Available online at www.sciencedirect.com
ScienceDirect
International Journal of Pharmaceutics 342 (2007) 33–39
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Cationic liposome (DC-Chol/DOPE = 1:2) and a modified ethanol injection method to prepare liposomes, increased gene expression

Yoshie Maitani [a,*], Saki Igarashi [a], Mamiko Sato [b], Yoshiyuki Hattori [a]

[a] *Institute of Medicinal Chemistry, Hoshi University, Ebara 2-4-41, Shinagawa-ku, Tokyo 142-8501, Japan*
[b] *Laboratory of Electron Microscopy, Japan Women's University, 2-8-1 Mejirodai, Bunkyo-ku, Tokyo 112-8681, Japan*

Received 12 December 2006; received in revised form 21 February 2007; accepted 24 April 2007
Available online 10 May 2007

Abstract

Cationic liposomes composed of 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) and dioleoylphosphatidylethanolamine (DOPE) (DC-Chol/DOPE liposome, molar ratio, 1:1 or 3:2) prepared by the dry-film method have been often used as non-viral gene delivery vectors. The formulation and preparation of DC-Chol/DOPE liposomes, as well as the formation of their lipoplexes were investigated in an attempt to improve transfection efficiency *in vitro*. A more efficient transfection in medium with serum was achieved using DC-Chol/DOPE liposomes (molar ratio, 1:2) than those (3:2), and preparation method by a modified ethanol injection than the dry-film. The most efficient DC-Chol/DOPE liposome for gene transfer was molar ratio (1:2) and prepared by a modified ethanol injection method. The enhanced transfection might be related to an increase in the release of DNA in the cytoplasm by the large lipoplex during incubation in optiMEM, not to an increased cellular association with the lipoplex. The use of a modified ethanol injection method might enhance the role of DOPE that is aid in destabilization of the plasma membrane and/or endosome. These findings suggested that cationic liposomes rich in DOPE prepared by a modified ethanol injection method will help to improve the efficacy of liposome vector systems for gene delivery.

Keywords: Cationic liposome; Gene transfection; DOPE; DC-Chol; Ethanol injection method; Dry-film method

1. Introduction

Cationic liposome-mediated transfer of DNA is a promising approach, because of low immunogenicity and toxicity, ease of preparation, and potential applications for active targeting. The disadvantages include poor efficiency of transfection in vivo. Therefore, cationic lipids and improved formulations of liposome have been developed for the efficient delivery of DNA into cells (Gao and Huang, 1991; Vigneron et al., 1996). Notably, liposomes composed of 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol) together with dioleoylphosphatidylethanolamine (DOPE) (DC-Chol/DOPE liposome) have been classified as one of the most efficient vectors for the transfection of DNA into cells (Zhou and Huang, 1994; Farhood et al., 1994, 1995) and in clinical trials (Nabel et al., 1993, 1994).

It has been demonstrated that a 3:2 or 1:1 molar ratio of DC-Chol/DOPE liposome result in high transfection efficiency (Farhood et al., 1995). The correlation between structure and transfection efficiency of them was reported (Congju et al., 2004; Kiefer et al., 2004). A number of investigations (Farhood et al., 1994; Zuidam and Barenholz, 1998; Colosimo et al., 1999) including our own (Maitani et al., 2006) used this molar ratio as a control to develop novel cationic liposomes (Wiseman et al., 2003; Mukherjee et al., 2005). In these cases, liposomes were mostly prepared by the dry-film method, but there were few attempts to examine the optimal ratio of DC-Chol/DOPE liposome prepared by other methods to our knowledge.

Liposome-mediated gene delivery is dependent on numerous factors, such as the formulation of the liposomes including the cationic lipid/neutral lipid ratio, how the liposomes are prepared, the cationic liposome/DNA charge ratio of the complex of cationic liposome and DNA (lipoplex), and the method used to produce the lipoplex. Recently, it was reported that the way in which a liposome is prepared affects transfection efficiency (Tranchant et al., 2004). Also, large lipoplexes were

* Corresponding author. Tel.: +81 3 5498 5048; fax: +81 3 5498 5048.
E-mail address: yoshie@hoshi.ac.jp (Y. Maitani).

0378-5173/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2007.04.035

reported to increase the efficiency of transfection (Felgner et al., 1994; Zhang et al., 1997; Ross and Hui, 1999; Turek et al., 2000; Almofti et al., 2003; Hattori et al., 2005). Including these information, to further improve the transfection efficiency, it is necessary to evaluate DC-Chol/DOPE liposome from formulation and preparation method of liposome to formation method of their lipoplex.

We report that greater transfection efficiency was obtained in human cervical carcinoma HeLa cells in medium with serum, using (1) DC-Chol/DOPE liposomes (molar ratio, 1:2) than liposomes (1:1 or 3:2), and (2) a modified ethanol injection method to prepare liposomes than the dry-film method. The present findings support the notion that cationic liposomes rich in DOPE, and a modified ethanol-based method to prepare liposomes will help to improve the efficacy of liposome vector systems for gene delivery.

