Gene Transfection into HeLa Cells by Vesicles Containing Cationic Peptide Lipid

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Lipid vesicles are potentially useful as microcapsules for drug and/or gene delivery. We developed cationic lipid vesicles consisting mainly of sorbitan monooleate (Span 80) and cationic peptide lipid (CPL), and evaluated the CPL vesicles as gene transfection vectors. The optimum CPL concentration for gene transfection into HeLa cells was found to be 20 wt % of total lipid, and such CPL vesicles did not exhibit significant cytotoxicity. Co-culture of Poly-L-lysine and plasmids prior to making CPL vesicle-plasmid complexes was effective. Lipofection using LipofectAMINE was suppressed in 10% serum-supplemented medium. The transfection efficiency of 20 wt % CPL vesicles, however, was not affected by serum in the medium when plasmids were treated with poly-L-lysine.

Key words: cationic lipid vesicle; lipofection; gene transfection; HeLa cell; luciferase

Gene transfection into eukaryotic cells has become an important technique for analysis of gene function, production of recombinant gene products, and gene therapy. Both viral and nonviral systems have been used for gene transfection, and each provides unique advantages. Viral-mediated gene transfer has a number of advantages over nonviral methods, such as higher DNA uptake and gene expression efficiencies. But, these methods also present problems associated with parent viruses, such as immunogenicity and oncogenicity.1,2 On the other hand, cationic liposomes have frequently been used as nonviral DNA carriers for gene transfection.3–8 Various cationic lipids have been developed over the past several years, resulting in that several are capable of achieving gene transfer. The lipids used in preparation of cationic lipid vesicles vary greatly and have both monocation and polycation head groups.9,10 Cationic liposome-mediated gene transfer is safer and simpler than virus-mediated transfection,11–13 and offers higher transfection efficiencies than other nonviral gene delivery methods, such as electroporation or calcium phosphate precipitation.14,15 But, there are numerous issues that must be resolved before the application of cationic liposomes to therapeutic gene delivery. These include the finite lifetime of the transfection complex, inactivation of the gene/liposome complex by serum protein, degradation in the endosome compartment, and transfer to the nucleus. In order to overcome these problems, several lipid and non-lipid chemical substances have been developed. For example, DOSPA-based formulations are available commercially and have been used extensively in both in vivo and in vitro studies.16

Adapting this system for efficient in vivo gene transfection requires major improvements in order to obtain small, stable DNA/liposome complexes.

In the present study, we developed a lipid-based transfection method that uses cationic lipid vesicles. For practical applications, cationic lipid vesicles of submicrometer size composed of inexpensive, commercially available, artificial amphiphilic lipids have been used. Vesicles composed of non-ionic surfactant, Span 80 (sorbitan monoooleate), Tween 80 (polyoxyethylene (20)
sorbitan monooleate), soybean lecithin, cholesterol, and cationic lipids were prepared by a modified two-step emulsification procedure. In terms of physicochemical properties, the resulting vesicles were similar to phospholipids liposomes, and thus may be regarded as members of the liposome family or inexpensive liposome alternatives. These lipid vesicles are potentially useful as microcapsules for drug and/or gene delivery, and we have reported the usefulness of immovesicles for target-specific drug delivery.

In this paper, a newly synthesized cationic peptide lipid (CPL) was used in the preparation of cationic peptide-lipid vesicles. It has been reported that amphiphiles containing an amino acid residue, interposed between the polar head group and the aliphatic double chain, form stable single-walled vesicles in aqueous media. We used N,N-dihexadecyl-Nor-l-seryl-(trihexylammonio)hexanyloyl-l-alaminamide bromide (CPL) as a vesicle-forming lipid. The physicochemical properties of the resulting single-walled vesicles, such as vesicle diameter, bilayer thickness, number of lipid molecules in the outer and inner surfaces of the vesicles, and phase transition behavior, are comparable to those of naturally occurring phospholipids such as egg lecithin liposomes, although CPL vesicles are much more stable morphologically than those of egg lecithin. In the present study, we investigated the efficiency of DNA transfection using Span 80 and CPL vesicles.

