et al., 2007).

The luciferase in the lung was measured 24 h after intratracheal injection. Mice were anesthetized with ethyl ether and the lung was perfused with 10 ml of PBS through the left ventricle to remove the blood. The lung was collected with minimal main bronchi and immediately homogenized in 500 μl of cold lysis buffer (Promega Co., Madison, WI, USA). The homogenate samples were centrifugated at 15,000 rpm for 5 min at 4 °C and the luciferase assay was done as described above.

For TNF-α measurement, lysates were prepared exactly as described for the luciferase assay in the lung. TNF-α levels were determined using a mouse TNF-α ELISA kit (R&D, Minneapolis, MN, USA).

## 2.7. Immunohistochemistry of luciferase in the lung

0.01% (molar percentage of lipids) rhodamine-DHPE (*N*-(lissamine rhodamine B sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt) labeled MHAPC liposomes (MHAPC/DOPE=1/2, molar ratio) were prepared with MHAPC concentration of 4.5 mM as described in Section 2.3. The lipoplex was prepared with 20 μg of DNA at a charge ratio (+/−) of 3/1. The lipoplexes were intratracheally injected into mouse lung, and the lung was collected at 24 h. The frozen lungs were cryosectioned into 12 μm slices. The sections were fixed with 70% ethanol and washed in PBS before incubation with primary goat anti-luciferase pAb (1:100) (Promega Co.). The sections were then incubated in bovine serum albumin (BSA) to reduce non-specific binding of a secondary antibody. Finally, they were incubated with rabbit

anti-goat IgG–HRP (Santa Cruz Biotech, Santa Cruz, CA, USA) for 2 h. The color was developed using a peroxidase substrate kit DAB SK-400 (Vector Lab, Inc. Burlingame, CA, USA).

## 3. Results and discussion

### 3.1. DOPE content in liposomes affected gene transfection

DOPE played a very important role in the destabilization of liposomes upon contact with cellular membranes and/or endosomes. In the formulations of cationic liposomes containing DOPE as a helper lipid, most studies used cationic lipids/DOPE at a molar ratio of 2/1, 3/2 or 1/1 as the optimum formulation (Miller, 2003). Recently we reported that DC-Chol/DOPE (1/2, molar ratio) liposomes, which were prepared by a modified ethanol injection method, were more effective for gene transfection than DC-Chol/DOPE = 1/1 and 3/2 liposomes (Maitani et al., 2007). To optimize the CCD liposomes, therefore, at first the DOPE content in liposomes was investigated in an attempt to determine the most effective formulations. As shown in Fig. 2, all the lipoplexes showed higher gene transfection in A549 cells with increasing molar ratio of DOPE (CCD/DOPE = 1/2, molar ratio) at a charge ratio (+/−) of 3/1, lipoplexes with less DOPE content (CCD/DOPE = 1/1 and CCD/DOPE = 2/1) were not effective enough to transfect A549 cells. Therefore, a liposome formulation rich in DOPE content, CCD/DOPE = 1/2-liposomes, was used for further studies. The lipoplexes were named DC-Chol, DMAPC, DMHAPC, HAPC and MHAPC lipoplexes.

### 3.2. Characterization of lipoplexes and nanoplexes

Although liposomes rich in DOPE had higher gene transfection ability than those poor in DOPE, they were only 1/10 to 1/50 as effective as LA-2000 (Fig. 2). Since a nanoparticle formulation with OH-Chol and Tween 80 has shown excellent gene transfection (Hattori et al., 2007), we prepared CCD nanoparticles using Tween 80 as well as CCD liposomes and investigated their use for gene transfection in A549 cells at three charge ratios (+/−) of 1/1, 3/1 and 5/1.

The liposomes and nanoparticles prepared by modified ethanol injection had a mean particle size of about 200 nm, with zeta-potential from +40 to +60 mV, except for OH-Chol/DOPE (1/2, molar ratio) liposomes, which were about 400 nm in size.

