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Physicochemical characterization and gene transfection efficiency of lipid emulsions with various co-emulsifiers

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Abstract

Transfection systems based on complexes of DNA and lipid emulsions were evaluated with respect to their effectiveness, toxicity, physicochemical characteristics, and cell-type dependence. The potential of a series of co-emulsifiers to serve as vectors was investigated. The co-emulsifiers examined included 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), Tween, cholesterol, stearylamine, and polyethylenimine (PEI). Squalane and 1,2-dioleoyl-*sn*-glycero-3-trimethylammonium-propane (DOTAP), respectively, were the main oil phase and cationic lipid added to the lipid emulsions. Cell viability was reduced after inclusion of either of the two cationic components of stearylamine and PEI. DOPE and cholesterol showed both higher transfection activity and cell viability as compared to the other co-emulsifiers. The incorporation of DOPE and cholesterol also prevented droplet aggregation of the emulsions after long-term storage. Results of the transfection of COS-1, A549, or HaCat cell lines with lipid emulsions indicated differences in transfection activities of each formulation for the different cell lines. It is concluded that DOPE and cholesterol as co-emulsifiers for DOTAP were preferable for stability and DNA transfection of emulsions.

Keywords: Lipid emulsion; Transfection; Gene therapy; DOTAP; Squalane

1. Introduction

Human gene therapy is promising for the advancement of medicine, however its efficacy has not been sufficient in clinical trials (Kurisawa et al., 2000). The establishment of gene therapy may be achieved by de-

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signing sophisticated gene vectors. Viruses have successfully been used to achieve efficient gene expression; however, several disadvantages with the use of viral delivery systems such as unacceptable immune responses and large-scale manufacturing control have limited the success of these carriers (Rungsardthong et al., 2001). Non-viral delivery systems for genes have increasingly been proposed as a safer alternative to viral vectors (Mao et al., 2001). The most commonly used non-viral vectors are liposomes, which are composed of cationic lipids. However, the gene expression induced by cationic liposomes is usually low in serum-containing medium and is often toxic in vitro and in vivo (Filion and Phillips, 1998). The instability of liposome–DNA complexes is also the main obstacle to their becoming a successful gene carrier. To overcome these problems, cationic emulsions have been developed which are physically stable and can facilitate the efficient transfer of genes (Liu et al., 1996; Kim et al., 2001, 2002).

The cationic emulsifier most frequently used for emulsions in gene delivery is 1,2-dioleoyl-*sn*-glycero-3-trimethylammonium-propane (DOTAP). However, DOTAP shows a high level of toxicity in many cells at higher doses (Filion and Phillips, 1998). DOTAP is also very costly because of a difficulty of purification. Hence, selection of co-emulsifiers in the emulsions is important for resolving such problems. The transfection activity also greatly depends on the composition of the co-emulsifiers. Some investigations have focused on the use of certain co-emulsifiers as helpers for gene delivery (Liu et al., 1996; Kim et al., 2001, 2002). However, relatively little work has completely and comprehensively compared various types of co-emulsifiers with each other. The aim of the present study was to screen a series of co-emulsifiers in lipid emulsions to increase the efficiency of transgene expression and improve the stability of the vectors, while decreasing their toxicity. The emulsion systems used in this study contain squalane as the core oil and DOTAP as the cationic lipid. A series of co-emulsifiers including l,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), Tween, cholesterol, stearylamine, and polyethylenimine (PEI) was also used as additives. The transfection target was COS-1 cells in the absence or presence of serum. The toxicity of the emulsions was evaluated by cell viability using the 3-[4,5]-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. The physical properties of the emulsions–DNA complexes were assessed by gel electrophoresis, dynamic light scattering, zeta potential, and pH titration.

2. Materials and methods

2.1. Materials

DOTAP and DOPE were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Squalane, cholesterol, stearylamine, PEI (0.8, 2, and 750 kDa), and MTT were obtained from Sigma Chemical (St. Louis, MO, USA). Tween 20 and Tween 80 were from Kanto Chemical (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were supplied by Biowest (Nuaillé, France). A Maxi-V500 plasmid extraction system was obtained from Viogene (Taipei, Taiwan). A β-galactosidase kit was from Gene Therapy System (San Diego, CA, USA).

