Surfactant-mediated gene transfer for animal cells

Jun You, Masamichi Kamihira & Shinji Iijima

Department of Biotechnology, Graduate School of Engineering, Nagoya University, Chikusa-ku, Nagoya 464-01, Japan

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Abstract

A commercially available cationic surfactant, dimethyl-dioctadecyl ammonium bromide (DDAB), was used for making lipid vesicles. DDAB easily dissolved in water at 60 °C and formed lipid vesicles at room temperature. The lipid vesicles showed very low cytotoxicity compared with other cationic surfactants. After the lipid vesicles were mixed with plasmid DNA solution, the solution was added to mammalian cells. The addition of a nonionic surfactant (Tween 80) to the cationic lipid vesicles at the weight ratio of 1:1 enhanced transfection efficiency. Adding more or less than the optimal amounts of DNA and lipid vesicles resulted in decreased transfection efficiency. With the optimal amounts of DNA (pCMV β) and lipid vesicles, about 90–95% of CHO-K1 and BHK-21C13 cells transiently expressed β -galactosidase activity 24 h after transfection. By this procedure, stable transformants around 10⁵ cells corresponding to 10% efficiency could be obtained by one batch transfection.

Abbreviations: DDAB – dimethyl-dioctadecyl ammonium bromide; HTAB – hexadecyltrimethyl ammonium bromide; DMB – decamethonium bromide; CPC – cetylpyridinium chloride; MTAC – methyltrioctyl ammonium chloride; CDAB – cetyldimethylethyl ammonium bromide; CHO – Chinese hamster ovary; BHK – baby hamster kidney; CMV – cytomegalovirus

Introduction

DNA transfection of animal cells has become an important technique for analyses of gene function, production of recombinant gene products and the development of gene therapy. Although animal cells can take up exogenous DNA under appropriate conditions, the spontaneous uptake of intact DNA into cells and its subsequent expression in a nucleus is a very inefficient process due to the multitude of enzymatic and membrane barriers imposed by cells. Thus, a number of methods have been developed to facilitate this process to obtain both transient and stable expression of exogenous DNA. These include the use of polycations, liposomes, reconstituted viral envelopes and reagents such as calcium phosphate and DEAE-dextran (Klebe *et al.*, 1984; Bond and Wold, 1987; Mannino and Fould-Fogerite, 1988). Physical approaches for introducing DNA such as microinjection, particle gun

and electroporation (Potter *et al.*, 1984) have been also used successfully. However, these procedures require a special device.

A recent addition to the repertoire of DNA transfection methodologies is cationic liposome-mediated transfection (lipofection) (Felgner *et al.*, 1987; Felgner and Ringold, 1989; Malone *et al.*, 1989; LeVentis and Silviu, 1990). Cationic liposomes spontaneously form complex with plasmid DNA or RNA in solution and facilitate fusion between the complex and cells in culture, resulting in the efficient transfer of nucleic acids to a wide variety of animal cell types. The possible use of cationic liposomes in the transfection has been the subject of numerous studies, and promising results from a human clinical study were published recently where the technique was employed *in vivo* (Nabel *et al.*, 1993).

In the present study, we report a new DNA-transfection procedure using commonly available inex-

pensive reagents for further development of alternative DNA transfer method for wide variety of purpose.

Materials and methods

Cell lines, media and plasmid DNAs

A Chinese hamster ovary CHO-K1, a baby hamster kidney BHK-21C13, and an African green monkey kidney COS-7 cell lines were obtained from RIKEN cell bank and used as host cells for gene transfection. CHO-K1 and COS-7 cells grew with an anchorage dependent manner and BHK-21C13 cells subcloned from anchorage dependent BHK-21 cells grew in suspension.

CHO-K1 cells were cultured in Ham's F12 medium (Gibco BRL Co., Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (JRH Sciences, Lenexa, KS, USA). COS-7 cells were cultured in minimum essential medium (MEM) (Gibco BRL Co.) supplemented with 10% FBS. BHK-21C13 cells were cultured in MEM medium supplemented with 10% FBS and 1% non-essential amino acids (NEAA). The cells were cultured at 37 °C in a 5% CO₂ incubator. Media for all cultures routinely included 100 units ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin.