To establish the relationship between gene transfection and particle size, the size of lipoplexes (Fig. 3A) and nanoplexes (Fig. 3B) was measured after incubation with RPMI-1640 culture medium. CCD lipoplexes or nanoplexes showed a mean

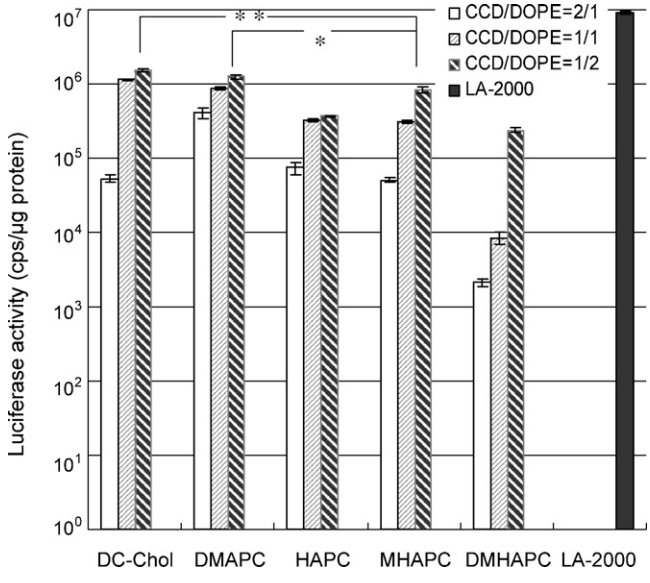


Fig. 2. Effect of DOPE composition in CCD lipoplexes on transfection efficiencies. LA-2000 is lipofectamine-2000. Charge ratio (+/−) was 3/1 and the amount of DNA was 1 μg/well. The values are expressed as mean ± S.D. ($n = 3$). *$P < 0.05$, **$P < 0.01$, Student's $t$ test.