2.2. Preparation of lipid emulsions

The DOTAP and co-emulsifier were weighed and mixed with deionized distilled water. The mixture was sonicated until clear using a probe sonicator at 10 W (VCX600, Sonics and Materials, Danbury, CT, USA) in an ice–water bath. Squalane (10%) was added to the aqueous phase and sonicated for 5 min in an ice–water bath. The concentration of DOTAP and co-emulsifier was represented against squalane and water. The DOTAP liposomes (1.5%) were also prepared for comparison. A 1.5% DOTAP was mixed with double-distilled water and sonicated for 5 min at room temperature.

2.3. Preparation of plasmid DNA

The plasmid pCMV-LacZ was amplified in *Escherichia coli* (strain DH5-α) and purified by column chromatography. The purity of the plasmid was measured by OD_{260}/OD_{280} (1.85∼1.90; OD, optical density) as well as by electrophoresis in a 0.8% agarose gel. Purified plasmid DNA was resuspended in sterile double-distilled water and frozen in aliquots at a concentration of 0.5 mg/ml.

2.4. Agarose gel electrophoresis

Interactions between plasmid DNA and lipid emulsions were investigated by electrophoresis on agarose gels. The plasmid (2 μg, 0.5 μg/μl) and 0.4 μl of emulsion were mixed, and serum-free DMEM was added to give a total volume of 65 μl. The incubation time for forming complexes was 10 min. The complexes were then loaded onto 0.6% agarose gels containing ethidium bromide (0.5 mg/ml). The gel running buffer was 40 mM Tris acetate and 1 mM EDTA. The gel was run at 45 V for 120 min. The trapping efficiency of the plasmid to lipid emulsion was determined as [(the free DNA of the non-complex group − the free DNA of the complex)/the free DNA of the non-complex group] × 100%. The band intensity of gel electrophoresis was quantified by the method of densitometry (Molecular Dynamics Personal Densitometer SI, Amersham Biosciences, USA).

2.5. In vitro transfection

COS-1, A549, and HaCaT cell lines were individually cultured in DMEM supplemented with 10% FCS at 37 °C in a 5% CO_2 incubator. Cells were seeded at 6×10^4 cells per well onto 24-well plates 12 h before transfection. After washing the cells with serum-free DMEM, 65 μl of complex and 145 μl of serum-free DMEM were added to each well. To examine the effect of serum, 145 μl of FCS was added instead of serum-free medium. After 3 h of incubation, cells were fed with DMEM containing 10% FCS and cultured for 24 h. The transfected cells were assayed using a β-galactosidase kit by a photometric assay at 595 nm according to the manufacturer's instructions.

2.6. Cytotoxicity assay

Cytotoxicity was evaluated by the MTT assay. Cells were seeded at a density of 1×10^4 cells/well in 96-well plates, and incubated 12 h before treatment. The emulsion (0.1 ml) and 52.4 μl of DMEM medium were added to the well and incubated for 3 h at 37 °C. After incubation, 50 μl of the 5 mg/ml MTT solution in PBS was added and incubated for an additional 2 h at 37 °C. MTT-containing medium was aspirated off, and 125 μl of DMSO was added to dissolve the formazan crystal formed by live cells. The absorbance was measured at 545 nm.

2.7. Particle size and zeta potential analysis

The mean particle size and zeta potential of the lipid emulsions were measured by photon correlation spectroscopy (Nano ZS® 90, Malvern, UK) using a helium–neon laser with a wavelength of 630 nm. The emulsion or emulsion/DNA complex was diluted with double-distilled water by 100-fold before the measurement. The determination was repeated three times per sample for three samples comprising an identical composition. The size and zeta potential of the emulsions were also monitored after storing at 50 °C for 49 days.

2.8. Acid–base titration

The buffering capacity of the lipid emulsion with various co-emulsifiers was determined by acid–base titration. The lipid emulsion was titrated with increasing volumes of 10^{-8} mol HC1 or NaOH, and the pH was measured by the pH meter at every point.

