Macromolecular Research, Vol. 15, No. 3, pp 280-283 (2007)

Preparation of Cationic Liposome Containing a Novel Water-Soluble Detergent and Its Application to Gene Delivery *In Vitro*

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Received October 11, 2006; Revised Jnauary 26, 2007

Introduction

As gene delivery carrier vectors, various synthetic nonviral vectors have been developed over the past decade.[1,2] The synthetic vehicles for encapsulation and delivery of therapeutic genes are cationic liposomes and polymers, or the combination of both vectors. Some recent studies show that they express sufficient amounts of therapeutically active compounds in distal tumors, causing reduced tumor growth.[3] For the synthetic carriers studied so far, liposome system is one of the most promising carriers for delivering therapeutic genes as well as biologically active molecules into mammalian cells.[4-8] However, low efficiency of synthetic nonviral gene carriers including liposome has been the major limitation for their successful clinical applications. In addition, for liposomal gene delivery systems, the stability problem of the liposome as well as liposome/DNA complex has been raised for systemic gene delivery and the "Stealth" liposomal system concept was introduced for the efficient liposomal drug/gene delivery applications.[9-13] As well as the problems in physicochemical properties of the liposomal gene carriers, the membranous structure of cells also gave the main barriers for gene delivery using nonviral carriers.[14]

Here, we report the novel cationic detergent-containing liposomal system and its application to gene delivery *in vitro*. The cationic detergent contains hydroxyl functional groups on its hydrophilic group, which provide the potential conjugation sites for introducing multifuntionality such as, TAT-derived peptides,[15] membrane-disrupting peptides,[16] or nucleus targeting signals.[17,18]

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Experimental

Materials. Polyethylenimine (PEI, 25 kDa), polyamidoamine dendrimer (PAMAM, generation 4), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and ethidium bromide were purchased from Sigma-Aldrich Korea. Luciferase 1000 Assay kit and Reporter Lysis Buffer were purchased from Promega (Madison, WI). The luciferase expression plasmid (pCN-Luci) was prepared as reported previously.[19] Fetal bovine serum (FBS), and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco (Gaithersburg, MD). Micro BCA™ protein assay kit was purchased from Pierce (Rocford, Il). DHDA (trade name: PB123) was kindly supplied by Homecare Research Center, LG Household & Health Care Ltd., and 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE) was supplied by Doosan Company, Korea.

Liposome Preparation. DOPE was solubilized in chloroform and dried to a thin film using a rotary evaporator. DHDA solubilized in pure water was added to the dried DOPE film and vortex mixed vigorously (molar ratio of DHDA : DOPE = 1 : 1). After overnight incubation at 4 °C for hydration, the mixture was sonicated using a bath-type sonicator. The size distribution of the liposome was determined by dynamic light scattering as described below.

Gel Retardation Assay. Liposome/plasmid complexes were prepared at various charge ratios ranging from 2 to 18 in Hepes buffered saline (HBS, 20 mM Hepes, 150 mM NaCl, pH 7.4). After 30 min incubation at room temperature for complex formation, the samples were subject to electrophoresis on a 0.7% (w/v) agarose gel containing ethidium bromide (0.5 μg/mL of the gel). After electrophoresis, the gel was analyzed on a UV illuminator to show the location of the DNA.

Dynamic Light Scattering. The hydrodynamic diameter of DHDA : DOPE liposome (1 mg/mL) was determined by dynamic light scattering.[19] The size distribution was measured using a Zetasizer 3000HS (Malvern Instruments, UK). The laser used is a nominal 5 mW He-Ne laser having a 633 nm wavelength. Scattered light was detected at a 90° angle. The refractive index (1.33) and the viscosity (0.89) of ultrapure water were used at 25 °C for measurements. Zetasizer 3000 (Advanced) Size mode v1.61 software was used for data acquisition. Data analysis was performed in automatic mode. The measured value is presented as the average size ± standard deviation of 5 runs.