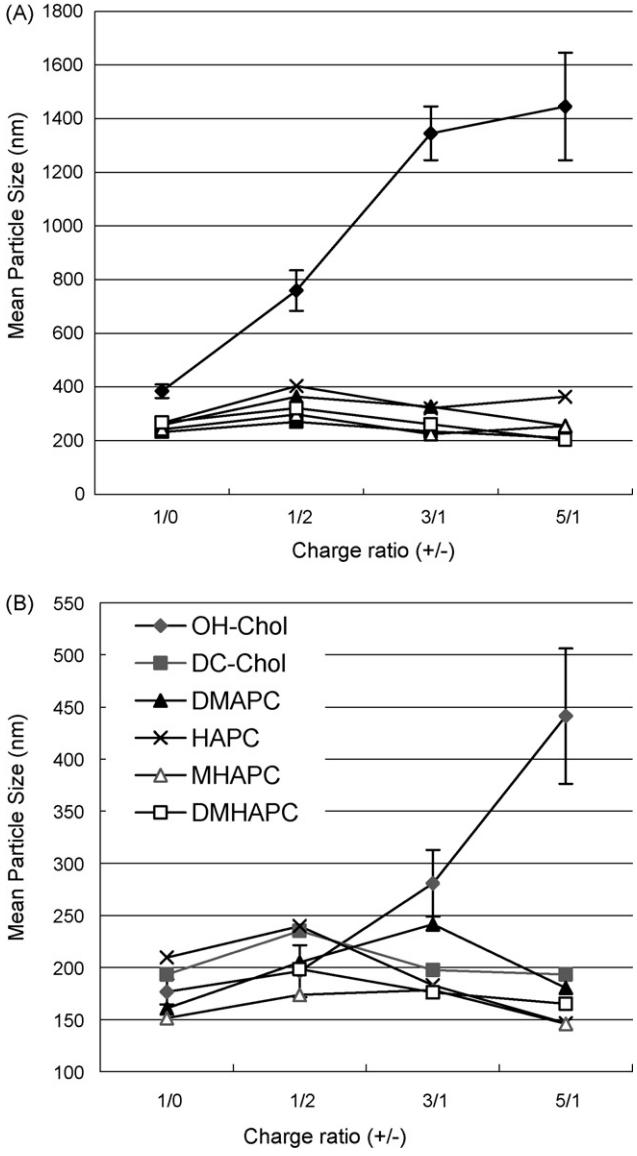


Fig. 3. Particle size of lipoplexes (A) and nanoplexes (B) after incubation with RPMI-1640 medium. The size of each lipoplex and nanoplex in water was measured after incubation with RPMI-1640 medium for 15 min. The values for OH-Chol were expressed as mean ± S.D., other values are expressed as mean values ($n = 3$).

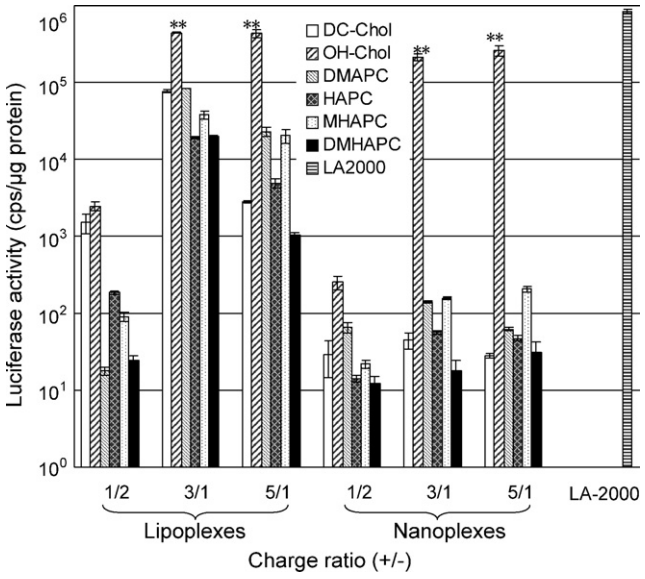


Fig. 4. Transfection efficiencies of lipoplexes and nanoplexes in A549 cells. The values were expressed as mean ± S.D. ($n = 3$). **Significant difference between OH-Chol and other CCDs in the same charge ratio group ($P < 0.01$, Student's $t$ test). The values are expressed as mean ± S.D. ($n = 3$).

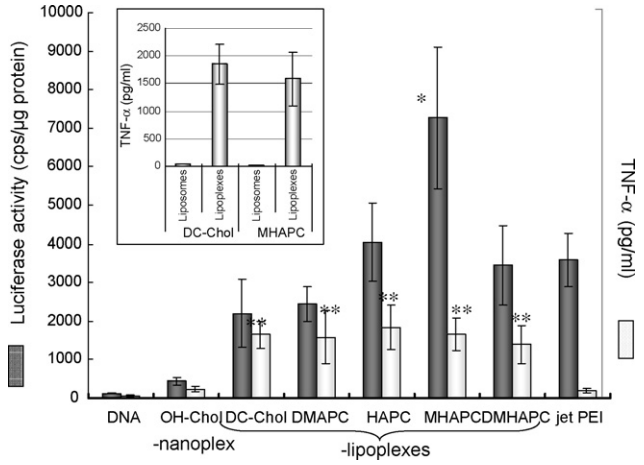


Fig. 5. Luciferase activity (closed columns) and TNF-α (open columns) in the lung at 24 h after intratracheal injection of lipoplexes and nanoplexes (+/− = 3/1). *Significant difference from OH-Chol nanoplex, jet-PEI, DC-Chol and DMHAPC lipoplexes ($P < 0.05$, Student's $t$ test). **Significant difference from OH-Chol nanoplex and jet-PEI ($P < 0.01$, Student's $t$ test). The values are expressed as mean ± S.D. ($n = 3$). The inset showed the TNF-α values of DC-Chol and MHAPC liposomes or lipoplexes in the lung at 24 h after intratracheal injection.

particle size from 150 to 300 nm at a charge ratio (+/−) of 3/1. The OH-Chol liposomes and nanoparticles formed large particles with DNA at charge ratios (+/−) of 3/1 and 5/1, and, therefore, for the *in vivo* study, we selected OH-Chol nanoplexes only at a charge ratio (+/−) of 3/1 as the optimum formulation.