Recombinant plasmids, pCMV β (Macgregor and Caskey, 1989) and pCH110 (Hall *et al.*, 1983), encoding *lacZ* gene under the control of cytomegalovirus (CMV) early promoter and SV40 early promoter, respectively, were used for transient expression. A recombinant plasmid pMC1Neo (Toyobo Co., Osaka, Japan) encoding *neo* gene under the control of TK promoter was used for stable expression.

Preparation of cationic lipid vesicles

Commercially available cationic surfactants, dimethyldioctadecyl ammonium bromide (DDAB), hexadecyltrimethyl ammonium bromide (HTAB), decamethonium bromide (DMB), cetylpyridinium chloride (CPC), methyltrioctyl ammonium chloride (MTAC) and cetyldimethylethyl ammonium bromide (CDAB) were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and used for making cationic lipid vesicles as a DNA carrier. Nonionic surfactants, NP-40 (Sigma Chemical Co.), Pluronic F-68 (BASF Japan Co., Osaka, Japan), Triton X-100 (Wako Pure Chemical Industries Co., Osaka, Japan), Tween 20 (Wako Pure Chemical Industries Co.) and Tween 80 (Wako Pure

Chemical Industries Co.) were employed as an additive for improvement of stability of lipid vesicles and transfection efficiency.

The surfactant was dissolved in water at 60 °C or by sonication at room temperature. Then the surfactant solution (1 mg ml⁻¹) was autoclaved at 120 °C for 20 min, and preserved in a refrigerator. The solution was prewarmed and homogenized before use.

Cytotoxic test of surfactants

Cytotoxicity of cationic surfactant reagents was measured as follows. CHO-K1 cells were plated at the cell density of 1.5×10^5 cells per a 35 mm-dish and incubated at 37 °C for 24 h. Then the medium was changed in the presence of 2 µM or 20 µM cationic surfactant reagents. After 24 h, the cells were trypsinized and stained with 0.1% trypan blue. The living cells were counted under a microscope.

Transient transfection assay

Cultured dishes with 50 to 70% confluent cells were rinsed twice with serum-free opti-MEM medium (Gibco BRL Co.) to remove serum from the culture. In the case of BHK-21C13 cells, the cells were collected and washed by centrifugation. The opti-MEM medium was removed just before the addition of the solution containing lipid vesicle/DNA complex. Typically, 3 µg of DNA and 36 µg of lipid vesicles which contained DDAB and Tween 80 at the weight ratio of 1:1 were used for transfection. They were diluted separately in 0.75 ml opti-MEM medium, gently mixed and then stood at room temperature for about 30 min to form the complex. The solution containing the complex was added to the rinsed cells in a 35 mm diameter tissue culture dish (Iwaki Grass Works Co., Tokyo, Japan). After 6 h incubation, the DNA/lipid vesicle solution was removed and growth medium was added. Assay of transient expression of β -galactosidase was performed at 24 to 48 h post transfection.

In situ staining was performed to identify β -galactosidase expressed cells. Cells were rinsed twice with phosphate buffered saline (PBS), fixed with 2 ml of 2% (v/v) formaldehyde and 0.2% glutaraldehyde in PBS for 5 min at room temperature, rinsed twice with PBS, and then stained for 2 h to overnight with 2 ml of X-gal solution: 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl₂, and 1 mg ml⁻¹ 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (Wako Pure Chemical Indus-

tries Co.) in PBS. Stained cells were counted under a microscope and the percentage of stained cells (transfection efficiency) was determined as the average of counts in three different fields.

Stable transfection assay

Complexes of plasmid DNA and lipid vesicles were prepared as mentioned for transient transfection. CHO-K1 cells were plated at the cell density of 1.5×10^5 cells per a 35 mm-dish on the day before transfection and incubated for 6 h with DNA/lipid vesicle solution containing 3 μ g pMC1Neo DNA and 36 μ g of lipid vesicles in 1.5 ml opti-MEM serum free medium. Then the medium was changed to Ham's F12 medium supplemented with 10% FBS. Two days after the transfection the cells were diluted to 10^{-5} and seeded in a 96-wells tissue culture plate. Transformants were selected in the medium containing 400 μ g ml $^{-1}$ geneticin (Sigma Chemical Co.). After 2 weeks, cell colonies were fixed and stained with the solution containing 10% formaldehyde and 0.2% crystal violet in PBS for 2 h at room temperature. The plate was rinsed with water and the number of cell colony was counted after drying.