Cytotoxicity Assay. The cytotoxicity of the reagents was measured by MTT assay.[20] 293 cells were seeded at a density of 2×10^4 cells/well in a 96-well microassay plate (Fal-

con), and incubated for 1 day before assay. Cells achieving 70-80% confluence were incubated with the media containing the reagents at various concentrations for 24 h. Then, 25 μL of stock solution of MTT (2 mg/mL in DPBS) were added to each well. After 4 h of incubation at 37 °C, each medium was removed and 150 μL of DMSO was added to each well to dissolve the formazan crystal formed by proliferating cells. Absorbance was measured at 570 nm using VersaMax microplate reader (Molecular Devices) and recorded as a percentage relative to the untreated control cells.

***In Vitro* Transfection.** Human embryonic kidney 293 cells were maintained in DMEM medium supplemented with 10% FBS in a 5% CO_2 incubator. For the transfection studies, 293 cells were seeded at a density of 2×10^5 cells/well in 24-well plates (Falcon), and grown for one day to reach 70-80% confluence prior to transfection. The cells were then treated with each complex solution containing 1 μg of plasmid DNA at different charge ratios for 24 h at 37 °C in the presence of 10% FBS.

PEI/pCN-Luci and PAMAM/pCN-Luci complexes were prepared at a 7/1 charge ratio (N/P, nitrogen/phophate) and at a 4/1 N/P ratio, respectively. DHDA/DOPE liposomes were mixed with pCN-Luci at charge ratios of 8, 10, and 12. After 30 min incubation at room temperature, each complex solution was added to the cells. The amount of pCN-Luci was fixed at a 1 μg/well. The cells were then incubated for one day in the incubator.

Luciferase Assay. After transfection experiments, the medium was removed and cells were washed with DPBS, followed by the addition of 150 μL of reporter lysis buffer (Promega, Madison, WI) to each well. After 30 min of incubation with mild shaking at room temperature, the cells were harvested and transferred to microcentrifuge tubes. After 15 sec of vortexing, the cells were centrifuged at 12,000 rpm for 5 min. Each lysate was transferred into a luminometer tube and luciferase activity was integrated over 10 sec with a 2 sec measurement delay in a Lumat LB 9507 luminometer (Berthold, Germany) with 50 μL of Luciferase Assay Reagent. The protein concentrations of the extracts were determined by using Micro BCA™ protein assay kit. Luciferase activity was measured in terms of relative light units (RLU) and the final values were reported as RLU/mg of total protein in the lysate.

Results and Discussion

Preparation and Features of DHDA/DOPE Liposome. The chemical structures of DHDA and DOPE are presented in Figure 1. DHDA is considered to adopt a cone-like structure similar to other amphiphilic reagents with the geometry of a large hydrophilic head group and a small hydrophobic area that form micelles in water. DOPE is also known to be one of the distinctive nonbilayer-forming lipids with a small

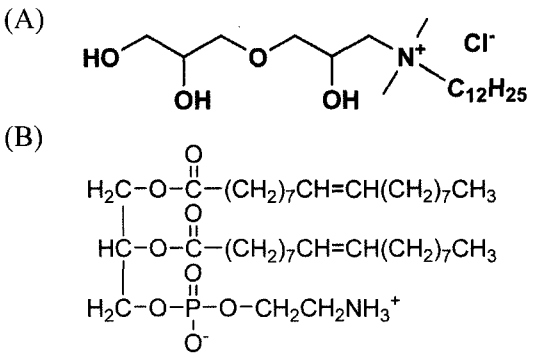

Figure 1. The chemical structures of (A) [3-(2,3-dihydroxypropoxy)-2-hydroxypropyl]dimethyl dodecyl ammonium chloride (DHDA) and (B) 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE).

head group and a large hydrophobic area. So, the complementary combination of these two amphiphilic reagents of different molecular geometry could form a bilayer structure like a liposome vesicle.[21] Another particular structural feature of the cationic detergent DHDA is that the water-soluble propylene glycol and glycerol moieties are directly linked to the quaternary ammonium head group. This property contributes to the increased water solubility of DHDA/DOPE liposome as well as the cationic detergent itself. In addition, the oligo-hydroxyl groups located on the surface of liposomes are also considered to enhance the stability in aqueous solution and may act as potential sites for chemical modification to introduce various functional moieties. We checked the liposomal solution after liposome preparation for over 8 months stored in room temperature, and there was no aggregation or precipitation due to the instability of the liposome. As shown in Figure 2, the mean diameter of DHDA/DOPE liposome was observed to be 226.5 ± 3.4 nm by dynamic light scattering method.