3. Results and discussion

3.1. The effect of DOTAP concentration in lipid emulsions

DOTAP was the main lipid used for forming lipid emulsions in this study. DOTAP at various concentrations was used to prepare the emulsions for determining the physicochemical properties and transfection activity. Essential requirements for a vector of transfection are that it should bind DNA sufficiently strongly and rapidly. To verify complex formation, gel electrophoresis was performed. As shown in Fig. 1, plasmid DNA exists as a mixture of both supercoiled and open, circular forms. The densitometry only detected the band of the supercoiled, circular form for quantifying the trapping efficiency as depicted in Table 1. When the lipid emulsion was mixed with DNA, free DNA disappeared when the concentration of DOTAP increased. Plasmid bands with emulsion were completely retained in the gel loading slots at a concentration of 4%. This

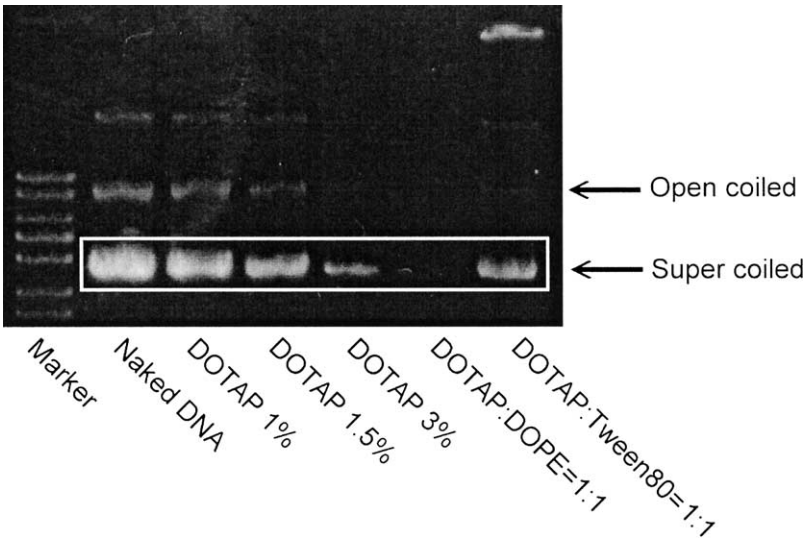

Fig. 1. Gel electrophoresis of the complex formed between the lipid emulsion and DNA. The unbound free DNA in the agarose gel was visualized under UV light.

indicates the partial dissociation of DNA from the complexes with DOTAP at lower doses. The liposomes with 1.5% DOTAP showed an approximated entrapment to the emulsions with DOTAP at the same concentration.

The safety of new formulations must be well established before being used in clinical situations. From the cell proliferation assay using MTT, the cytotoxicity of the lipid emulsions was measured (Table 2). At higher DOTAP doses, the emulsions decreased the cell viability in the absence of serum. However, cells remained viable in all emulsions with various DOTAP concentrations in the presence of serum. Cells incubated under serum-free conditions with emulsions showed significant levels of cell death due to a lack of growth factors provided by the serum that are required for cell survival (Dodds et al., 1998; Yi et al., 2000). The liposomes showed lower cell viability than the emulsions with 1.5% DOTAP.

Table 1
The trapping efficiency of DNA interacted with lipid emulsions with various co-emulsifiers determined by agarose gel electrophoresis

Formulations	Concentration (%, w/v)	Trapping efficiency (%)
DOTAP liposomes	1.5	39.62
DOTAP	1.0	15.87
DOTAP	1.5	32.29
DOTAP	2.0	88.86
DOTAP	3.0	62.54
DOTAP	4.0	101.03
DOTAP + DOPE	1.5:1.5	82.05
DOTAP + Tween 20	1.5:1.5	86.57
DOTAP + Tween 80	1.5:1.5	36.31
DOTAP + cholesterol	1.5:1.5	85.17
DOTAP + stearylamine	1.5:1.5	98.47
DOTAP + PEI (750 kDa)	1.5:1.5	91.69
DOTAP + DOPE	2.0:1.0	89.95
DOTAP + DOPE	1.0:2.0	13.78
DOTAP + cholesterol	2.0:1.0	80.62
DOTAP + cholesterol	1.0:2.0	41.11

