

Acta Medica Okayama

Volume 51, Issue 3

1997

Article 6

JUNE 1997

Gene transfection by cationic liposomes: comparison of the transfection efficiency of liposomes prepared from various positively charged lipids

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Abstract

We compared the transfection efficiency of four types of positively charged liposomes composed of (i) N-(α -trimethylammonioacetyl)-didodecyl-D-glutamate chloride (TMAG), dilauroylphosphatidylcholine (DLPC), and dioleoylphosphatidylethanolamine (DOPE) (1:2:2 molar ratio); (ii) 3 β [N-(N', N'-dimethylaminoethane)-carbonyl] cholesterol (DC-Chol) and DOPE (3:2 molar ratio); (iii) dimethyldioctadecylammonium bromide (DDAB) and DOPE (1:2.2 molar ratio); (iv) N-[1-(2,3-dioleoyloxy) propyl] -N,N,N-trimethylammonium chloride (DOTMA) and DOPE (1:1, w/w; lipofectin). Luciferase gene was used as a reporter gene. Among the cationic liposomes used, the liposomes composed of TMAG, DOPE and DLPC showed a much higher efficiency of plasmid DNA entrapment than the other cationic liposomes tested. In the absence of serum, the cationic multilamellar vesicles (MLV) and small unilamellar vesicles (SUV) composed of TMAG, DOPE and DLPC gave highly efficient transfection. On the other hand, MLV, dehydration-rehydration vesicles (DRV), and SUV liposomes prepared with the mixtures of DC-Chol and DOPE showed similar levels of transfection efficiency. However, the cationic liposomes composed of DDAB and DOPE showed inferior efficiency, whether in the form of DRV, SUV or MLV. The transfection efficiency of lipofectin was also low. In the presence of serum, on the other hand, a considerable (about 30-50%) amount of transfection activity was still observed at 10% fetal calf serum in the cationic MLV and SUV composed of TMAG, DOPE and DLPC. Cationic MLV, composed of TMAG, DOPE and DLPC, can transfect plasmid DNA, not only in the adherent cell lines but also in the suspension cell lines. These findings indicate that the transfection efficiency of cationic liposomes is affected by the lipid composition, the type of liposome, or the presence or absence of serum. They also indicate that the cationic liposomes containing TMAG, DOPE and DLPC are efficient vectors for gene transfer into cells.

KEYWORDS: cationic liposome, luciferase, plasmid DNA, transfection efficiency

*PMID: 9227794 [PubMed - indexed for MEDLINE]

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Gene Transfection by Cationic Liposomes: Comparison of the Transfection Efficiency of Liposomes Prepared from Various Positively Charged Lipids

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We compared the transfection efficiency of four types of positively charged liposomes composed of (i) *N*-(α -trimethylammonioacetyl)-didodecyl-*D*-glutamate chloride (TMAG), dilauroylphosphatidylcholine (DLPC), and dioleoylphosphatidylethanolamine (DOPE) (1:2:2 molar ratio); (ii) 3 β [N-(*N*', *N*'-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) and DOPE (3:2 molar ratio); (iii) dimethyldioctadecylammonium bromide (DDAB) and DOPE (1:2.2 molar ratio); (iv) *N*-[1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTMA) and DOPE (1:1, w/w; lipofectin). Luciferase gene was used as a reporter gene. Among the cationic liposomes used, the liposomes composed of TMAG, DOPE and DLPC showed a much higher efficiency of plasmid DNA entrapment than the other cationic liposomes tested. In the absence of serum, the cationic multilamellar vesicles (MLV) and small unilamellar vesicles (SUV) composed of TMAG, DOPE and DLPC gave highly efficient transfection. On the other hand, MLV, dehydration-rehydration vesicles (DRV), and SUV liposomes prepared with the mixtures of DC-Chol and DOPE showed similar levels of transfection efficiency. However, the cationic liposomes composed of DDAB and DOPE showed inferior efficiency, whether in the form of DRV, SUV or MLV. The transfection efficiency of lipofectin was also low. In the presence of serum, on the other hand, a considerable (about 30-50%) amount of transfection activity was still observed at 10% fetal calf serum in the cationic MLV and SUV composed of TMAG, DOPE and DLPC. Cationic MLV, composed of TMAG, DOPE and DLPC, can transfect plasmid DNA, not only in the adherent cell lines but also

in the suspension cell lines. These findings indicate that the transfection efficiency of cationic liposomes is affected by the lipid composition, the type of liposome, or the presence or absence of serum. They also indicate that the cationic liposomes containing TMAG, DOPE and DLPC are efficient vectors for gene transfer into cells.