2. Materials and methods

2.1. Materials

DOPE was obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA). DC-Chol was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Chloroquine diphosphate was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Lipofectamine 2000 was purchased from Invitrogen Corp. (Carlsbad, CA, USA). pCMV-luc was constructed using a cDNA fragment (589 bp) coding for a cytomegalovirus (CMV) promoter amplified by PCR with a pEGFP-C1 plasmid (Clontech, CA, USA) containing a green fluorescent protein (GFP) reporter gene under the control of the CMV promoter as a template, and the following CMV promoter-specific primers: CMV promoter forward primer (5′-ATGGTACCTAGTTATTAATAGTAATCAA-3′) and CMV promoter reverse primer (5′-TCAAGCTTGATCTGACGGTTCACTAAAC-3′). The forward and reverse primers, respectively, contained KpnI and HindIII restriction sites. After the amplification, the cDNA was digested with KpnI and HindIII and ligated into a KpnI/HindIII-digested pGL3-enhancer (Promega, Madison, WI, USA). The FITC-labeled 20-mer randomized oligodeoxynucleotide (FITC-ODN) was synthesized with a phosphodiester backbone as described previously (Hattori and Maitani, 2005). The protein-free preparation of the plasmid was purified following alkaline lysis using maxiprep columns (Qiagen, Hilden, Germany). All other reagents were of analytical grade.

2.2. Preparation of liposomes

Liposomes were prepared by two methods: a modified ethanol injection (MEI) method (Maitani et al., 2001) or the dry-film method (Bangham et al., 1965). Liposomes prepared by MEI are abbreviated as ML and liposomes prepared by the dry-film method as DL. Regarding the MEI method, all lipids were dissolved in about 5 ml of ethanol, and the ethanol was removed with a rotary evaporator leaving behind about 2 ml of the ethanol solution. Next, a constant volume of sterile water was added to the ethanol solution. Liposomes formed spontaneously after further evaporation of the residual ethanol. The liposome suspension was immediately filtered through 0.45-μm Millex-HA filters (Millipore, Cork, Ireland) for sterilization. For the dry-film method, all lipids were dissolved in chloroform and the solution was dried with N_2 gas to remove the chloroform solvent. The dried film was vacuum desiccated for at least 10 min. Water was added, and after sufficient hydration, the film was suspended by vortexing. The samples were then sonicated for 10 min in a bath type sonicator. The particle size distribution of liposomes was determined using a dynamic light-scattering instrument, and the zeta-potential of them were determined by the electrophoresis light-scattering method (Model ELS-800, Ostuka Electronics Co. Ltd., Japan), at 25 °C after by diluting of dispersion to an appropriate volume with water. Before transfection in the process of formation of lipoplex, the size of liposomes and lipoplexes was determined to be diluted in water within 5 min and 20 min after incubating liposomes and lipoplexes in optiMEM (Invitrogen Corp.), respectively as described in the following section.

2.3. Cell culture

HeLa cells were kindly provided by Toyobo Co., Ltd. (Osaka, Japan) and grown in DMEM supplemented with 10% fetal bovine serum at 37 °C in a humidified 5% CO_2 atmosphere. Human prostate cancer LNCaP cells were supplied by the Department of Urology, Keio University Hospital (Tokyo, Japan). Human hepatoblastoma HepG2 cells were obtained from the Riken Cell Bank (Ibaraki, Japan). Cell cultures were prepared by plating cells in 35-mm culture dishes 24 h prior to each experiment.

2.4. Formation of lipoplex and transfection

Lipoplexes at charge ratios (+/−) of 1/1–11/1 of cationic lipid to DNA were formed by direct mixing or dilution. The former involves adding 3.16–11.1 μl of liposome suspension (1–3.75 mg total lipid/ml water) to 1 μg of DNA with gentle shaking and leaving at room temperature for 10–15 min. The optimal lipoplexes (1 μg of DNA) were as follows: for the liposome composed of DC-Chol/DOPE (molar ratio, 1:0 = 1/0): 11.1 μl of liposome suspension (1 mg lipid/ml water) at a charge ratio of (+/−) of 7:1, for the liposome composed of DC-Chol/DOPE (3:2 = 3/2): 3.16 μl of liposome suspension (1.92 mg total lipid/ml water) at a charge ratio of (+/−) of 2:1, and for the liposome composed of DC-Chol/DOPE (1:2 = 1/2): 3.16 μl of liposome suspension (3.75 mg total lipid/ml water) at a charge ratio of (+/−) of 2:1. With the dilution method, the liposomes and DNA were diluted separately to 125 μl in optiMEM, allowed to stand for 5 min, mixed, and incubated at room temperature for a further 20 min. The lipoplex was diluted with MEM containing 10% serum to a final concentration of 2 μg of DNA per 1 ml of medium per well, and incubated with the cells for 24 h in the medium. For the treatment with chloroquine, cells were incubated in MEM containing 10% serum and a 100 μM chloroquine aqueous solution for 1 h. After this medium was

discarded, the lipoplex prepared by the above procedure was added.

2.5. Luciferase assay

The plasmid pCMV-luc was transfected into cells using the liposomes. After 24 h of incubation, the cells were washed twice with phosphate buffered-saline (pH 7.4, PBS) and harvested with 125 μl of cell culture lysis reagent (Toyo Ink, Tokyo, Japan). Luciferase expression was quantified with 10 μl of centrifuged lysate supernatant using a picagene luciferase assay kit (Toyo Ink, Tokyo, Japan) as described previously (Hattori et al., 2005). BCA protein assay reagent was purchased from Pierce (Rockford, IL, USA). Light emission, expressed in counts per second (cps), was normalized to the protein concentration of each sample, determined using BCA protein assay reagent.