### 3.3. Comparison between lipoplexes and nanoplexes for gene transfection in A549 cells

As shown in Fig. 4, the lipoplexes were far more effective than nanoplexes at the same charge ratio, especially at charge ratios (+/−) of 3/1 and 5/1. This may be explained by the contribution of the membrane destabilization role of DOPE in the liposomes. Among the six kinds of cationic cholesterol derivatives, OH-Chol lipoplexes and nanoplexes exhibited significantly elevated gene transfection, which may have been due to many factors, such as the amido linker and hydroxyethyl group in the structure of OH-Chol (Okayama et al., 1997), and some physical characteristics of the OH-Chol lipoplex and nanoplex, such as large particle size as shown in Fig. 3. The large particle size of OH-Chol lipoplex and nanoplex contributed partly to the high gene transfection in A549 cells, since large particles were more readily endocytosized into cells.

Furthermore, by comparing various lipoplexes at a charge ratio (+/−) of 3/1, we can see that the non-hydroxyethylated cationic cholesterol derivatives, DC-Chol and DMAPC, were more effective than hydroxyethylated ones (except for OH-Chol), namely DMHAPC, HAPC and MHAPC. Moreover, MHAPC, which bears a tertiary amine in the headgroup, exhibited a little higher transfection ability than HAPC and DMHAPC at the optimized formulation (CCD/DOPE = 1/2, molar ratio). Based on the higher gene transfection of lipoplexes than nanoplexes in A549 cells, we selected lipoplexes at a charge ratio (+/−) of 3/1 for further *in vivo* research.

### 3.4. Luciferase expression and TNF-α levels in the lung

Bolus intratracheal injection through the exposed bronchui of mouse was employed for all the *in vivo* studies. This administration method guarantees that 100% of the injected solution reaches the lung instantly (Driscoll et al., 2000). As long as the lipoplexes and nanoplexes can be stabilized under the mucus during distribution of the suspension in the lung, they have a chance to transfect the epithelial cells and even the alveolar cells in the lung.

As shown in Fig. 5, use of the lipoplexes resulted in much higher gene expression in the lung than use of OH-Chol nanoplexes, which were the most effective in A549 cells. One possible reason for this may be the insufficient DNA-protecting ability of OH-Chol nanoplexes in the presence of mucus and surfactants in the lung, while the lipoplexes might be able to encapsulate DNA in highly ordered multilammellar structures. Among these lipoplexes, DMHAPC, HAPC, and MHAPC lipoplexes, which were all hydroxyethylated in the cationic terminal, showed higher luciferase level than DC-Chol and DMAPC lipoplexes, which were not hydroxyethylated. In contrast to the *in vitro* data, the lipoplexes containing hydroxyethylated cationic cholesterol derivatives most strongly promoted gene expression in the lung. Although it is unclear how a hydroxyethyl group at the amine headgroup improves transfection, the hydroxyethyl moiety may affect the interaction between DNA and the cationic lipid membrane, and assist cellular association or some steps after internalization into the cells (Nakanishi and Noguchi, 2001).

Interestingly, the use of MHAPC lipoplexes, which contained a hydroxyethylated tertiary amine as the cationic part, resulted in 2- and 60-fold higher gene expression than the use of jet-PEI and naked DNA, respectively. The exact mechanism by which

the hydroxyethyl group in the cationic part and the tertiary amine in MHAPC increased gene expression is not known, but might be related to the stability of MHAPC lipoplexes in the presence of mucus and/or increased release of DNA from the lipoplexes in the acidic endosomal compartment.