Materials and Methods

Reagents. Span 80 (MW 700) and Tween 80 (MW 1,207) were purchased from Wako Pure Chemical (Osaka, Japan). Cholesterol was purchased from Sigma (St. Louis, MO). Crude soybean phospholipids were obtained from Wako Pure Chemical and purified using the acetone precipitation method. The crude soybean phospholipids were purified and analyzed by HPLC using a silica column (Wakosil C-200, Wako Pure Chemical) to yield 30 wt % phosphatidylethanolamine, 25 wt % phosphatidylcholine, 17 wt % phosphatidylinositol, 11 wt % phosphatidic acid, and 3 wt % lysophosphatidylcholine. The remaining lipids were glycolipids and natural lipids. N,N-dihexadecyl-Nor-l-seryl-(trihexylammonio)hexanyloyl-l-alaminamide bromide (CPL) was prepared according to methods reported previously. The molecular structure of CPL is shown in Fig. 1.

Plasmid vector. The expression vector was constructed by insertion of luciferase as a reporter gene into a pcDNA3 vector (Invitrogen, Carlsbad, CA). Green fluorescence protein (GFP) expression plasmid (pEGFP) was purchased from Clontech (Mountain View, CA). The vectors were amplified in E. coli and purified using a Plasmid Maxi kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocols.

Preparation of cationic lipid vesicles. Micrometer-sized lipid vesicles were prepared by a two-step emulsification technique, as described previously. The buffer solution for vesicle preparation was TE (10 mM Tris, 1 mM EDTA). In the first step, 4.0 ml of n-hexane solution containing 132 mg Span 80, 12 mg soybean lecithin, 6 mg cholesterol, and CPL (13 mg to 40 mg) was added to a test tube (20 mm diameter, 95 mm height). TE (0.3 ml) was then added dropwise and the system was sonicated using a supersonicator (US-150U; Nihon Seiki, Japan) three times at 15-s intervals. The inner aqueous phase of the water-in-oil emulsion was composed of TE. This emulsion was then transferred to a round bottom flask, and n-hexane was removed in a rotary evaporator, leading to the formation of a creamy layer of water and lipid, which adhered to the inner wall of the flask. After complete evaporation of n-hexane, 4.0 ml of TE containing 10 mM Tween 80 was added as a second emulsifying agent. The mixture was stirred by homomixer at a constant speed of 1,000 x g for 1 min, and the resulting heterogeneous suspension was transferred to a 5 ml brown bottle and stirred by magnetic stirrer for 3 h. The solution was centrifuged overnight to remove n-hexane completely. Finally, the solution was centrifuged to remove the oil mass.

Transfection to HeLa cells. HeLa cells were purchased from ATCC and used as host cells for gene transfection. Cells were cultured in Modified Eagle’s Medium (MEM) supplemented with 10% fetal bovine serum (FBS), 2 mM l-glutamine, 0.375% sodium bicarbonate, 100 U/ml penicillin, and 100 μg/ml streptomycin, and incubated in a humidified 5% CO2 atmosphere at 37°C.

In this study, LipofectAMINE (Invitrogen, Carlsbad, CA) was used as a positive control for lipofection. HeLa cells were prepared in a 12-well culture plate (3 x 105/ ml/well) 1 d before transfection. PLUS reagent (Invitrogen) and 0.7 μg of pcDNA3-luciferase or pEGFP were mixed and then incubated for 15 min at room temperature. The plasmid solution was added to 2 μl of LipofectAMINE or the optimal volume of the vesicles and the mixture was incubated at room temperature for 20 min. Before transfection, cells were washed once with PBS, and 400 μl of OPTI-MEM (Invitrogen) was added to each well. One hundred microliters of plasmid/vesicle complex solution was added to each well and the mixture was incubated at 37°C for 3 h. After incubation, 500 μl of MEM supplemented with 20% FBS was added to each well, and the cells were cultured for a further 48 h in a CO2 incubator.
Nucleic acid binding proteins. Protamine, poly-L-Lysine (M.W. 2,000–4,000), and histone proteins were purchased from Sigma. These reagents were used to produce plasmid/vesicle complexes instead of PLUS reagent at the final concentrations of 75 mg/ml of protamine, 0.5 mM of poly-L-Lysine, and 12.5 mg/ml of histone proteins respectively.