Key words: cationic liposome, luciferase, plasmid DNA, transfection efficiency

Liposomes are bilayer vesicles composed of amphiphilic phospholipids. They are non-toxic, biodegradable and poorly immunogenic. Furthermore, liposome-entrapped materials are protected from enzymatic attack until they reach their target sites.

Although liposomes have been used for *in vitro* gene transfer in the cells in culture (1-6), the greatest potentiality for liposome-mediated gene transfer seems to lie in its *in vivo* applications. The potential usefulness of liposomes as a vehicle for gene transfer has attracted considerable interest during the last few years (7-11). In particular, positively charged liposomes, which are composed of cationic lipids, are efficient tools for gene transfer into cells (12-18). Thus far, experiments involving gene delivery *in vitro* and *in vivo* to cells with various cationic liposomes have been reported (12-29). However, the respective transfection efficiency of various methods has not been comparatively studied as yet. Therefore, it is important to elucidate the transfection efficiency of various cationic liposomes into cells for their practical application to gene transfer *in vitro* and *in vivo*. The present paper

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reports the transfection efficiency of positively charged liposomes composed of synthetic cationic lipids, such as *N*-(α -trimethylammonioacetyl)-didodecyl-*D*-glutamate chloride (TMAG), 3β [*N*-(*N*', *N*'-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol), dimethyldioctadecylammonium bromide (DDAB), or *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTMA).

Materials and Methods

Materials. TMAG was received from Sogo Pharmaceutical Co., Ltd., Tokyo, Japan; dioleoylphosphatidylethanolamine (DOPE) was purchased from Avanti Polar Lipids, Birmingham, AL, USA; DDAB from Kanto Chemical Co., Inc., Tokyo, Japan; dilauroylphosphatidylcholine (DLPC) from Sigma (Sigma Chemical Co., St. Louis, MO, USA); 4', 6-diamidino-2-phenylindole (DAPI) from Nacalai Tesque, Inc., Kyoto, Japan. Lipofectin came from Gibco BRL (Gibco BRL, Gaithersburg, MD, USA). DC-Chol was synthesized according to the method of Gao and Huang (17). Plasmid pSR α /L- Δ 5', (a gift from Dr. Y. Takebe, National Institute of Health Japan, Tokyo, Japan), which allows expression of firefly luciferase driven by the SR α promoter (30), was amplified in *Escherichia coli* JM109 and purified by the cesium chloride gradient centrifugation method.

Cell lines. COS-1 cells (African green monkey cell line CV transfected with simian virus 40), bovine embryonic kidney (BEK) cells, fetal lamb kidney (FLK) cells and C8 cells (cat cells containing the murine sarcoma virus genome) were used. They were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal calf serum (FCS) and antibiotics. The human lung cancer cell line, H226Br, and a mouse myeloma cell line, P3-X63-Ag8-U1 (P3U1), were also used in the present study. They were maintained in RPMI-1640 medium supplemented with 10% FCS and antibiotics.

Preparation of liposomes. Cationic liposomes entrapping plasmids were prepared from a lipid mixture solution containing TMAG, DLPC and DOPE in a molar ratio of 1:2:2 (total amount, 0.5 μ mol), DC-Chol and DOPE in a molar ratio of 3:2 (total amount, 0.5 μ mol), and DDAB and DOPE in a molar ratio of 1:2.2 (total amount, 0.96 μ mol), respectively.

For the preparation of multilamellar vesicles (MLV) containing plasmids, the lipids dissolved in chloroform-methanol (1/1, v/v) were dried in a conical flask under

reduced pressure and stored *in vacuo* for 30 min. Next, 250 μ l of sterilized phosphate-buffered saline (PBS, pH 7.2) containing 20 μ g of plasmids was added to a lipid film composed of TMAG, DLPC and DOPE (molar ratio 1:2:2), and DC-Chol and DOPE (molar ratio 3:2). Alternatively, 480 μ l of sterilized PBS containing 38.4 μ g of plasmids was added to a lipid film composed of DDAB and DOPE (molar ratio 1:2.2). They were left at room temperature for 30 min, then vortexed for 2 min. Untrapped plasmids were removed by centrifugation at 25,000 $\times g$ (for liposomes composed of TMAG, DLPC and DOPE, and liposomes consisting of DC-Chol and DOPE) or 120,000 $\times g$ (for liposomes composed of DDAB and DOPE) for 30 min.