2.6. Flow cytometry

The HeLa cells were prepared by plating in a 35-mm culture dish 24 h prior to each experiment. Each liposome suspension (2.5–4.8 mg total lipid/ml water) was mixed with 2 μg of FITC-ODN and then diluted in 1 ml of the medium containing 10% serum. The cells were incubated with the nanoplex for 1, 2 and 6 h in the medium containing 10% serum. After incubation, the cells were washed two times with 1 ml of PBS to remove any unbound lipoplexes, detached with 0.25% trypsin, and resuspended with PBS containing 0.1% BSA and 1 mM EDTA. The suspended cells were directly introduced into a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with a 488-nm argon ion laser. Data for 10,000 fluorescent events were obtained by recording forward scatter (FSC), side scatter (SSC), and green (530/30 nm) fluorescence.

2.7. Cryotransmission electron microscopy

Specimens for electron microscopy were prepared on Quantifoil® R1.2/1.3 holey carbon grids at a Leica EM CPC cryo-preparation station. A drop of 2.5 μl of the solution was applied to the grid, excess liquid was blotted by touching with a piece of filter paper and the grid immediately plunged into liquid ethane kept at −165°C. The grid was then transferred to the microscope by a cryo-transfer device. Cryo-electron microscopy was performed on a JEOL JEM-3100FFC transmission electron microscope (cryo-TEM). It is equipped with field emission gun (FEG), helium temperature specimen stage, omega-type energy filter and Gatan MegaScan 795 2Kx2K CCD camera. For improved contrast of ice-embedded specimens we employed a novel Zernike-type phase plate at the back focal plane of the objective lens (Danev and Nagayama, 2006). It provides a true phase contrast regime revealing details in the image which are hidden in the conventional defocus phase contrast mode. All images were taken by the CCD camera with the TEM operated at 300 kV acceleration voltage, zero-loss energy filter mode, ×60,000 indicated magnification and employing the phase plate. At that magnification the specimen resolution at the CCD is 3.0 Å/pix. To minimize electron beam damage we employed a minimum dose protocol which irradiates the area of interest only during the image exposure. The total dose to the specimen was about 6 $e^{-}/Å^{2}$.

2.8. Cell viability assay

The cell viabilities upon transfection using DC-Chol/DOPE(3/2), (DL3/2) and DC-Chol/DOPE(1/2), (ML1/2) at a charge ratio of (+/−) of 2:1 were evaluated with a WST-8 assay (Dojindo, Kumamoto, Japan). HeLa 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 lipoplex of 2 μg/mL plasmid 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. Statistical analysis

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

3. Results and discussion

3.1. Characterization of liposomes

We prepared three formulations of liposomes composed of DC-Chol and DOPE at a molar ratio of 1:0, 3:2 and 1:2 using the MEI (ML1/0, ML3/2, ML1/2) or dry-film method (DL1/0, DL3/2, DL1/2). Pure DC-Chol showed vesicles, in agreement with findings of Lendemans et al. (2005). Since large particle/DNA complexes are often more efficient in transfecting cells *in vitro*, the size of liposomes should be similar when comparing transfection efficiency. Liposomes prepared by both methods were about 150–230 nm in size and about 54–59 mV in zeta-potential. By the dry-film method, DL was prepared by briefly sonicating liposomes until a homogeneous size distribution ranging from 150 to 230 nm was obtained (Fig. 1). ML produced by the MEI method had a homogeneous size distribu-

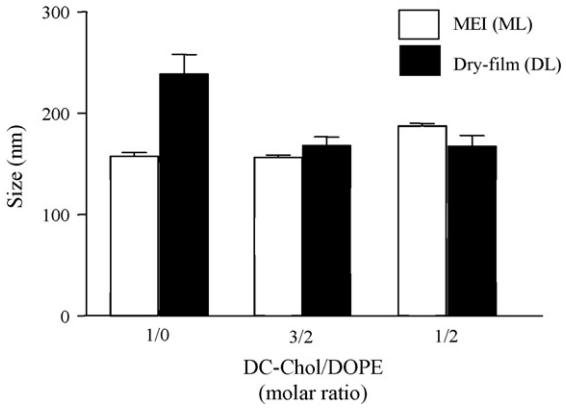

Fig. 1. Effect of preparation method of liposomes on their size. ML1/0, ML3/2, ML1/2, and DL1/0, DL3/2, DL1/2 were prepared by the MEI and dry-film method, respectively. The size of the liposome was measured in water. Each result represents the mean ± S.D. ($n = 3$).