The ability of each emulsion to transfect mammalian cells was examined using a COS-1 cell model. The transfection efficiency was commonly determined by quantitating β-galactosidase expression and measuring cell viability as a function of total protein in cell lysates (Hofland et al., 1996). Since there is a good correlation between cytotoxicity as assessed by the decrease in cellular protein levels and results obtained using the MTT assay (Gebhart and Kabanov, 2001), the percentage of cell viability instead of protein levels was used to calculate the calibrated β-galactosidase activity as shown in Fig. 2. The transfection of DNA alone without complexing to lipid emulsions produced no measurable β-galactosidase activity (data not shown). In the absence of serum, it was apparent that the transfection activity was increased as DOTAP concentrations increased above 3% (Student's *t*-test,

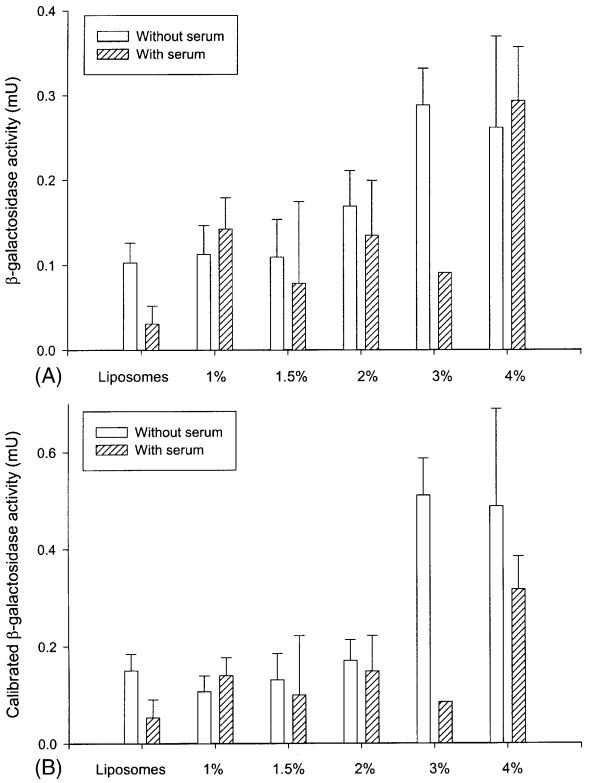

Fig. 2. In vitro transfection efficiency of lipid emulsions with various DOTAP concentrations determined by β-galactosidase activity (A) and calibrated β-galactosidase activity. The DOTAP concentration in liposomes was 1.5%. Each value represents the mean ± S.D. ($n = 4$). The calibrated β-galactosidase activity means β-galactosidase activity (mU) divided by cell viability (%).

$p < 0.05$). The low interaction of the DNA–emulsion complexes at lower DOTAP concentrations may have contributed to this result, since disassembly of the complex in the extracellular environment would significantly limit its efficacy (Bonadio, 2000). In developing gene carriers, those that are efficient in vitro often fail to show the same efficiency when applied in vivo. The main reason for the poor efficacy in vivo is the sensitivity to serum (Yi et al., 2000). Gene transfer mediated by liposomes, the commonly used non-viral vector for DNA, is adversely affected by serum (Fig. 2). The presence of serum did not interfere with the transfection ability of DNA–emulsion complexes at lower DOTAP concentrations (Fig. 2). However, additional barriers to gene delivery in the presence of serum were decisive at higher DOTAP concentrations (3% and 4%). The higher DOTAP ratios may provide higher positive charges for emulsions, and are therefore capable of binding plasma membranes through non-specific electrostatic interactions (Bonadio, 2000).