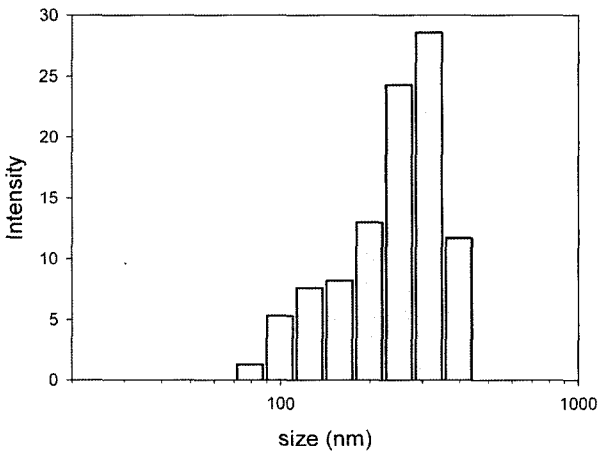

Figure 2. Dynamic light scattering of DHDA/DOPE liposome.

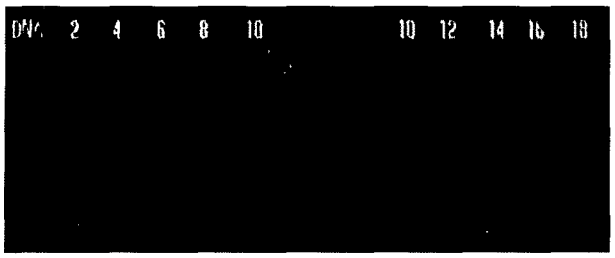

Figure 3. Analysis of complex formation of DHDA/DOPE and plasmid DNA at various charge ratios by agarose gel electrophoresis. DNA lane is 0.5 μg of pCN-Luci plasmid DNA only (Lane 1), and each number marked in respective lanes indicates the charge ratio (N/P) of DHDA/DOPE per plasmid DNA.

Analysis of Complex Formation by Agarose Gel Electrophoresis. Agarose gel electrophoresis was performed to characterize the complex formation of DHDA/DOPE with plasmid DNA. As expected, the binding and complex formation efficiency of the cationic detergent was found to be low due to the long hydrophilic constituent coupled to the ammonium group. As shown in Figure 3, complete retardation of plasmid DNA started at around charge ratio 10 (N/P, nitrogen/phosphate). This value is higher than those of conventional cationic lipids reported so far.[22-25]

Cytotoxicity Experiments *In Vitro*. The cytotoxicity of DHDA/DOPE liposome was compared with those of PEI and PAMAM at various concentration ranges for 293 cells (Figure 4). Each cell was incubated for 24 h at various concentration levels of the reagents in the presence of serum. The results showed that PEI was toxic to cells even at low concentration levels but PAMAM had much less toxicity over the experimental concentration ranges. The PEI-mediated toxicity was expected to result from the non-biodegradable feature, and high charge density with relatively high molecular weight. On the other hand, the cytotoxicity of DHDA/DOPE liposome showed much lower levels at a relatively low concentration range (below 50 μg/mL) compared with that of PEI. However, the liposome displayed high toxicity as much as PEI over 80 μg/mL. We presumed that this is related with the interaction of the cationic detergent with the cell membrane making it vulnerable to the disturbance of the plasma membrane.