Luciferase assay. Luciferase assay was performed using the Luciferase Assay System (Promega, Madison, WI) according to the manufacturer’s instructions, and its activity was measured by Luminescencer JNR (ATTO, Tokyo, Japan). The transient levels of luciferase expression in the cells were represented in terms of relative luciferase units (RLU) based on the chemiluminescence of the lysis solution.

Expression of GFP. Expression of GFP was detected by flow cytometry (FACS Calibur; BD Biosciences, San Jose, CA), and analyzed using CellQuest software (BD Biosciences).

XTT assay. XTT (2,3-bis[3-methoxy-4-nitro-5-sulfo-phenyl]-2H-tetrazolium-5-carboxanilide inner salt) was used to evaluate cell growth. This colorimetric assay is based on the bioreduction of a tetrazolium salt to an intensely colored formazan, which is directly related to the activity of cellular mitochondrial enzymes. XTT solution was added to the culture medium and incubated for 6 h in a CO\textsubscript{2} incubator at 37°C. The spectrophotometric absorbance of the culture medium was measured at 450 nm, and was detected using a model 550 microplate reader (Bio-Rad, Hercules, CA).

Results and Discussion

Characteristics of the CPL vesicles

Using our emulsification technique (see “Materials and Methods”) with soybean phospholipids, cholesterol, Span 80, Tween 80, and CPL, we obtained stable suspensions containing submicrometer-sized vesicles with diameters in the range of 50–200 nm. Previously we confirmed and reported that the vesicle boundaries are true bilayers, and in the case of w/o/w (water-in-oil-in-water) double emulsions, the boundaries are swollen bilayers that contain an oily bilayer interior. In both cases the trapped volume is mainly aqueous. Because the content of oily compounds in the present study was rather low (sorbitan trioleate in Span 80, and PEG(20) sorbitan trioleate in Tween 80), it is likely that the suspension prepared was not a w/o/w double emulsion, but rather contained vesicles, with a slightly extended bilayer thickness.

Optimization of conditions for gene transfer by CPL vesicles

The transfection efficiency of the cationic lipid vesicles composed of Span 80 and CPL were tested using HeLa cells as a target. In preliminary experiments, we confirmed that maximum gene transfection was obtained by pre-conjugation of plasmid and CPL vesicles for 15 min followed by treatment of the HeLa cells with the plasmid/CPL vesicle complex for 3 h before the addition of 20% FBS-MEM medium (data not shown).

Evaluation of cytotoxicity and transfection efficiency of CPL vesicles

The cytotoxicity of cationic lipid vesicles is one of the factors that interferes with effective transfection. The 20 wt % CPL vesicles were diluted with TE buffer to give solutions of 0.05, 0.5, and 2.0 at 600 nm absorbance. This series of solutions was added to Hela cells in 10% FBS-MEM at 0.4 v/v%, and the cells were cultured for a further 7 d. On day 7, cell viability was determined by XTT assay, as described in “Materials and Methods”. As shown in Fig. 4, no cytotoxicity was observed at any of the vesicle concentrations tested.
Effects of nucleic acid binding proteins on gene transfection by CPL vesicles

It is well-known that several nucleic acids enhance the transfection efficiency of the plasmid/liposome complex. In our preliminary experiments, we tested and compared several nucleic acid binding proteins, including protamine, poly-L-lysine, and histone, in order to determine their capacities to enhance gene transfection. As Fig. 5A suggests, poly-L-lysine induced higher transfection efficiency than the other proteins we tested (protamine and histone), almost equivalent to