Dehydration-rehydration vesicles (DRV) containing plasmids were prepared as follows: The lipids were mixed in a conical flask. After evaporation of the solvent by a rotary evaporator and then by a vacuum pump, 0.5 ml of sterilized distilled water was added, and the lipid film was dispersed by vortexing for 2 min. After addition of plasmids, the liposomal suspension was freeze-dried. The dried lipid mixture was hydrated with sterilized PBS. After incubation for 2 h at room temperature, unencapsulated plasmids were removed by centrifugation at 15,000 $\times g$ for 30 min.

Small unilamellar vesicles (SUV) were prepared by sonication of the lipid suspension. Briefly, a thin lipid film was dispersed in 0.5 ml of PBS by vortexing, and sonicated with a probe-type sonicator (Branson Sonifier 250, Branson Sonic Power Co., Danbury, CT, USA) at 20 W for 5 min. For preparation of SUV-plasmid complexes, plasmid DNA was mixed with the SUV suspension. Uncaptured plasmids were removed by centrifugation at 120,000 $\times g$ for 30 min. The pellets were resuspended in sterilized PBS.

DNA determination. The amount of DNA entrapped in liposomes or complexed with liposomes was determined by the method described previously (31). Briefly, a 20 μ l suspension of liposome-entrapped plasmids or standard DNA solution containing 0.5–2 μ g of DNA was added to 200 μ l of 1.5 M NaCl/30 mM ethylenediaminetetraacetic acid (EDTA, pH 8.0), followed by vortex mixing. The resulting solution was mixed with 300 μ l of a mixture of chloroform and methanol (2:1, v/v). After centrifugation at 13,000 $\times g$ for 5 min, the aqueous phase (200 μ l) was mixed with 1 ml of 10 mM Tris-HCl buffer (pH 7.0) containing 100 ng/ml DAPI, 100 mM NaCl and 100 mM EDTA, and the fluorescence

of DNA-DAPI complexes was subsequently measured at 450nm with excitation at 360nm with a Hitachi fluorescence spectrophotometer F-3010 (Hitachi Co., Ltd., Tokyo, Japan).

Liposome-mediated transfection. Cells (1×10^5) were plated on 24 well plates in 0.4ml of medium supplemented with 10 % FCS and incubated at 37°C for 24h. After incubation at 37°C for 24h, the plates were divided into two groups as follows. One was transfected in the presence of 10 % FCS; after incubation, 0.1µg of DNA-containing liposomes were added to the cells, followed by addition of 0.4ml of medium containing 10 % FCS, and then the cells were further incubated at 37°C for 48h. The other was transfected in the absence of serum; after incubation, the medium was exchanged with 0.4ml of fresh medium without serum, and then DNA-containing liposomes were added to the cells. After incubation at 37°C for 3h, 0.4ml of medium supplemented with 20 % FCS was added. After the cells were further incubated at 37°C for 48h, they were harvested and used for luciferase assay.

Transfection with lipofectin was performed according to the supplier's instructions. The ratio of plasmid DNA to lipofectin reagent was 10µg of DNA/50µl reagent. The cells were treated in the same manner as described above.

Luciferase assay. Transfection efficiency was evaluated by the luciferase assay using a luciferase assay system kit (Promega Co., Madison, WI, USA). After 48h of incubation, the cells were washed twice with PBS, covered with 50µl of cell culture lysis reagent (25mM Tris-phosphate, pH 7.8, containing 2mM dithiothreitol, 2mM 1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid, 10 % glycerol, and 1 % Triton X-100), and incubated for 15min at room temperature. The cell suspension was centrifuged briefly in a microcentrifuge. The supernatant was then subjected to an assay for luciferase activity and protein quantification.

The enzymatic activity of luciferase was measured as follows: 100µl of luciferase assay reagent [20mM Tricine, 1.07mM (MgCO₃)₄Mg(OH)₂ · 5H₂O, 2.67mM MgSO₄, 0.1mM EDTA, 33.3mM dithiothreitol, 270µM coenzyme A, 470µM luciferin, and 530µM ATP] was added to 15µl of the supernatant in a 96 well microplate (MS-8496K, Sumitomo Bakelite, Tokyo, Japan). Integrated light output (luciferase activity) was measured for 1min with a micro lumino reader (CORONA MLR-100, Corona Electric Co., Ltd., Hita-

chinaka, Japan) and expressed as "unit/µg protein/min".

The protein concentration of the cell lysate was quantitated using Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA, USA) and bovine plasma gamma globulin was used as a standard.

Statistical analysis. Student's *t*-test was performed for statistical evaluation of the results. Results are expressed as the arithmetic mean with the standard deviation of the mean (mean ± SD).