Transfection Efficiency Experiments for 293 Cell Lines. To investigate the gene transfection effectiveness of DHDA/DOPE liposome, we performed *in vitro* transfection tests with human embryonic kidney 293 cell lines in the presence of serum. The amount of PEI (25 kD) and PAMAM dendrimer was adjusted to the condition in which the optimum transfection efficiency was observed. After 24 h transfection, the cell lysate was cultivated for protein assay and the expressed luciferase assay. For DHDA/DOPE liposome, the charge ratios were selected around 10 where complete retardation of the plasmid was observed by agarose gel electrophoresis. The results displayed in Figure 5 demonstrate that DHDA/DOPE exhibited the highest level of gene expression at charge ratio 8, and the more liposomes are used, the less efficient in the reporter gene expression level. A decreased efficiency at high charge ratio 12 seemed to be due to the toxicity of the elevated concentration of the cationic detergent.

Conclusions

The cationic liposome vesicle was efficiently constructed using novel cationic micelle-forming DHDA and fusogenic helper lipid, DOPE. The hydrophilic parts of DHDA reagent are composed of quaternary ammonium groups, and glycerol

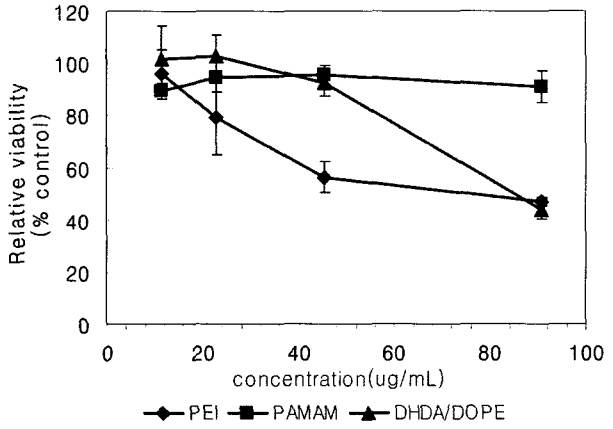

Figure 4. Effect of PEI, PAMAM, and DHDA/DOPE liposome on 293 cells viability. Relative cell viability was calculated, assuming the absorbance at 570 nm of intact control cells to be 100%. Each value is the average ± standard deviation of three different measurements.

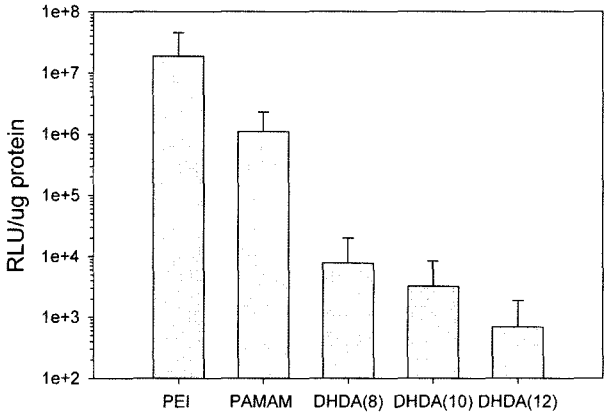

Figure 5. Transfection efficiency of polyethylenimine (25 kD), PAMAM dendrimer (G4), and DHDA/DOPE liposome (each number means a charge ratio). Each value is the mean ± standard deviation of three experiments.

and propylene glycol moieties that provide three additional hydroxyl functional groups. The structural features of DHDA contribute to the increased water-solubility and stability of DHDA/DOPE liposome in water. Because the cationic head groups are sterically hindered by such hydrophilic groups of DHDA, high charge ratios around 10 were required for effective complex formation with plasmid DNA. Even though DHDA/DOPE liposomes show some toxicity at much elevated concentration levels and much reduced transfection efficiency compared to other conventional polymeric gene carriers, the hydroxyl groups located on the surface of liposome may be useful for additional introduction of cell-specific targeting ligands and other functionally active compounds to extend the utility of DHDA/DOPE liposome.

Acknowledgements. This study was financially supported by research fund of Chungnam National University in 2005 and by the Korea Science and Engineering Foundation (R01-2006-000-10617-0) in Korea.