![Graph](image_url)

**Fig. 3.** Optimum Concentration of CPL Vesicles for Gene Transfection.

CPL vesicles were diluted with TE buffer and turbidity was measured at 600 nm. Each CPL vesicle solution was mixed with 0.7 μg of pcDNA3-luciferase treated with PLUS reagent. HeLa cells were treated with plasmid/CPL vesicle complex for 3 h in serum-free OPTI-MEM. Following transfection, MEM containing 20% FBS was added to each well, the cells were cultured for 48 h, and luciferase assay was performed. Data represent means ± SD of three independent measurements.

![Graph](image_url)

**Fig. 4.** Cytotoxicity of CPL Vesicles.

HeLa cells were cultured for 7 d in the presence of various concentrations of CPL vesicles. Viable cell density was evaluated by XTT assay. Data represent means ± SD of three independent measurements.

![Graph](image_url)

**Fig. 5.** Effects of Nucleic Binding Proteins on CPL Vesicle-Mediated Gene Transfer.

A, 0.7 μg of plasmids were treated with 7.5 μg/ml of protamine, 50 μM poly-L-lysine, and 1.25 μg/ml of histone or PLUS reagent prior to mixing with CPL vesicles. HeLa cells were treated with gene/CPL vesicle complex for 3 h in serum-free OPTI-MEM. Following transfection, MEM medium containing 20% FBS was added to each well, the cells were cultured for 48 h, and luciferase assay was performed. B, Combination of protamine, poly-L-lysine, histone. Data represent means ± SD of three independent measurements. Pro, protamine; PLL, poly-L-lysine; H, histone.
that of PLUS reagent in LipofectAMINE and CPL vesicles. The viability of transfected cells was almost 80% in LipofectAMINE treated cells and in CPL vesicle treated cells (data not shown). Combination of poly-L-lysine with other proteins did not show any synergistic effects on transfection efficiency (Fig. 5B). The major role of these proteins is thought to be the formation of a complex with DNA, which can have a compact structure, and then to deliver DNA into the nucleus. Previous reports suggest that cell uptake of DNA is facilitated by poly-L-lysine,26,27) and this might be why poly-L-lysine induced high transfection efficiency. Based on those results, we decided to use poly-L-lysine as a transfection enhancer.

Effect of serum on gene transfection by CPL vesicles

It is well-known that serum in the medium inhibits the transfection efficacy of lipofection,28–30) and this represents a serious problem for in vivo gene delivery systems. As shown in Fig. 6, the transfection efficiency of LipofectAMINE was clearly suppressed by the coexistence of 10% FBS in the medium, but the transfection efficiency of CPL vesicles was not affected by FBS when plasmids were treated with poly-L-lysine. As Fig. 7 shows, the expression intensity of GFP in Hela cells treated with CPL vesicle was higher than that of those treated with LipofectAMINE under 10% FBS conditions. These results suggest that CPL vesicles are able efficiently to transduce genes into cells in serum-containing medium, and that CPL vesicles might make for better transfection efficiency in vivo.

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![Fig. 6. Effect of FBS on Transduction Efficiency Determined by Luciferase Activity.](image)

CPL vesicles were mixed with 0.7 μg of plasmid treated with poly-L-lysine or PLUS reagent. Gene transfection was performed in the absence or presence of 10% FBS for 3 h at 37 °C, and the cells were cultured for 48 h. The open and closed bars show the absence and presence of serum respectively. Data represent means ± SD of three independent measurements.

![Fig. 7. Effect of FBS on Transduction Efficiency Determined by GFP Expression.](image)

CPL vesicles were mixed with 0.7 μg of plasmid treated with poly-L-lysine. Gene transfection was performed in the presence of 10% FBS for 3 h at 37 °C, and the cells were cultured for 48 h. Expression of GFP was detected and analyzed by the flow cytometry technique.


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