Novel Liposomes for Efficient Transfection of β -Galactosidase Gene into COS-1 Cells*

Takuya KOSHIZAKA, Yutaka HAYASHI, and Kunio YAGI**

*Institute of Applied Biochemistry, Yagi Memorial Park,
Mitake, Gifu 505-01, Japan*

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Summary A pCH110 plasmid encoding the β -galactosidase gene of *E. coli* was effectively entrapped in novel positively charged liposomes consisting of N-(α -trimethylammonioacetyl)-didodecyl-D-glutamate chloride, dilauroyl phosphatidylcholine, and dioleoyl phosphatidylethanolamine (1 : 2 : 2, molar ratio) by an improved reverse-phase evaporation method. The DNA entrapping efficiency of these liposomes was 5-70 fold higher than that of liposomes containing no positively charged lipid. By using such positively charged liposomes, we could achieve transfection of the gene into COS-1 cells by simply adding the liposomes to the medium covering the cells; and the transfection efficiency exceeded that obtained by the calcium phosphate precipitation method, especially when a small amount of DNA was used.

Key Words: positively charged liposomes, gene transfection, β -galactosidase, COS-1 cells

Liposomes have been regarded as a useful carrier for gene transfer [1-12], and this approach became practical by the development of the reverse-phase evaporation method by Szoka and Papahadjopoulos [13] for the preparation of large unilamellar vesicles into which DNA can be encapsulated. Since the method alters DNA to some extent during the preparation procedure, mainly during the sonication step, we improved the method so as to obtain less altered DNA entrapped in liposomes [14]. In parallel with this work, we have been devoting our efforts to obtain better liposomes in terms of their entrapment and transfection of DNA, since the efficiency of gene transfer by the use of liposomes is rather low without certain aids, such as glycerol shock [3, 4], polyethylene glycol treatment [3], or

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** To whom correspondence should be addressed.

the addition of viral fusion proteins [11].

Considering that both DNA and the cell surface possess negative charges, we thought that positively charged liposomes should be suitable both for efficient entrapment of DNA and for efficient transfection of DNA into cells. Thus we screened liposomes containing various positively charged lipids for the expression of the bacterial β -galactosidase gene, and found that these liposomes can entrap a large amount of DNA as compared with commonly used liposomes and that the expression of the gene can be achieved in mammalian COS-1 cells transfected with only a small amount of the DNA entrapped in these liposomes.

MATERIALS AND METHODS

Materials. Positively charged lipids, N-(α -trimethylammonioacetyl)-dilauroyl-D-glutamate chloride (TMAG) and N-methyldidodecylamine (MDDA), were purchased from Sogo Pharmaceutical Co., Ltd., Tokyo. Dilauroyl phosphatidylcholine (DLPC), dilauroyl phosphatidylethanolamine (DLPE), dimyristoyl phosphatidylethanolamine (DMPE), dimyristoyl phosphatidylcholine (DMPC), dioleoyl phosphatidylcholine (DOPC), bovine brain phosphatidylserine (PS), egg yolk phosphatidylcholine (PC), cholesterol (Chol), *o*-nitrophenyl- β -D-galactopyranoside (ONPG), and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Sigma Chemical Co., St. Louis, MO. Dioleoyl phosphatidylethanolamine (DOPE) was obtained from Avanti Polar Lipids, Birmingham, AL. The pCH110 plasmid (7.3 kilo bases) constructed by the fusion of the *Escherichia coli lacZ* gene (β -galactosidase) to the simian virus 40 early promoter [15] was obtained from Pharmacia, Uppsala. Ficoll 400 was also obtained from Pharmacia. Dulbecco's modified Eagle's medium (DMEM) was purchased from GIBCO, Grand Island, NY. Deoxyribonuclease I (DNase) and Dulbecco's phosphate-buffered salts without Mg and Ca (DPBS-) were procured from Takara, Kyoto.