Although the use of lipoplexes resulted in much higher gene expression in the lung, the lung seemed to have some inflammatory response to the lipoplex suspensions. The lipoplexes induced higher TNF-α secretion than OH-Chol nanoplexes and jet-PEI. The strong inflammatory response to lipoplexes was thought to be related to the lipoplexes themselves, since only low levels of TNF-α were detected with the DNA alone and DC-Chol and MHAPC liposomes alone (Fig. 5, inset). Furthermore, the high DOPE content in the lipoplexes might also have been responsible for the inflammatory response, since both OH-Chol nanoplexes and MHAPC nanoplexes induced low levels of TNF-α (data not shown). The present data were in accord with a report showing that lung toxicity observed with lipoplexes could be increased by the addition of DOPE, although DOPE suspension alone caused a negligible inflammatory response (Scheule et al., 1997).

### 3.5. Charge ratio of MHAPC lipoplex affected gene transfection

As shown in Fig. 3, the positively charged (+/− = 3/1) MHAPC lipoplexes were far more effective than negatively charged ones (+/− = 1/2) in A549 cells. Since most reports showed that nearly neutral or negatively charged lipoplexes/nanoplexes can produce higher gene expression in tumor tissues than positively charged ones (Miller, 2003; Hattori et al., 2007), we investigated the effect of the charge ratio (+/−) of MHAPC lipoplexes on gene transfection in the lung. In Fig. 6, it can be seen very clearly that the *in vivo* result corresponded to the *in vitro* one, with positively charged (+/− = 3/1) lipoplexes being significantly more effective than negatively charged ones. Since there are large amounts of surfactants and proteins in the lung lavage fluids and much mucin covers the epithelial cells in the lung, negatively charged lipoplexes might have a small chance of being retained as intact particles to transfect epithelial cells, whereas positively charged lipoplexes might be stable enough to exhibit gene expression ability (Rosenecker et al., 2003).

### 3.6. Distribution of MHAPC lipoplexes in the lung and localization of luciferase by immunostaining

Since MHAPC lipoplexes at a charge ratio (+/−) of 3/1 produced the highest gene expression in the lung, we investigated the distribution of rhodamine-labeled MHAPC lipoplexes and the location of the luciferase expression they produced after intratracheal injection. By observing the fluorescence of rhodamine in the cryosections, it was clear that rhodamine was mainly distributed throughout the bronchi and bronchioles, and some had even diffused to the alveolar cells (Fig. 7a and b). However, the distribution of lipoplexes was not homogeneous throughout the lungs: no fluorescence of rhodamine was

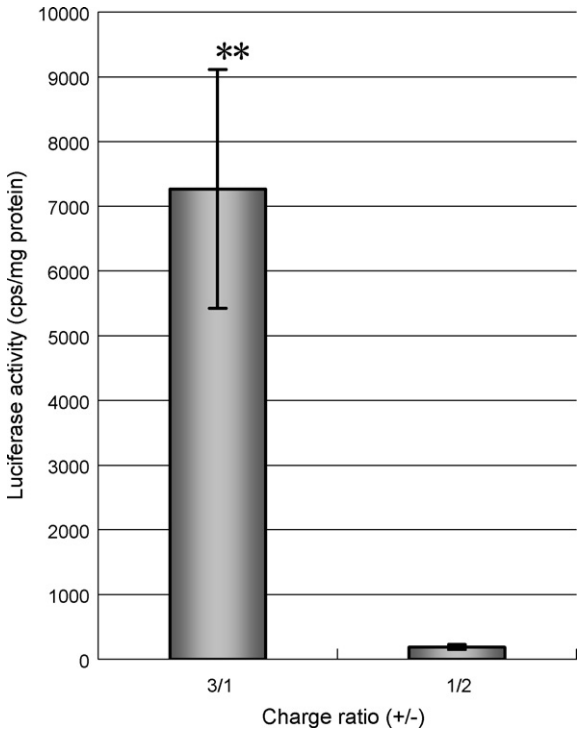


Fig. 6. Effect of charge ratio (+/−) of MHAPC lipoplexes on gene expression in the lung. **, Significant difference ($P < 0.01$, Student's $t$ test). The values are expressed as mean ± S.D. ($n = 3$).