Other measurement

Average diameter of lipid vesicles was determined by a dynamic light scattering device (DLS-700, Ohtsuka Electronics Co., Osaka, Japan) and expressed as a Contin diameter.

Results and discussion

Cytotoxicity and transfection efficiency of cationic surfactants

Figure 1 shows cytotoxicity of six commercially available cationic surfactants for CHO-K1 cells. Except for DDAB and DMB, the four surfactants exhibited serious toxic effect for the cells at 20 μ M and most of cells died within a day. The toxic effect was alleviated very much by the decrease of concentration to 2 μ M. However, CPC and MTAC still exhibited cytotoxicity and the cell viability was less than 20%. The other surfactants were applied for gene transfection. For the transient transfection, plasmid DNA (pCMV β) and the respective surfactants were mixed, and the solution was added to the cell culture dish where the final sur-

Figure 1. Comparison of cationic surfactants on cytotoxicity of CHO-K1 cells. The cationic surfactants were added to the culture medium at the concentration of 2 μ M (□) and 20 μ M (▨). The cell viability was measured 24 h after the addition.

factant concentration was 2 μ M. Only when DDAB was used as a DNA carrier, the cells took up the DNA and expression of *lacZ* gene was observed. Thus, we assumed that DDAB formed a kind of complex with the plasmid DNA and promoted the cellular uptake of DNA.

Many surfactant mediated gene transfection procedures, which use commercial lipofection reagents (Holmen *et al.*, 1995), synthetic cationic surfactants and natural phospholipids (Bennett *et al.*, 1995), are now available. However, some of such reagents exhibited cytotoxic effect even in the effective concentration for gene transfection (Yagi *et al.*, 1993). DDAB has been also used for gene transfection as a DNA carrier (Rose *et al.*, 1991; Lappalainen *et al.*, 1994). In those cases, DDAB was mixed with dioleoyl-phosphatidylethanolamine (DOPE) at molar ratio of 1:1 or 1:2.5 to enhance transfection efficiency, and toxic effect on CaSki cells at 10 μ M was reported (Lappalainen *et al.*, 1994). In addition, the procedure for making lipid vesicles requires an evaporator and a sonicator.

As shown in Fig. 1, DDAB itself seemed to have low cytotoxicity for animal cells compared with other cationic surfactants. Therefore, the effect of DDAB concentration on viability of CHO-K1 cells was examined as shown in Fig. 2. More than 95% of the cells was viable at the concentrations up to 20 μ M. The cell viability decreased when the concentration increased

Figure 2. Cytotoxicity of DDAB lipid vesicles on CHO-K1 cells. Lipid vesicles consisting of only DDAB (□) or DDAB + Tween 80 (1:1, w/w) (■) were added. The cell viability was measured 24 h after the addition.

more than 40 μM . Almost all the cells tested died with 160 μM of the surfactant. Nevertheless, DDAB had low cytotoxicity to animal cells compared with the surfactants used in gene transfection including commercial lipofection reagents and natural phospholipids (Yagi *et al.*, 1993; Lappalainen *et al.*, 1994).

Effect of nonionic surfactant addition on transfection efficiency

The mechanism of gene expression in surfactant mediated gene transfer is assumed as follows. Cationic surfactants form lipid vesicles which have positive charges on the surface. Since DNAs have negative charges, the lipid vesicles form some complex with DNAs. Normally animal cell surface also has negative charges derived from sialic acid residues, hence the positive charges of lipid vesicles interact with cell surface and lipid vesicle/DNA complex binds to cell surface. Then cells take up the complex by endocytosis. The incorporated DNA is transferred to nucleus of a cell and gene product is expressed. Generally, the processes of the formation of lipid vesicle/DNA complex and the endocytosis of the complex are important in surfactant mediated gene transfection. In this regards, dispersion condition of lipid vesicles seems to be crucial for the complex formation. DDAB lipid vesicles tended to aggregate at high concentration especially