Preparation of liposomes. For the preparation of liposome-entrapped plasmids, an improved procedure [14] of the reverse phase evaporation method [13] was used to enable the entrapment of plasmids in liposomes without damage to DNA due to sonication. In the improved procedure described briefly below, reverse-phase micelles were prepared first by sonication, and then the plasmids were added to form plasmid-containing liposomes.

The mixture of positively charged lipid and phospholipids (1 μ mol of total lipids) was placed in a 10-ml test tube, and their solvent evaporated. The lipids were dissolved in 20 μ l of chloroform and 100 μ l of diethylether, and then 30 μ l of sterilized DPBS- was added, followed by sonication in a bath-type sonicator (Branson, model B-220, Shelton, CT) to form a lipid micelle dispersion. The organic solvent was evaporated at 450 mmHg until the mixture became a gel. Next, 30 μ l of DPBS- containing 20 μ g of pCH110 plasmid and 5% glycerol was mixed with the gel by vortexing, and then evaporation at 720 mmHg was carried out until an aqueous suspension was obtained. Finally sufficient DPBS- was added to give

a final suspension volume of 0.5 ml. The liposomes that had entrapped DNA were separated from unentrapped DNA by floatation on Ficoll gradients [3].

For the preparation of liposomes consisting of lipids without TMAG, the same procedure was adopted except that a larger amount of lipids (10 μ mol) was used, since the entrapping efficiency is low in the case of these liposomes.

Liposome-mediated transfection of cells. For the transfection experiments, COS-1 (ATCC CRL 1650) cells were inoculated at a density of 1×10^5 cells per 35-mm plate, incubated at 37°C for 1 day in DMEM supplemented with 10% (v/v) fetal bovine serum, penicillin G (100 U/ml), and streptomycin (100 μ g/ml) under an atmosphere of 5% CO₂ and 95% air; and a suspension of liposome-entrapped plasmids was added to 2 ml of the medium covering the cells. After incubation for 16 h at 37°C, the medium was replaced with 2 ml of fresh medium, and the cells were further incubated for 56 h at 37°C and then harvested by scraping.

The calcium phosphate precipitation method was carried out as described by Graham and van der Eb [16] by treatment with 15% glycerol for 2 min at room temperature.

β -Galactosidase assay. Lysates of harvested cells were prepared by three freeze-thaw cycles in 300 μ l of DPBS—. Assay of the enzyme was made according to Miller [17]: 50 μ l of the lysate, 200 μ l of 100 mM sodium phosphate buffer (pH 7.0) containing 10 mM KCl, 1 mM MgCl₂, and 50 mM β -mercaptoethanol, and 50 μ l of 4 mg/ml ONPG in 100 mM sodium phosphate buffer (pH 7.0) were mixed and incubated at 37°C for 30 min. The reaction was stopped by the addition of 200 μ l of 1 M Na₂CO₃. The optical density of the reaction mixture was measured at 420 nm. The enzyme activity was expressed as nmol of ONPG cleaved per min per mg protein, and the amount of protein was determined according to Markwell *et al.* [18].

DNA determination. The amount of DNA entrapped in liposomes was determined according to Brunk *et al.* [19]: to a 100- μ l suspension of liposome-entrapped plasmids or standard DNA solution containing 0.05–2 μ g of DNA was added 200 μ l of 1.5 M NaCl/30 mM EDTA (pH 8.0), followed by vortex mixing. The resulting solutions were mixed with 300 μ l of a mixture of chloroform and methanol (2 : 1, v/v), and the organic solvent was removed after centrifugation at 12,000 rpm 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 10 mM EDTA, and the fluorescence of DNA-DAPI complexes was subsequently measured at 450 nm with excitation at 360 nm.