Results and Discussion

Efficiency of DNA entrapment. The efficiency of liposomes for encapsulating plasmid DNA was examined using different cationic liposomes.

As shown in Table 1, the cationic liposomes composed of TMAG, DOPE, and DLPC (TMAG-liposomes) displayed a much higher efficiency for plasmid DNA entrapment than the others tested, *i.e.*, liposomes composed of DC-Chol and DOPE (DC-Chol-liposome) and of DDAB and DOPE (DDAB-liposome), regardless of the type of liposomes tested, *i.e.*, MLV, DRV or SUV. In addition, the encapsulating efficiency of DNA by TMAG-liposome was not much different according to their forms. These observations may be ascribed to the strong electrostatic interaction between negatively charged phosphate group of DNA and the positively charged surface of TMAG-liposome, because TMAG has strongly positive charges (14, 25). On the other hand, in the DC-Chol-liposome and DDAB-liposome, their encapsulating efficiency for DNA was quite different according

Table 1 Efficiency of different cationic liposomes for entrapment of DNA

| Form ^b | Efficiency (%) ^a | | |
|-------------------|-----------------------------|------|------|
| | I ^c | II | III |
| MLV | 90.8 | 23.1 | 8.3 |
| DRV | 78.5 | 64.5 | 10.5 |
| SUV | 83.2 | 76.1 | 36.2 |

^a: The efficiency of liposomes for entrapment of DNA is expressed as the percentage of DNA recovered to the total DNA used.

^b: MLV, multilamellar vesicles; DRV, dehydration-rehydration vesicles; SUV, small unilamellar vesicles.

^c: I, liposomes composed of TMAG, DLPC and DOPE (1:2:2 molar ratio); II, liposomes composed of DC-Chol and DOPE (3:2 molar ratio); III, liposomes composed of DDAB and DOPE (1:2.2 molar ratio).

Table 2 Transfection efficiency of different cationic liposomes^a

| Lipid composition | Form ^b | Luciferase activity (light units/mg protein/min) | |
|---------------------------------------|-------------------|--------------------------------------------------|----------------|
| | | Without serum | With serum |
| TMAG:DLPC:DOPE (1:2:2 molar ratio) | MLV | 4481.9 ± 785.4 | 2241.4 ± 254.8 |
| | DRV | 271.5 ± 40.8 | 75.8 ± 17.1 |
| | SUV | 2124.5 ± 197.8 | 726.1 ± 86.5 |
| DC-Chol:DOPE (3:2 molar ratio) | MLV | 868.3 ± 89.9 | 38.3 ± 8.4 |
| | DRV | 551.3 ± 86.9 | 187.6 ± 25.8 |
| | SUV | 595.4 ± 115.5 | 177.9 ± 21.3 |
| DDAB:DOPE (1:2.2 molar ratio) | MLV | 21.4 ± 7.1 | 9.0 ± 1.4 |
| | DRV | 8.6 ± 2.1 | 0.6 ± 0.2 |
| | SUV | 6.3 ± 2.3 | 4.2 ± 0.9 |
| Lipofectin | SUV | 27.4 ± 8.0 | 9.0 ± 1.5 |

^a: Transfection was performed with 0.1 μg of DNA-containing cationic liposomes. Data are given as mean ± SD.

^b: MLV, multilamellar vesicles; DRV, dehydration-rehydration vesicles; SUV, small unilamellar vesicles.

to their form. In both of these cationic liposomes, the efficiency of the SUV for DNA encapsulating was the highest (Table 1). This finding shows that, in the DC-Chol-liposome and DDAB-liposome, SUV reveals a higher entrapment efficiency than other type of cationic liposomes. Previously, in the case of complex formation between cationic liposomes and DNA, it was shown that SUV was superior to the others (18), which would explain the above observation.

Effect of lipid composition and form of liposomes on transfection activity. We studied the effect of lipid composition and type of liposome on gene transfer in the presence or absence of serum. The transfection activity of cationic liposomes was evaluated by luciferase assay. The data are summarized in Table 2.

In the absence of serum, the TMAG-liposome (MLV and SUV) showed highly efficient transfection. The transfection activity of the TMAG-liposome (DRV) was lower than that of the TMAG-liposome (MLV and SUV). On the other hand, DC-Chol-liposome showed a similar level of transfection efficiency regardless of the type of liposomes. However, the DDAB-liposome showed inferior efficiency, whether in the form of DRV, SUV or MLV. The transfection efficiency in the case of lipofectin was also low.