In order to elucidate this mechanism, the zeta potential of the emulsions was determined. As shown in Table 3, the zeta potential increased as DOTAP concentrations increased from 1.5% to 2% (t-test, $p < 0.05$), and it also leveled off above 2%. One report speculated that non-viral vectors bind to cells via their net positive charge, with adhesion being facilitated by the interaction between the positively charged surface and the negatively charged cell membrane (Mao et al., 2001). The discrepancy of surface changes is more significant when incorporating DNA into lipid emulsions. The zeta potentials of DNA–emulsion complexes were -21.94 ± 2.79, 8.36 ± 6.39, 25.11 ± 1.60, and 46.51 ± 5.16 mV for DOTAP concentrations ranging from 1.5% to 4%, respectively. It was proposed that a portion of the DNA is displaced from the vesicle surface, and the DNA shields the positive charges of the vesicle (Ishiwata et al., 2000). The loss of transfection activity of emulsions with less DOTAP may be due to neutralization of the positive charges on the DNA complex. These positive charges were thought to be important for the binding of the complex to the cell surface. At higher DOTAP concentrations, the complex may also exhibit strong interactions with negatively charged components in the serum, resulting in a reduction of transfection in the presence of FCS (Fig. 2).

The mean droplet size generally increased as the DOTAP concentration increased and leveled off above 3%. The plateau appeared to possibly have been due to the saturation of the adsorption of DOTAP onto the squalane droplets. The physical stability of the carrier and its complex with DNA has been regarded as one of the important factors in its use (Zelphati et al., 1998; Yi et al., 2000). The size and zeta potential of the emulsions were monitored for 49 days at 50 °C to investigate the stability of the lipid emulsions. The size and zeta potential charge are shown in Table 3. The enlargement of droplet size was significant at lower DOTAP concentrations after 49 days of incubation. This may indicate that sufficient DOTAP is required in order to avoid aggregation of droplets in the lipid emulsions. The electrostatic repulsion of the positively charged

Table 2
The cell viability of COS-1 after treatment of lipid emulsions with various co-emulsifiers determined by MTT assay

Formulations	Concentration (%, w/v)	Cell viability without serum (%)	Cell viability with serum (%)
DOTAP liposomes	1.5	68.13 ± 15.15	57.43 ± 11.16
DOTAP	1.0	104.77 ± 27.70	100.97 ± 30.34
DOTAP	1.5	82.86 ± 25.90	78.33 ± 15.99
DOTAP	2.0	98.82 ± 28.36	89.81 ± 12.39
DOTAP	3.0	56.46 ± 5.23	106.33 ± 15.70
DOTAP	4.0	53.66 ± 17.43	92.74 ± 10.11
DOTAP + DOPE	1.5:1.5	78.97 ± 11.67	99.24 ± 9.62
DOTAP + Tween 20	1.5:1.5	87.30 ± 31.91	104.34 ± 22.46
DOTAP + Tween 80	1.5:1.5	58.64 ± 7.03	108.20 ± 5.08
DOTAP + cholesterol	1.5:1.5	77.08 ± 9.35	76.95 ± 9.58
DOTAP + stearylamine	1.5:1.5	74.27 ± 11.15	50.54 ± 7.20
DOTAP + PEI (750 kDa)	1.5:1.5	26.68 ± 7.64	48.09 ± 13.59
DOTAP + PEI (2 kDa)	1.5:1.5	32.15 ± 4.21	71.58 ± 14.56
DOTAP + PEI (0.8 kDa)	1.5:1.5	35.43 ± 5.04	50.07 ± 5.50
DOTAP + DOPE	2.0:1.0	79.53 ± 10.34	85.46 ± 19.38
DOTAP + DOPE	1.0:2.0	52.09 ± 19.57	96.45 ± 13.67
DOTAP + cholesterol	2.0:1.0	72.78 ± 5.73	70.56 ± 10.00
DOTAP + cholesterol	1.0:2.0	74.44 ± 5.26	46.19 ± 4.48

Each value represents the mean ± S.D. ($n = 4$).