RESULTS AND DISCUSSION

Transfection efficiency

We prepared various liposomes containing positively charged lipids by the procedure described under Materials and Methods. We found that liposomes

containing TMAG or MDDA gave highly efficient transfection. However, the liposomes containing MDDA were difficult to prepare, since the lipids and DNA tended to stick to the wall of the tube during preparation, yielding recoveries of DNA and lipids of less than 50%. Accordingly, we chose to examine liposomes containing TMAG. After repeated preliminary experiments, we found that TMAG/DLPC/DOPE in a molar ratio of 1:2:2 yielded the most efficient expression.

Using the above liposomes, we examined the relationship between the amount of the plasmid added and that of the enzyme expressed. For this purpose, different volumes of a suspension of liposome-entrapped plasmids ($24.2 \mu\text{g}$ DNA/ μmol lipids/ 0.5 ml PBS) were added to the medium (2 ml) covering the cells. After the incubation, expressed β -galactosidase was measured for its activity. As shown in Fig. 1, the amount of the enzyme expressed increased with increasing amount of the plasmid up to $0.25 \mu\text{g}$, but a further increase in DNA concentration resulted in a rather large decrease in the enzymatic activity. This fact may be ascribed to a toxic effect of the increased amount of lipids at higher plasmid levels, for when a large amount of lipids (above 50 nmol) was added to the medium (2 ml) covering the cells (1×10^5 cells) in the plate, the cells were found to be dead. In the case of the maximum transfection in Fig. 1, the amount of lipids added was 10 nmol , and the toxicity with this amount was not serious. However, some toxicity should be

Fig. 1. Expression of β -galactosidase activity in COS-1 cells transfected with liposome-entrapped pCH110 or with empty liposome-pCH110 complexes. Liposomes were composed of $1 \mu\text{mol}$ of lipids and $24.2 \mu\text{g}$ of pCH110. Empty liposome-pCH110 complexes were prepared by mixing of empty liposomes and pCH110 at the same ratio as that of liposome-entrapped pCH110. Open circles, liposome-entrapped pCH110; closed circles, empty liposome-pCH110 complexes.

expected even at this level, since at a yet lower concentration of lipids, the amount of expressed enzyme per amount of plasmid DNA was higher, as can be seen in Fig. 1.

Considering the above results, we performed the transfection experiment using the suspension of liposomes containing 0.25 μg DNA. As shown in Table 1, TMAG-containing liposomes, TMAG/DLPC/DOPE in a molar ratio of 1 : 2 : 2, revealed the highest transfection efficiency. This efficiency exceeds that of the calcium phosphate precipitation method performed with a glycerol shock. As mentioned above, even higher transfection efficiency could be obtained when a smaller amount of lipids was used.

Examination of Table 1 reveals that the transfection efficiency of the liposomes containing TMAG was affected by other components. When the DOPE in TMAG/DLPC/DOPE-liposomes was replaced with DOPC, a 6-fold drop in the expression level was found; whereas with DLPE or DMPE, a considerable expression level was still retained. Further, if DMPC was employed instead of DLPC, a 12-fold drop in potency occurred. Since the amounts of lipids in these liposomes entrapping the same amount of DNA were not much different (see Table 1), the difference in transfection efficiency described above cannot be ascribed to the toxicity of the lipids. The transfection efficiency in the case of liposomes prepared without TMAG dropped enormously. However, this drop was not due to the toxicity of lipids, even though a larger amount of lipids was used (data not shown). These results indicate the importance of the electrostatic interaction between the negatively charged cell surface and the positively charged

Table 1. Effect of lipid composition of liposomes on entrapment of pCH110 DNA and on expression of β -galactosidase activity in COS-1 cells.