In the presence of serum, the TMAG-liposome (MLV and SUV) showed high transfection efficiency. However, the TMAG-liposome (DRV) showed inferior efficiency. As for the DC-Chol-liposome, although DRV and SUV liposomes showed almost the same efficiency, MLV liposomes showed a reduced activity. The transfection

activity of the DDAB-liposome (MLV, DRV and SUV) and lipofectin was very low.

In this study, we found that the transfection efficiency of the cationic liposomes was affected by the lipid composition, type of liposomes, or the presence of serum. Cationic liposomes are efficient transfection reagents for cells *in vitro* (4, 12, 18, 20, 26–28). In the present study, the fact that the various cationic lipid-containing liposomes showed different transfection efficiencies points to the importance of the electrostatic interaction between the negatively charged cell surface and the positively charged surface of the liposomal membrane for transfection. Indeed, liposomes containing TMAG which has a strongly positive charge (14, 18), revealed the highest transfection efficiency. On the other hand, the transfection efficiency of DDAB-liposome and lipofectin was very low. This may be caused by the obvious cytotoxicity on the cells by DDAB-liposome and lipofectin (28, 32).

Transfection efficiency of various cationic liposomes for entrapment of plasmid DNA was also affected by their form, *i.e.*, MLV, DRV, SUV. This is thought to be due to a discrepancy in incorporation of liposomes into cells by endocytosis or cell fusion. A plausible explanation has not yet been found to explain this result.

Cationic liposomes are usually used only for *in vitro* studies because of their sensitivity to serum components. Most cationic liposome-mediated cellular transfections are performed in the absence of serum for an initial period of at least several hours (12, 20). Recently, however, it was reported that cationic liposomes can also be utilized for DNA delivery into cells *in vivo* (19, 22–25, 30). In order

Table 3 Transfection of different mammalian cell lines with TMAG-liposome^a

| Cell line | Luciferase activity (light units/mg protein/min) |
|-----------|-----------------------------------------------------|
| COS-1 | 2363.6 ± 128.3 |
| C8 | 397.5 ± 61.6 |
| H226Br | 41.0 ± 30.6 |
| FLK | 28.7 ± 6.6 |
| P3U1 | 1.6 ± 1.1 |

^a: MLV composed of TMAG, DLPC and DOPE (1:2:2 molar ratio).
MLV: See Table 2.

to investigate the effect of serum on the transfection efficiency of liposomes composed of various cationic lipids, therefore, cationic liposome-mediated transfection efficiency was evaluated in the presence or absence of serum. In this study, the 10 % FCS produced an inhibitory effect on transfection efficiency. In particular, serum produced inhibitory effects during transfection of cationic liposomes, such as DC-Chol-liposome, DDAB-liposome, and lipofectin. However, a considerable (about 30–50 %) amount of the transfection activity was still observed at 10 % FCS concentration in TMAG-liposome of SUV and MLV. This finding indicates that the TMAG-liposome can efficiently transfer DNA in the presence of serum. Moreover, the TMAG-liposome (MLV) maintained a 50 % level of transfection activity even in the presence of serum, suggesting that the TMAG-liposome (MLV) may be useful not only for gene transfer *in vitro*, but also for gene transfer into tissue cells *in vivo*. The findings that gene transfer mediated by cationic liposomes was reduced in the presence of serum may be due to the degradation of DNA by nucleases contained in the serum, since DNA forms only an unstable complex with cationic lipids (6). Another possible explanation is that the absorption and/or fusion of cationic liposomes with cell membranes may be interfered with by serum lipoproteins (6).

Transfection efficiency of different mammalian cell lines with TMAG-liposome (MLV). The transfection efficiency of the TMAG-liposome was measured in several cell lines, such as C8, H226Br, FLK and P3U1, in the presence of serum (Table 3).

The TMAG-liposome was able to mediate gene transfer in all cell lines tested. The transfection efficiency depended on the cell line. The highest luciferase expression occurred in COS-1 and C8 cells, while the lowest

expression was found in P3U1 cells. It is known that lymphoid cells are resistant to transfection (33, 34); they are usually transfected by the electroporation method (35–37). However, it is noteworthy that the TMAG-liposome was able to transfect luciferase activity into the suspension cell line P3U1, which belongs to lymphoid cell group, though the efficiency was low. This result indicates that plasmid DNA can be transfected by the TMAG-liposome not only in the adherent cell lines but also in the suspension cell lines.

Acknowledgments. We wish to express our appreciation to Ms. J. Inagaki, Ms. K. Kaihara and Ms. Tana for technical assistance.

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Received February 10, 1997; accepted March 14, 1997.