DOTAP may contribute to this effect. It has been reported that the stability of emulsions may be closely related to their transfection activity (Liu et al., 1996; Yi et al., 2000). This result was confirmed by our data, as the emulsion with higher DOTAP doses produced higher transfection abilities. The stability of the zeta potential showed an opposite result to that of size. The emulsions with higher DOTAP concentrations (above 1.5%) significantly (Student's t-test, $p < 0.05$) lost a part of their zeta potential after 49 days of incubation. This may have been because the excess DOTAP departed from the oil droplets following the increase in incubation time, thus reducing the zeta potential on the surface of the droplets.

Table 3
The size and zeta potential of lipid emulsions with various co-emulsifiers determined at 0th day and 49th day after preparation

Formulations	Concentration (%, w/v)	Size (nm)		Zeta potential (mV)	
		0th day	49th day	0th day	49th day
DOTAP	1.0	274.03 ± 16.34	572.16 ± 54.17	57.62 ± 0.77	63.56 ± 2.31
DOTAP	1.5	284.26 ± 11.26	667.52 ± 233.98	57.81 ± 1.14	48.37 ± 1.12
DOTAP	2.0	364.11 ± 20.86	409.02 ± 99.92	63.70 ± 2.71	49.21 ± 2.73
DOTAP	3.0	434.31 ± 58.67	482.23 ± 277.44	63.46 ± 1.36	56.42 ± 4.48
DOTAP	4.0	458.47 ± 140.42	500.66 ± 105.57	63.93 ± 1.82	51.27 ± 4.48
DOTAP + DOPE	1.5:1.5	633.37 ± 49.80	607.43 ± 105.93	70.37 ± 1.47	60.87 ± 3.09
DOTAP + Tween 20	1.5:1.5	654.76 ± 68.44	873.17 ± 95.94	47.31 ± 1.03	67.05 ± 1.67
DOTAP + Tween 80	1.5:1.5	348.13 ± 24.61	791.38 ± 195.01	65.79 ± 2.34	66.68 ± 1.82
DOTAP + cholesterol	1.5:1.5	433.31 ± 18.59	556.89 ± 68.03	60.76 ± 2.70	47.21 ± 2.58
DOTAP + stearylamine	1.5:1.5	310.49 ± 2.97	3344.00 ± 167.83	52.31 ± 5.04	33.76 ± 4.01
DOTAP + PEI (750 kDa)	1.5:1.5	395.40 ± 13.04	722.05	66.96 ± 2.44	41.45 ± 4.77
DOTAP + DOPE	2.0:1.0	661.64 ± 53.14	704.00 ± 92.32	74.39 ± 1.63	53.16 ± 5.45
DOTAP + DOPE	1.0:2.0	459.70 ± 33.12	417.91 ± 20.35	62.76 ± 2.33	60.05 ± 6.18
DOTAP + cholesterol	2.0:1.0	425.10 ± 11.96	499.08 ± 22.98	73.19 ± 1.05	67.09 ± 3.88
DOTAP + cholesterol	1.0:2.0	357.47 ± 1.23	430.78 ± 31.90	68.70 ± 0.40	60.63 ± 6.66

Each value represents the mean ± S.D. ($n = 3$).

3.2. Effect of various co-emulsifiers in lipid emulsions

All co-emulsifiers, except for Tween 80, prepared with DOTAP (1.5%:1.5%) for the emulsions were capable of forming complexes with DNA as judged by results from agarose gel electrophoresis (Table 1). As compared to the emulsion of 1.5% DOTAP alone (control group), the incorporation of co-emulsifiers enhanced the interaction between DNA and the emulsions (*t*-test, $p < 0.05$). The emulsions containing Tween 80 with a PEG moiety could not form tight complexes, and some of free DNA was released into the agarose gel (Fig. 1). It is evident that the PEG moieties in the Tween series showed a steric hindrance in the interactions of DNA binding to lipid formulations. This effect depends on the PEG chain content and the length (Rungsardthong et al., 2001). That is why Tween 20, which has a shorter alkyl length than Tween 80, did not show reduced trapping efficiency as compared to Tween 80.