Liposomes (molar ratio)	Entrapment efficiency (μg DNA/ μmol lipid)	Expression of β -galactosidase activity
TMAG : DLPC : DOPE (1 : 2 : 2)	24.2	93.2
TMAG : DLPC : DLPE (1 : 2 : 2)	23.7	51.0
TMAG : DLPC : DMPE (1 : 2 : 2)	23.8	37.7
TMAG : DLPC : DOPC (1 : 2 : 2)	26.4	14.9
TMAG : DMPC : DOPE (1 : 2 : 2)	19.5	7.9
TMAG : DLPC (1 : 4)	28.2	5.1
DLPC : DOPE (1 : 1)	4.2	3.7
DLPC : DLPE (1 : 1)	3.2	3.4
PC : PS : Chol (2 : 2 : 1)	2.6	2.9
PS : Chol (1 : 1)	0.4	3.8
Calcium phosphate (+glycerol)	—	72.7
Control	—	1.3

Transfections were carried out as described under Materials and Methods, with 0.25 μg of pCH110 DNA entrapped in liposomes. β -Galactosidase activity is expressed as nmol ONPG cleaved/min/mg protein.

surface of the liposomal membrane for the transfection. In addition, the fact that the various TMAG-containing liposomes showed different transfection efficiencies suggests that components of the liposomes other than TMAG also play an important role in the transfection process involving the incorporation of genes into the cells across the cellular membrane and transport of genes to the proper site of the cells. To explain these differences, we must examine the present liposomes from the point of view of endocytosis [20] or cell fusion [21, 22]. This awaits further investigation.

As a control, we conducted the same experiment with empty liposome-DNA complexes at the same ratio of amounts of DNA and lipids as that of liposome-entrapped DNA. As shown in Fig. 1, empty liposome-DNA complexes showed a definite transfection, and this can be ascribed to the similar effect as observed in lipofection [23]. However, the transfection efficiency of the complexes was lower than that of liposome-entrapped DNA. This indicates that the entrapment of DNA by the present liposomes is not only due to complex formation but also to encapsulation. In the case of the complexes, the expression level also started to decrease at 0.25 μ g of DNA (see Fig. 1). This can also be ascribed to the toxicity of lipids. A comparative study of the mode of transfection in the case of liposome-entrapped DNA with that in the case of liposome-DNA complexes might be useful to clarify the mechanism of transfection.

Entrapment efficiency

The efficiency of liposomes for entrapment of DNA was assessed by the ratio of the amount of DNA (μ g) to that of lipids (μ mol). As shown in Table 1, this ratio for liposomes containing TMAG exceeded the ratio of DNA to lipids used in the preparation. Since the recovery of DNA was nearly quantitative (data not shown), the above phenomenon indicates that the lipids were not recovered sufficiently during the preparation of the DNA-containing liposomes. The entrapment efficiency of one of the liposomes lacking TMAG, i.e., the PS/Chol liposomes, was similar to the value reported by Fraley *et al.* [4]. Compared with those lacking TMAG, the liposomes containing TMAG revealed a 5-70-fold higher efficiency for the DNA entrapment. The DNA entrapped in the liposomes containing TMAG was not susceptible to DNase treatment. On the contrary, in the liposomes lacking positive charges, i.e., DLPC/DOPE and DLPC/DLPE liposomes, their DNA content was susceptible to DNase digestion. Taking into account the strongly positive charge of TMAG, the high efficiency of the liposomes containing TMAG in terms of DNA entrapment can be mainly ascribed to the electrostatic interaction between DNA and TMAG, as is the case for lipofection [23]. It should be noted, however, that the entrapment efficiency did not parallel the expression efficiency.

All the data reported in the present paper indicate that the transfection and expression of DNA can be achieved by simply adding the liposome-entrapped plasmids to the medium covering the cells without any additional manipulations

such as glycerol shock. The expression level obtained by the present method was higher than that by the calcium phosphate method, especially in the case of a small amount of DNA. Another advantage of the liposomal vehicle is that a specific antibody or a ligand for a specific receptor can be attached to such liposomes in order to deliver the gene to the specific organ or tissue. Although the toxicity of these liposomes should be examined further, the presently reported method probably has a potential utility in gene therapy.

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