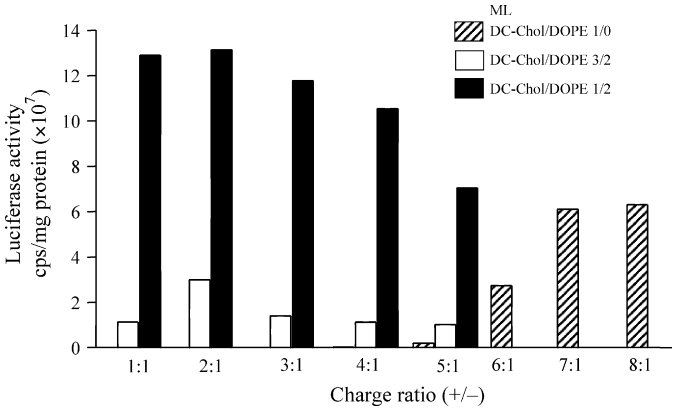

Fig. 2. Effect of the charge ratio (+/−) of ML to plasmid DNA on transfection efficiency in HeLa cells. MLs were prepared by the MEI method. Lipoplexes at a charge ratio (+/−) of 2:1 for ML3/2 and 1/2, and of 7:1 for ML1/0 were prepared by dilution of the liposome and DNA in optiMEM. Lipoplexes were diluted with MEM containing serum to a final concentration of 2 μg of DNA in 1 ml of medium per well, and the cells were incubated for 24 h in the medium. Each result represents the mean ($n = 2$).

tion ranging from 150 to 180 nm obtained without sonication. MEI is an easy method leading spontaneously to smaller particles with low polydispersity. Thus, use of the MEI method is suggested to obtain small liposomes.

3.2. Effects of the way to form the lipoplex and the charge ratio of liposome to DNA on transfection efficiency

To examine effects of the formation method of lipoplex on transfection efficiency, we used direct mixing of liposomes and DNA in water (non-dilution method) or dilution of liposomes and DNA separately in optiMEM (dilution method) for the formation of lipoplexes. In preliminary experiments, lipoplexes prepared by dilution with ML1/2 at a charge ratio (+/−) of 3 were significantly larger (2247 ± 352 nm) than these prepared by direct mixing (1427 ± 83 nm), showing more than two times greater transfection activity in HeLa cells in the presence of 10% serum. (data not shown). This finding suggested that the dilution method yielded a larger lipoplex and higher transfection efficiency than direct mixing. We decided to use the dilution method in subsequent experiments.

To investigate the optimal charge ratio of ML to DNA, we prepared lipoplexes by the dilution method at various charge ratios, and transfected them into HeLa cells (Fig. 2). The ML1/0-lipoplex showed a plateau of transfection efficiency at a charge ratio of (+/−) 7:1 whereas the ML3/2 and DL1/2-lipoplexes showed a maximum at (+/−) 2:1. The finding indicated that the optimal charge ratio of the ML1/0, ML3/2 and ML1/2-lipoplexes was 7:1, 2:1 and 2:1, respectively.

3.3. Transfection efficiency, size of liposome and lipoplex by dilution method

Using these optimal charge ratios of each liposome to DNA, the transfection efficiency of ML and DL was compared with that of Lipofectamine 2000 (Fig. 3A). ML1/2 and DL1/2 showed the greatest transfection efficiency with ML1/2 having comparable transfection efficiency to Lipofectamine 2000 ($P > 0.05$), one of the most efficient commercially available transfection agents. Good transfection efficiency by ML1/2 was also observed in HepG2 and LNCaP cells, corresponding to about one third of that of the Lipofectamine 2000 (data not shown), suggesting that ML1/2 is potentially useful for non-viral transfections.

When the lipoplex was formed by the dilution method, liposome and DNA were diluted separately in optiMEM, mixed, and then resulting lipoplex was incubated. During these processes, the size of ML after dilution in optiMEM for 5 min increased markedly with the increase of the DOPE ratio in ML unlike DL compared with that before dilution as shown in Fig. 1 (Fig. 3B). Furthermore, the size of the ML1/2- and DL1/2-lipoplexes after incubation in optiMEM for another 20 min increased further (Fig. 3C). This finding suggested that ML1/2 with its larger lipoplex showed greater transfection efficiency than DL1/2. Liposomes formulated without a neutral helper lipid have inferior rates of transfection. There-