in low temperature. When DDAB formed aggregation, effective lipid vesicle concentration reduced and transfection efficiency decreased. In order to stabilize DDAB lipid vesicles for prevention of the aggregation and facilitate endocytosis of the cells, the addition of nonionic surfactant to DDAB lipid vesicles was examined (Fig. 3). Commonly used five nonionic surfactants (NP-40, Triton X-100, Pluronic F-68, Tween 20 and 80) were added at the weight ratio of 1:1 to DDAB. After mixed with plasmid DNA (pCMV β) solution, transient transfection efficiency was measured with three cell lines. As shown in Fig. 3, the addition of nonionic surfactant to DDAB for the preparation of lipid vesicles influenced transfection efficiency; some nonionic surfactants enhanced the efficiency. However, these effects depended on cell line. Tween 80 and Pluronic F-68 had positive effects on transfection for all the cell lines tested. In particular, by the addition of Tween 80 to DDAB lipid vesicles, 85 and 92% of CHO-K1 and BHK-21C13 cells expressed the transfected gene. In addition to the improved transfection efficiency, the lipid vesicles were stabilized. Average diameter of DDAB lipid vesicles was 500–600 nm by dynamic light scattering method without Tween 80, but the diameter was decreased to 100–200 nm by the addition of the surfactant. This indicates that Tween 80 may act as a dispersing agent of DDAB lipid vesicles and the positive effects of Tween 80 may be due to the stabilization of the lipid vesicles. From these results, Tween 80 was used as an additive to DDAB lipid vesicles in further study.

To optimize the Tween 80 addition, we studied whether the transfection efficiency was influenced by the ratio of Tween 80 and DDAB (Fig. 4). In this experiment, the concentration of DDAB was fixed at 2 μM . As the ratio of Tween 80 increased to equal amount (Tween 80/DDAB = 1), transfection efficiency increased for all the cell lines tested. Above the equal ratio, the efficiency gradually decreased. The tendency was the same for the three cell lines and over the variety of DNA concentration, although the expression level was different with cell lines. In all cases tested, the maximum transfection efficiency was obtained when the ratio of Tween 80 to DDAB was around 1. Moreover, cytotoxicity of DDAB was not enhanced by the addition of Tween 80 (Fig. 2).

Optimization of transfection efficiency

As reported earlier (Felgner *et al.*, 1987; Felgner and Ringold, 1989), gene transfection using cationic lipo-

Figure 3. Effect of nonionic surfactant on transfection efficiency in the presence of DDAB lipid vesicles. Five nonionic surfactants were added at the weight ratio of 1:1 to DDAB. CHO-K1 (□), BHK-21C13 (▨) and COS-7 (▩) cells were applied for transfection of pCMV β .

Figure 4. Effect of Tween 80 and lipid vesicles ratio on transfection efficiency. (A) CHO-K1 cells were applied for transfection of 1 μ g (●), 3 μ g (○) and 5 μ g (■) pCMV β . (B) BHK-21C13 (●) or COS-7 (○) cells were applied for transfection of 3 μ g pCMV β .

somes as DNA carrier was influenced by many conditions such as concentrations of lipid vesicles and DNA, medium, cell density and cell type. In the present study, a serum-free medium should be used for incubation of the cells with lipid vesicle/DNA complexes to obtain high transfection efficiency. In the other transfection procedures including lipofection, the use of a serum-free medium is often crucial for obtaining good result (Felgner *et al.*, 1987). Some serum components may weaken interaction between lipid vesicles and DNA or cells. A variety of serum-free medium showed enhanced transfection efficiency with the present method. Among those, opti-MEM medium gave the best results with respect to cell viability during transfection, the efficiency and the reproducibility (data not shown).

For further optimization of transfection condition, the amounts of DNA and lipid vesicles added to the medium were examined (Fig. 5). In this experiment, Tween 80 was added to DDAB lipid vesicles at the same weight ratio. The optimal amount of lipid vesicles existed depending on the amount of DNA, indicating that the ratio of DNA and lipid vesicles was important. At low amounts of DDAB relative to DNA, the transfection efficiency increased with increase in the amount of lipid vesicles added. After reaching the maximum, it gradually decreased. Thus, excess lipid vesicles resulted in decreased transfection efficiency. These suggest that the lipid vesicles facilitate the DNA uptake, but the excess lipid vesicles interfere the DNA uptake. The optimal amounts of lipid vesicles in which transfection efficiency became the maximum were 24 and 36 μg for 2 and 3 μg DNA, respectively, per a 35 mm-dish. The optimal DNA/lipid vesicles ratio for 2 and 3 μg DNA was 0.083 (μg -DNA/ μg -lipid vesicles). Although the optimal amounts of DNA and lipid vesicles are changed by culture scale, DNA/lipid vesicles ratio may be a good index for scale-up or down of transfection.