observed in some other regions in the same slice (Fig. 7c and d). From the morphological observation of the lungs which received the MHAPC lipoplex suspension, only the upper and middle lobes exhibited an increased inflammatory response compared to normal lung tissue, indicating that the intratracheally injected lipoplex was mainly located in the upper and middle lobes (photos not shown).

After luciferase immunostaining (Fig. 7e–h), the fluorescence of rhodamine was markedly decreased after many rounds of washing. The fluorescence was only located in the epithelial cells of the bronchi and bronchioles (Fig. 7f and h), and luciferase was also only expressed in the epithelial cells (Fig. 7e, arrows). Although some fluorescence of rhodamine was seen in the alveolar cells (Fig. 7b), there was no luciferase expression in the alveolar cells (Fig. 7e). We suppose that the fluorescence in the alveolar cells was mainly caused by free rhodamine-DHPE that became separated from the lipoplexes in the lung.

Lipoplexes administered by intravenous injection can be captured by the vascular system in the lung and induce gene expression in the lung alveolar region (Scheule et al., 1997). Since intratracheal injection has limited injection volume (1–2 ml/kg weight), the injected lipoplexes are directly exposed to the mucus around the bronchi and bronchioles, resulting in gene expression only in the epithelial cells lining the bronchi and bronchioles. This mode of injection, however, avoids gene expression in other organs, making it possible to decrease the dose of lipoplexes and possibly to decrease toxic side effects, and provides a potentially effective way for gene therapy of cystic fibrosis and other lung diseases.