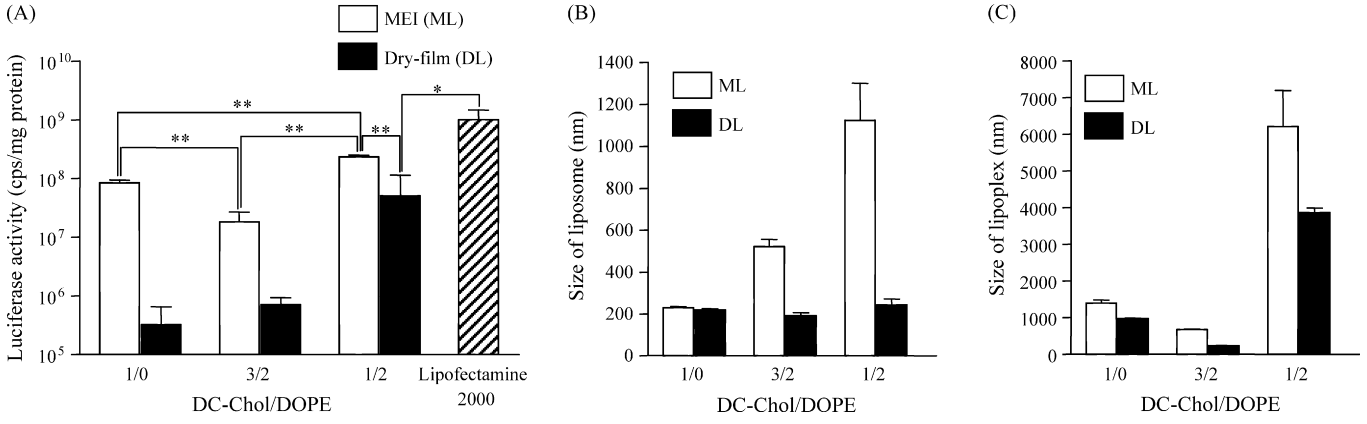

Fig. 3. Transfection efficiency of lipoplex prepared by dilution method of the liposome in HeLa cells (A), size of ML and DL after dilution in optiMEM within 5 min (B), and size of their lipoplex after incubation in optiMEM within further 20 min (C). Lipoplexes at a charge ratio (+/−) of 2:1 for ML3/2, DL3/2, ML1/2 and DL1/2, and of 7:1 for ML1/0 and DL1/0 were prepared by dilution of the liposome and DNA with optiMEM. Lipoplexes were diluted to a final concentration of 2 μg of DNA in 1 ml of medium containing serum per well, and the cells were incubated for 24 h in HeLa cells. The size of the liposome and lipoplex was determined in water. Each result represents the mean ± S.D. ($n = 3$). *$P < 0.05$. **$P < 0.01$ by ANOVA test.

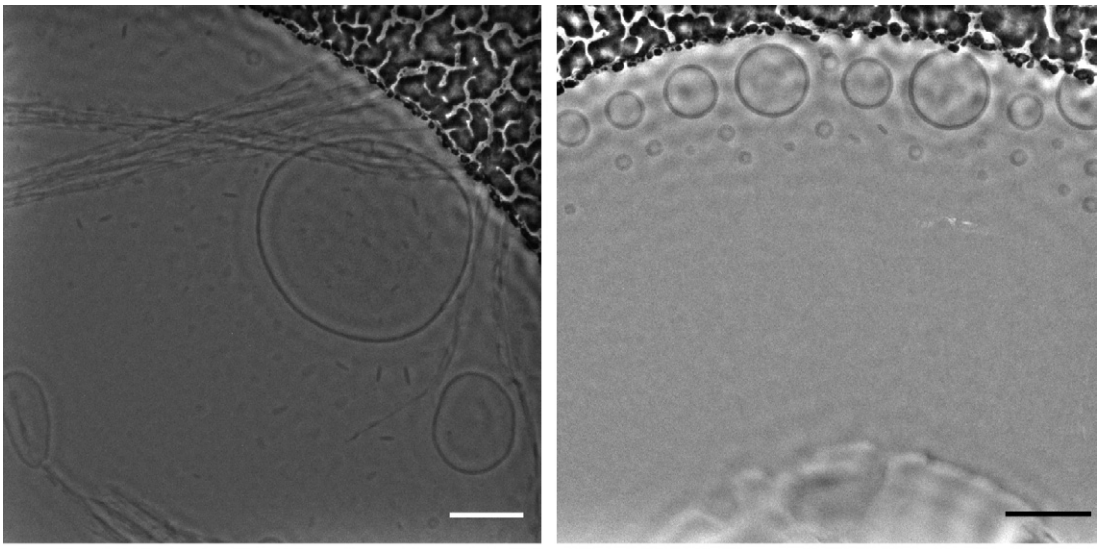

Fig. 4. Electron micrographs of ML1/2 and DL1/2 after cryofixation. Bar represents 100 nm. Concentration of total lipid = 3.75 mg/ml.

fore, cationic liposomes often contain the neutral helper lipid. DOPE increases remarkably the transfection efficiency of liposomes. From our experiment, the optimal ratio of DC-Chol to DOPE was 1:2 regardless of the type of liposome prepared (Fig. 3A), which was different from the value (3:2) reported (Farhood et al., 1995). The optimal DC-Chol content of the DOPE liposomes was about 50–60% (DC-Chol:DOPE = 3:2, 1:1) when 1 μg of plasmid DNA was added to 40 μM total lipid of cationic liposomes in DMEM at room temperature for 10–15 min to form a lipoplex (Farhood et al., 1995). Our lipoplex was prepared differently from theirs: it was prepared by mixing 10 μM total lipid and 2 μg of plasmid DNA at a DC-Chol+/DNA- ratio of 2:1 using the dilution method. The dilution method increased the size of the lipoplex and transfection efficiency. Our result was consistent with the finding that a larger lipoplex enhanced the efficiency of gene transfection into cultured mammalian cells (Zuidam and Barenholz, 1998).

For visualization of particle structures from different preparation method, ML1/2 and DL1/2 were investigated by cryo-TEM. Both liposomes showed one lamellar liposomal vesicles (Fig. 4). The difference in liposome preparation method did not influence the morphology of the liposomes. Both preparations showed similar pattern. It was reported that different ways of preparing liposomes such as the dry-film method (Bangham et al., 1965), and ethanol injection methods (Maitani et al., 2001) allow the formation of several particle types named multilamellar vesicles (MLV) and small unilamellar vesicles (SUV), respectively. However, in this case, the lipid formulation might dominate morphology of the liposome more than preparation process.