A cell viability of 70–80% is acceptable for the safety of a vector as a carrier for DNA. Non-ionic species such as DOPE, Tween 20, and cholesterol can maintain a high and tolerable viability of the COS-1 line (Table 2). However, Tween 80 can cause cell death to a certain extent in the absence of serum. Non-ionic surfactants such as Tween 80 are known to destabilize cell membranes since the lipid can extract proteins from the membrane, thus increasing the membrane's fluidity (Liu et al., 1996). In contrast to Tween 80, stearylamine showed low-cell viability in the presence of serum. PEI (750 kDa) in lipid emulsions was the most toxic co-emulsifier among all species tested.

β-Galactosidase expression was evaluated following transfection with emulsions as shown in Fig. 3. DOPE, cholesterol, stearylamine, and PEI (750 kDa) were generally more-active co-emulsifiers in the COS-1 cell line. Most of the vectors currently used for in vitro gene transfer are much less efficient in the presence of serum (Escriou et al., 1998). The advantage of using cationic emulsions as a gene carrier is the stability of the emulsion itself and of the emulsion/DNA complexes in the presence of serum (Chung et al., 2001). DOPE, cholesterol, and stearylamine in lipid emulsions can maintain high-in vitro transfection activities in serum comparable to the activity without serum. Because of the sufficient transfection in serum-containing medium, these emulsions are expected to be useful in vivo by utilizing systemic injections. However, the low-cell viability for stearylamine-containing emulsions in the presence of serum may limit the application of this emulsion. The same phenomenon was observed for Tween 80 and PEI (750 kDa). We also used PEI as a co-emulsifier with various MWs (0.8, 2, and 750 kDa) in the MTT assay. As shown in Table 2, cell viability was not improved by using PEI with different MWs. Although PEI (750 kDa) showed a sufficient calibrated transfection, it is not suitable for application because

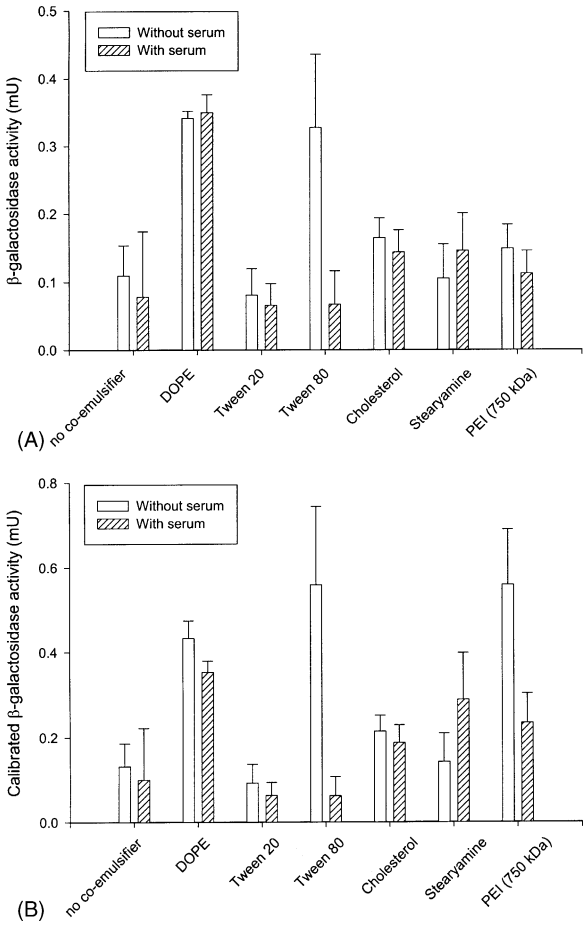

Fig. 3. In vitro transfection efficiency of lipid emulsions of DOTAP (1.5%) with various co-emulsifiers in a ratio of 1:1 (w/w) determined by β-galactosidase activity (A) and calibrated β-galactosidase activity. Each value represents the mean ± S.D. ($n = 4$). The calibrated β-galactosidase activity means β-galactosidase activity (mU) divided by cell viability (%).

of the safety to cells regardless of the MW of PEI incorporated into the emulsions.