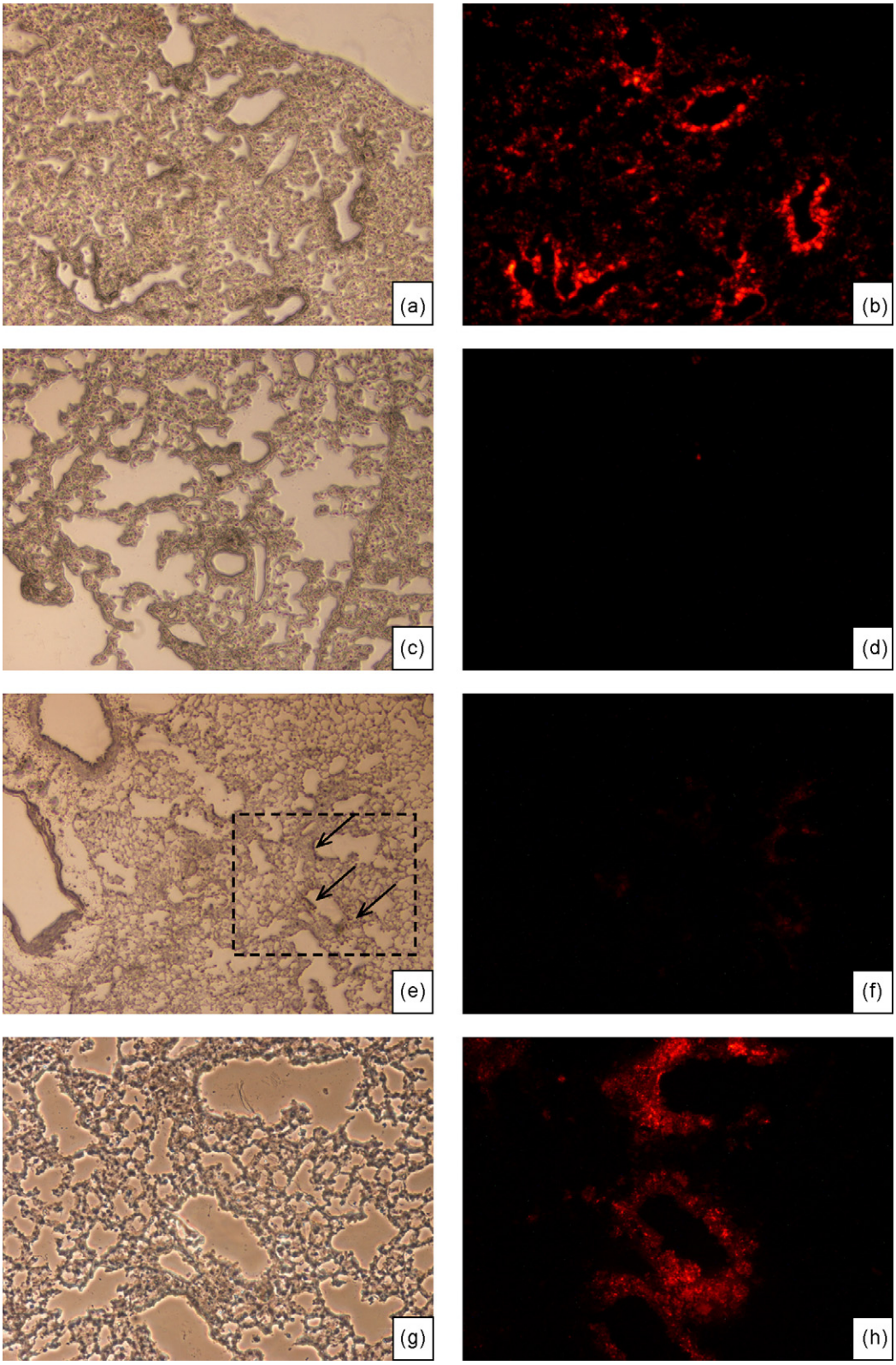


Fig. 7. Distribution of rhodamine-labeled MHAPC lipoplexes (a–d; before immunohistochemistry) and luciferase location in the lungs (e–h; after immunohistochemistry). a–f are shown at 40× magnification; g and h show 100× magnification of the regions in the dashed squares in e and f. Arrows in e indicate the luciferase (dark brown colored).

## 4. Conclusions

In the present work, three CCDs with a carbamate ester linker and a hydroxyethyl group were synthesized and formulated into liposomes and nanoparticles. In *in vitro* formulations, liposomes formulated with CCDs and DOPE of 1/2 molar ratio were more effective than the corresponding nanoparticles with CCDs and Tween 80 at all charge ratios. Furthermore, among the liposomal formulations, non-hydroxyethylated CCDs such as DC-Chol and DMAPC were more effective than hydroxyethylated ones in A549 cells. Gene transfection in the lung showed opposite results to those *in vitro*, with liposomes containing hydroxyethylated CCDs being more potent than ones containing non-hydroxyethylated CCDS. MHAPC liposomes, which contained a hydroxyethylated tertiary amine as the cationic part, showed the highest gene expression among CCD liposomes. All the lipoplexes caused higher TNF-α levels in the lung than the nanoplexes and jet-PEI and we considered the toxicity was largely caused by the lipoplex formulation, but our findings demonstrated that use of CCD lipoplexes with modification of the cationic cholesterol with a hydroxyethyl group at the tertiary amine headgroup, MHAPC, promoted gene expression in the lung without increasing the toxicity compared to other CCD lipoplexes. However, further efforts should be made to elucidate the mechanism of toxicity in the lung caused by CCD lipoplex and how to minimize the inflammation response of CCD lipoplex by optimizing the formulation.

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