ML was very stable in water; did not change its size for at least half a year and retained the ability to transfect (data not shown). However, when ML was incubated in optiMEM, DOPE increased the size of liposomes. The physicochemical properties of DC-Chol and DOPE mixed at a molar ratio of 3:2 or 1:1 (Sternberg et al., 1994; Wrobel and Collins, 1995; Ciani et al., 2004) have been studied extensively, but not so those of the 1:2 formulation. The size of the ML1/2-lipoplex was greatly increased. This finding might be similar to that the size of lipoplexes with not extruded liposomes through polycarbonate membrane increased up to more than 1 μm after 24 h whereas that with extruded ones exhibited steady values (Clement et al., 2005). Homogeneity of ML such as distribution of DOPE in liposomes might be different from DL although polydispersity of size of ML was lower than that of DL. MEI method might cause DOPE to be distributed heterogeneously at the surface of the liposome.

3.4. Association of ML-lipoplexes with HeLa cells

To compare the cellular association of the DNA transfected by liposomes, we examined the amount of DNA associated with the HeLa cells at different time points by flow cytometry. An analysis of flow cytometric profiles and mean intensities clearly indicated that the kinetics of the amount of DNA differed remarkably among MLs (Fig. 5). The uptake of FITC-ODN by ML1/0-lipoplexes was faster and greater than that by the ML3/2- and ML1/2-lipoplexes, suggesting that the high cationic charge of ML1/0-lipoplexes allowed them to interact with cell membranes. This indicated that ML3/2 and ML1/2 showed a similar association with the cells.

3.5. Cytotoxicity

We examined the cytotoxicity of lipoplex of DNA with novel formulation, ML1/2 and conventional formulation, DL3/2 at a charge ratio of (+/−) 2:1 after transfection (data not shown). The concentration of the cationic lipid used in the cell line experiment was same in both liposomes since the charge ratio of (+/−) of DC-Chol and DNA was same, resulting in

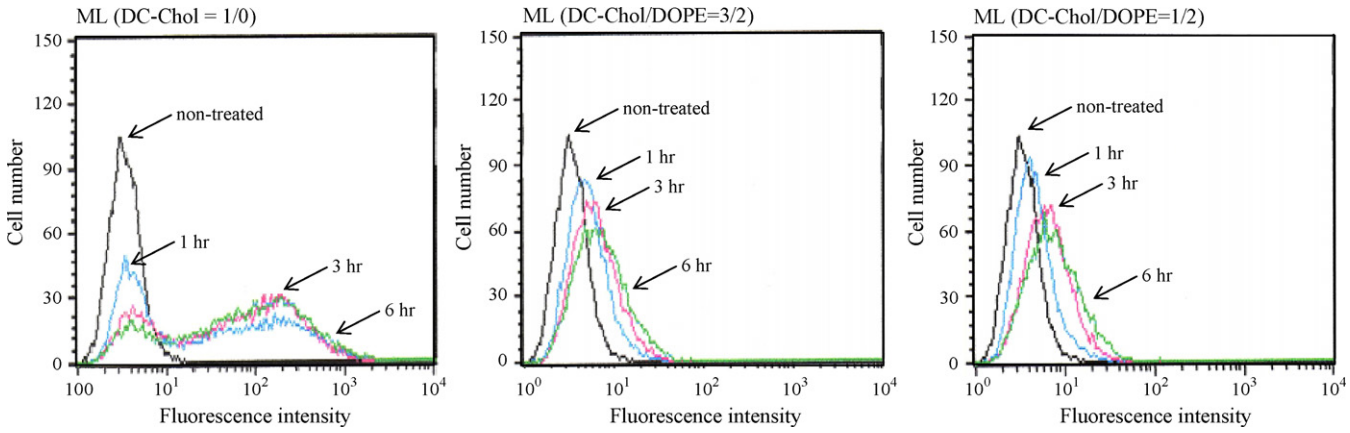

Fig. 5. The cellular association with ML-lipoplexes of FITC-ODN. The kinetics of the cellular association of FITC-ODN transfected with ML1/0, ML3/2 and ML1/2 prepared by the MEI method was evaluated by flow cytometry. Lipoplexes at a charge ratio (+/−) of 2:1 for ML3/2 and ML1/2, and of 7:1 for ML1/0 were prepared by dilution of the liposome and DNA in optiMEM. Each lipoplex was incubated with HeLa cells for 1, 3 and 6 h in medium containing serum.

a cell viability of 78.9 ± 3.8% for ML1/2, and 80.7 ± 3.3% for DL3/2 (mean ± S.D., $n = 7$). This finding indicated that cationic liposomes rich in DOPE prepared by a modified ethanol injection method did not accelerate the cytotoxicity of the lipoplexes.

3.6. Effect of chloroquine on transfection efficiency

To estimate the influence of a lipoplex is ability to avoid or escape the endo/lysosomal pathway on the overall expression of the luciferase reporter gene, we tested the effects of chloroquine on transfection efficiency. Pretreatment with chloroquine reduced significantly the transfection efficiency of ML1/2-lipoplex prepared by the dilution method (Fig. 6). This result was not observed with ML1/0 and ML3/2. Chloroquine is a lysosomotropic agent known to interfere with endocytosis by raising the endosomal and lysosomal pH and by inhibiting the maturation of endosomes (Farhood et al., 1995). It is suggested that the extensive transfection by ML1/2 is due to the