As shown in Fig. 5, the optimal amounts of lipid vesicles for each DNA amount were almost the same independent of cell line, but the behavior of the transfection efficiency on the variation of lipid vesicles amount was slightly different between the cell lines; the efficiency strongly depended on the amount of DNA and lipid vesicles with CHO-K1 cells, whereas the dependency was less with BHK-21C13 cells. To obtain the maximum transfection efficiency, appropriate amount of DNA is necessary, because low DNA amount causes lack of DNA and high DNA causes toxic effect for cells. In all cases, 3 μg of plasmid DNA per a

Figure 5. Effect of DNA and lipid vesicles amounts on transfection efficiency. CHO-K1 (A), BHK-21C13 (B) or COS-7 (C) cells were applied for transfection of 1 μg (○), 2 μg (●), 3 μg (□) and 4 μg (■) pCMV β .

35 mm-dish and 36 μg of lipid vesicles gave the highest efficiency. Under the optimal condition, the maximum transfection efficiency more than 90% was obtained for CHO-K1 and BHK-21C13 cells (Fig. 6). The level of the transfection efficiency was more than 10-fold higher than conventional calcium phosphate method and comparable to commercial lipofection procedure.

Figure 6. Photographs of β -galactosidase expressing cells after transfection (Stained with X-gal). CHO-K1, BHK-21C13 and COS-7 cells were plated at the cell density of 1.5×10^5 , 2×10^6 and 1.5×10^5 cells per a 35 mm-dish on the day before transfection. pCMV β (3 μ g) and lipid vesicles (36 μ g) consisting of DDAB and Tween 80 at the weight ratio of 1:1 were used for transfection. (A) CHO-K1, (B) BHK-21C13, (C) COS-7.

Table 1. Transfection efficiency under the optimal conditions

Plasmid DNA	Transfection efficiency (%)		
	CHO-K1	BHK-21C13	COS-7
pCMV β	90	95	36
pCH110	23	86	19

Transfection conditions: CHO-K1, BHK-21C13 and COS-7 cells were plated at the cell density of 1.5×10^5 , 2×10^6 and 1.5×10^5 cells per a 35 mm-dish on the day before transfection. The plasmid DNA (3 μ g) and lipid vesicles (36 μ g) consisting of DDAB and Tween 80 at the weight ratio of 1:1 were used for transfection.

This procedure was to applied to other vectors, pCH110 for transient expression and pMC1Neo for stable expression. Table 1 summarizes transfection efficiencies for transient expression under the optimal conditions determined by using pCMV β . Although the maximum transfection efficiency was different depending on plasmids and cell lines, the expression level was still high. The transfection procedure could be applied for stable expression and a great number of stable transformants around 10^5 corresponding to about 10% efficiency could be obtained in one batch transfection (per a 35 mm-dish) by using pMC1Neo plasmid and CHO-K1 cells.

In conclusion, we developed a surfactant mediated gene transfection procedure using commonly available cationic and nonionic surfactants. The advantages of this transfection procedure are as follows. (1) The preparation of lipid vesicles as DNA carrier is very easy. (2) The lipid vesicles are autoclavable for sterilization. (3) The transfection procedure is also very simple without a special device. (4) Under the optimal conditions, high transfection efficiency could be obtained. (5) The lipid vesicles are low toxic for animal cells. Using these advantages, we will modify the lipid vesicles with some ligands for enhanced and/or cell-specific gene transfection. Furthermore, *in vivo* gene transfection will be also possible due to the low toxicity.

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Address for correspondence: Masamichi Kamihira, Department of Biotechnology, Graduate School of Engineering, Nagoya University, Chikusa-ku, Nagoya 464-01, Japan.