References

(1) D. Luo and W. M. Saltzman, *Nat. Biotechnol.*, **18**, 33 (2000).
(2) V. Vijayanathan, T. Thomas, and T. J. Thomas, *Biochemistry*, **41**, 14085 (2002).
(3) J. D. Hood, M. Bednarski, R. Frausto, S. Guccione, R. A. Reisfeld, R. Xiang, and D. A. Cheresh, *Science*, **296**, 2404 (2002).
(4) R. I. Mahato, *Adv. Drug Deliver. Rev.*, **57**, 699 (2005).
(5) V. P. Torchilin, *Nat. Rev. Drug. Discov.*, **4**, 145 (2005).
(6) V. P. Torchilin, T. S. Levchenko, R. Rammohan, N. Volodina, B. Papahadjopoulos-Sternberg, and G. G. D'Souza, *Proc. Natl. Acad. Sci. USA*, **100**, 1972 (2003).
(7) S. Y. Joo, J. S. Kim, H. J. Park, and E. K. Choi, *Macromol. Res.*, **13**, 293 (2005).
(8) S. M. Lee, J. S. Kim, Y. K. Oh, Y. B. Lee, and H. Sah, *Macromol. Res.*, **13**, 218 (2005).
(9) A. Gabizon, H. Shmeeda, A. T. Horowitz, and S. Zalipsky, *Adv. Drug Deliver. Rev.*, **56**, 1177 (2004).
(10) K. W. Mok, A. M. Lam, and P. R. Cullis, *Biochim. Biophys. Acta*, **1419**, 137 (1999).
(11) P. Tam, M. Monck, D. Lee, O. Ludkovski, E. C. Leng, K. Clow, H. Stark, P. Scherrer, R. W. Graham, and P. R. Cullis, *Gene Ther.*, **7**, 1867 (2000).
(12) M. A. Monck, A. Mori, D. Lee, P. Tam, J. J. Wheeler, P. R. Cullis, and P. Scherrer, *J. Drug Target.*, **7**, 439 (2000).
(13) J. S. Choi, J. A. MacKay, and F. C. Szoka, Jr., *Bioconjugate Chem.*, **14**, 420 (2003).
(14) M. Nishikawa and L. Huang, *Hum. Gene Ther.*, **12**, 861 (2001).
(15) C. H. Tung and R. Weissleder, *Adv. Drug. Deliver. Rev.*, **55**, 281 (2003).
(16) P. Schoen, A. Chonn, P. R. Cullis, J. Wilschut, and P. Scherrer, *Gene Ther.*, **6**, 823 (1999).
(17) J. S. Choi, K. S. Ko, J. S. Park, Y. H. Kim, S. W. Kim, and M. Lee, *Int. J. Pharm.*, **320**, 171 (2006).
(18) J. A. Gruneich, A. Price, J. Zhu, and S. L. Diamond, *Gene Ther.*, **11**, 668 (2004).
(19) J. S. Choi, K. Nam, J. Y. Park, J. B. Kim, J. K. Lee, and J. S. Park, *J. Control. Release*, **99**, 445 (2004).
(20) T. Mosmann, *J. Immunol. Methods*, **65**, 55 (1983).
(21) I. M. Hafez and P. R. Cullis, *Adv. Drug Deliver. Rev.*, **47**, 139 (2001).
(22) J. S. Choi, E. J. Lee, H. S. Jang, and J. S. Park, *Bioconjugate Chem.*, **12**, 108 (2001).
(23) S. Kim, J. S. Choi, H. S. Jang, H. Suh, and J. S. Park, *Bull. Korean Chem. Soc.*, **22**, 1069 (2001).
(24) S. Han, R. I. Mahato, and S. W. Kim, *Bioconjugate Chem.*, **12**, 337 (2001).
(25) Y. Lee, H. Koo, Y. B. Lim, Y. Lee, H. J. Mo, and J. S. Park, *Bioorg. Med. Chem. Lett.*, **14**, 2637 (2004).