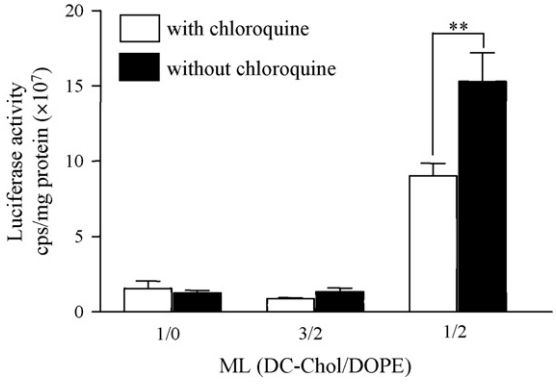

Fig. 6. The change in transfection efficiency of ML on pretreatment with chloroquine for 1 h. ML1/0, ML3/2 and ML1/2 were prepared by the MEI method. Lipoplexes at a charge ratio (+/−) of 2:1 for ML3/2 and 1/2, and of 7:1 for ML1/0 were prepared by dilution of the liposome and DNA in optiMEM. Each lipoplex was incubated with HeLa cells for 24 h in medium containing serum. $^{**}P < 0.01$, compared to pretreatment with chloroquine.

contribution of DOPE acting to release DNA from the lipoplex into the cytoplasm.

The entry into the cytoplasm is the first important step for liposome-mediated transfection. The ability to get DNA to bind with the cell surface, which is negatively charged, is dependent on the cationic charge of the liposome. The association of ML3/2- and ML1/2-lipoplex was similar (Fig. 5), but ML1/2 showed significantly greater transfection efficiency than ML3/2. These findings suggest that the release of DNA from the lipoplex may play the key role in the transfection, not the increase in the cellular association of the lipoplex.

Since cationic charge is needed for DNA-complexation and cellular uptake, reduced cationic charge is anticipated to cause a drop in transfection potency through reduced cellular uptake. However, the surface potential of ML1/2 was similar with that of ML3/2 and uptake of ML1/2 was similar with that of ML3/2.

This was consistent with our previous report that a large complex of nanoparticle and DNA increased transfection efficiency by inducing the release of DNA in endosomes (Hattori et al., 2005). The role of DOPE is to facilitate membrane fusion and aid in destabilization of the plasma membrane or endosome (Farhood et al., 1995; Zuidam and Barenholz, 1998). In this case, DOPE seemed to best do the latter.

ML1/2 was likely to deliver DNA into the cytoplasm, and release more DNA than ML1/0 or ML3/2 due to its larger lipoplex. The optimal ratio of DC-Chol to DOPE involved more DOPE, compared with that reported previously (Farhood et al., 1995). Distribution of DOPE in cationic liposomes seemed to be susceptible to preparation method. These findings, taken together, indicated that the most important lipid component in cationic liposomes is DOPE; only a minimal amount of cationic lipid is needed to provide a means for the liposomes to bind the negatively charged cell surface. Also, liposome preparation method is important for vectors as well as formulation of liposomes.

These findings suggested that cationic liposomes rich in DOPE prepared by a modified ethanol injection method are

a remarkable non-viral vector for gene transfection and gene therapy.

Acknowledgements

We thank Prof. Kuniaki Nagayama in Okazaki Institute for Integrative Bioscience National Institutes of Natural Sciences for Electron Microscopy, and Mr. Masahiko Furuhata, Mr. Atushi Yamada and Mr. Takashi Yoshizawa for their assistance. This project was supported in part by a grant from The Promotion and Mutual Aid Corporation for Private Schools of Japan, and by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

References

Almofti, M.R., Harashima, H., Shinohara, Y., Almofti, A., Li, W., Kiwada, H., 2003. Lipoplex size determines lipofection efficiency with or without serum. Mol. Membr. Biol. 20, 35–43.

Bangham, A.D., Standish, M.M., Watkins, J.C., 1965. Diffusion of univalent ions across the lamellae of swollen phospholipids. J. Mol. Biol. 13, 238–252.

Ciani, L., Ristori, S., Salvati, A., Calamai, L., Martini, G., 2004. DOTAP/DOPE and DC-Chol/DOPE lipoplexes for gene delivery: zeta potential measurements and electron spin resonance spectra. Biochim. Biophys. Acta 1664, 70–79.

Clement, J., Kiefer, K., Kimpfler, A., Garidel, P., Peschka-Suss, R., 2005. Large-scale production of lipoplexes with long shelf-life. Eur. J. Pharm. Biopharm. 59, 35–43.

Colosimo, A., Serafino, A., Sangiuolo, F., Di Sario, S., Bruscia, E., Amicucci, P., Novelli, G., Dallapiccola, B., Mossa, G., 1999. Gene transfection efficiency of tracheal epithelial cells by DC-chol-DOPE/DNA complexes. Biochim. Biophys. Acta 1419, 186–194.

Congju, A., Pozzi, D., Esposito, C., Castellano, C., Mossa, G., 2004. Correlation between structure and transfection efficiency: a study of DC-Chol-DOPE/DNA complexes. Colloids Surf. B 36, 43–48.

Danev, R., Nagayama, K., 2006. Applicability of thin film phase plates in biological electron microscopy. Biophysics 2, 35–43.

Farhood, H., Gao, X., Son, K., Yang, Y.Y., Lazo, J.S., Huang, L., Barsoum, J., Bottega, R., Epand, R.M., 1994. Cationic liposomes for direct gene transfer in therapy of cancer and other diseases. Ann. NY. Acad. Sci. 716, 23–34, discussion 34–35.

Farhood, H., Serbina, N., Huang, L., 1995. The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer. Biochim. Biophys. Acta 1235, 289–295.

Felgner, J.H., Kumar, R., Sridhar, C.N., Wheeler, C.J., Tsai, Y.J., Border, R., Ramsey, P., Martin, M., Felgner, P.L., 1994. Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations. J. Biol. Chem. 269, 2550–2561.

Gao, X., Huang, L., 1991. A novel cationic liposome reagent for efficient transfection of mammalian cells. Biochem. Biophys. Res. Commun. 179, 280–285.

Hattori, Y., Kubo, H., Higashiyama, K., Maitani, Y., 2005. Folate-linked nanoparticles formed with DNA complexes in sodium chloride solution enhance transfection efficiency. J. Biol. Nanotechnol. 1, 176–184.

Hattori, Y., Maitani, Y., 2005. Folate-linked nanoparticle-mediated suicide gene therapy in human prostate cancer and nasopharyngeal cancer with herpes simplex virus thymidine kinase. Cancer Gene Ther. 12, 796–809.

Kiefer, K., Clement, J., Garidel, P., Peschka-Suss, R., 2004. Transfection efficiency and cytotoxicity of nonviral gene transfer reagents in human smooth muscle and endothelial cells. Pharm. Res. 21, 1009–1017.

Lendemans, D.G., Myschik, J., Hook, S., Rades, T., 2005. Cationic cage-like complexes formed by DC-cholesterol, Quil-A, and phospholipid. J. Pharm. Sci. 94, 1794–1807.

Maitani, Y., Soeda, H., Wang, J., Takayama, K., 2001. Modified ethanol injection method for liposomes containing β-sitosterol β-D-glucoside. J. Liposome Res. 11, 115–125.

Maitani, Y., Yano, S., Hattori, Y., Furuhata, M., Hayashi, Y., 2006. Liposome vector containing biosurfactant-complexed DNA and entrapping DNA as herpes simplex virus thymidine kinase gene delivery system. J. Liposome Res. 16, 359–372.

Mukherjee, K., Sen, J., Chaudhuri, A., 2005. Common co-lipids, in synergy, impart high gene transfer properties to transfection-incompetent cationic lipids. FEBS Lett. 579, 1291–1300.

Nabel, G.J., Nabel, E.G., Yang, Z.Y., Fox, B.A., Plautz, G.E., Gao, X., Huang, L., Shu, S., Gordon, D., Chang, A.E., 1993. Direct gene transfer with DNA-liposome complexes in melanoma: expression, biologic activity, and lack of toxicity in humans. Proc. Natl. Acad. Sci. U.S.A. 90, 11307–11311.

Nabel, E.G., Yang, Z., Muller, D., Chang, A.E., Gao, X., Huang, L., Cho, K.J., Nabel, G.J., 1994. Safety and toxicity of catheter gene delivery to the pulmonary vasculature in a patient with metastatic melanoma. Hum. Gene. Ther. 5, 1089–1094.

Ross, P.C., Hui, S.W., 1999. Lipoplex size is a major determinant of in vitro lipofection efficiency. Gene Ther. 6, 651–659.

Sternberg, B., Sorgi, F.L., Huang, L., 1994. New structures in complex formation between DNA and cationic liposomes visualized by freeze-fracture electron microscopy. FEBS Lett. 356, 361–366.

Tranchant, I., Thompson, B., Nicolazzi, C., Mignet, N., Scherman, D., 2004. Physicochemical optimisation of plasmid delivery by cationic lipids. J. Gene Med. 6, S24–S35.

Turek, J., Dubertret, C., Jaslin, G., Antonakis, K., Scherman, D., Pitard, B., 2000. Formulations which increase the size of lipoplexes prevent serum-associated inhibition of transfection. J. Gene Med. 2, 32–40.

Vigneron, J.P., Oudrhiri, N., Fauquet, M., Vergely, L., Bradley, J.C., Basseville, M., Lehn, P., Lehn, J.M., 1996. Guanidinium-cholesterol cationic lipids: efficient vectors for the transfection of eukaryotic cells. Proc. Natl. Acad. Sci. U.S.A. 93, 9682–9686.

Wiseman, J.W., Goddard, C.A., McLelland, D., Colledge, W.H., 2003. A comparison of linear and branched polyethylenimine (PEI) with DCChol/DOPE liposomes for gene delivery to epithelial cells in vitro and in vivo. Gene Ther. 10, 1654–1662.

Wrobel, I., Collins, D., 1995. Fusion of cationic liposomes with mammalian cells occurs after endocytosis. Biochim. Biophys. Acta 1235,296–304.

Zhang, Y.P., Reimer, D.L., Zhang, G., Lee, P.H., Bally, M.B., 1997. Self-assembling DNA-lipid particles for gene transfer. Pharm. Res. 14,190–196.

Zhou, X., Huang, L., 1994. DNA transfection mediated by cationic liposomes containing lipopolylysine: characterization and mechanism of action. Biochim. Biophys. Acta 1189, 195–203.

Zuidam, N.J., Barenholz, Y., 1998. Electrostatic and structural properties of complexes involving plasmid DNA and cationic lipids commonly used for gene delivery. Biochim. Biophys. Acta 1368,115–128.