Since it is preferable in practical use to find a balance between transfection activity and toxicity of a particular emulsion, DOPE and cholesterol may be feasible for this purpose. DOPE is a preferred helper lipid for cationic liposome-mediated DNA transfer, as it contains a phosphoethanolamine headgroup to facilitate transfer of DNA from endosomes to the cytosol (Liu et al., 1996; Wong et al., 2002). The transfection efficiency of lipopolymers as a gene vector is enhanced by cholesterol's moiety (Lee et al., 2003). Cholesterol is taken up by cells through receptor-mediated endocytosis or transfer from lipoproteins. The same effect may be also observed in lipid emulsions in the present study.

The incorporation of Tween 80 into the lipid emulsion produced a small droplet size, which might have been due to the steric repulsion of the PEG moiety in Tween 80 preventing aggregation between droplets (Table 3). This effect was not detected for Tween 20, whose structure has a shorter chain length. On the other hand, the electrostatic repulsion by the two positive co-emulsifiers, stearylamine and PEI (750 kDa), contributed to the small size of both emulsions. There was no correlation between the size distribution and transfection efficiency of lipid emulsions, indicating that the size of the emulsions did not affect their transfection activities. Interactions between serum components and emulsions, such as electrostatic and hydrophobic interactions, may increase the size and change the properties of droplets (Mao et al., 2001). FCS was used as the medium to examine the size of emulsions with DOPE and Tween 80 in the presence of serum. No significant particle growth was found for DOPE (777.87 ± 82.20 nm) or Tween 80 (298.57 ± 6.11 nm) compared to the size in the absence of serum. Since the transfection ability of DOPE and Tween 80 in serum greatly differed, the stability of size in the presence of serum did not govern the process of transfection. The key point for the maintenance of size stability may be the incorporation of squalane or DOTAP but not the co-emulsifiers in the lipid emulsions (Chung et al., 2001).

As compared to the emulsion with 1.5% DOTAP alone, the addition of co-emulsifiers at 1.5% only slightly changed the zeta potential of droplet surfaces (Table 3). The DOPE-containing emulsion showed the highest zeta potential of 70.37 ± 1.47 mV. Since DOPE itself possesses neutral charges, the enhanced charges on the droplet surface may have been because DOPE in the oil droplet accumulated free DOTAP onto the surface of the droplet due to presence of the same functional group (l,2-dioleoyl-*sn*-glycero-3-) of DOPE and DOTAP. When adding DNA to form complexes, however, the zeta potential was greatly reduced to -29.34 ± 7.71 mV for DOPE emulsions. *When FCS was used as the medium for measuring zeta potential of DOPE*, it also showed a negative charge value of -10.81 ± 1.17 mV. The cationic complexes supposedly bind to glycosaminoglycans on the cell surfaces by electrostatic interactions (Mislick and Baldeschwieler, 1996). The negatively charged complexes of DOPE may interact with different sites of the cell surface from those interacting with positively charged ones. Some studies reported that negatively charged complexes show effective transfection (Fasbender et al., 1995; Ishiwata et al., 2000). It is proposed that the highly negative charge of DOPE emulsion may strongly interact with the binding site of cells, resulting in higher DNA expression. The addition of neither the positively charged stearylamine nor PEI (750 kDa) significantly increased the positive charges of the lipid emulsions (Table 3). The Tween 80-containing emulsion showed a zeta potential of -1.62 ± 0.25 mV in the presence of serum. The size of the emulsion was significantly smaller than that of DOPE (*t*-test, $p < 0.05$).

After storage at 50 °C for 49 days, stearylamine greatly destabilized the emulsion with a 10-fold increase in droplet size (Table 3). The great reduction in the zeta potential after 49 days caused the loss of electrostatic repulsion between droplets. In contrast to stearylamine, no size change was observed for the DOPE-containing emulsion for 49 days. It has been reported that stability-enhanced vectors have better transfection activity, especially under serum and in vivo conditions (Ren et al., 1999; Kim et al., 2001). Our results confirmed this hypothesis. The zeta potential of DOPE-containing emulsions also showed a minor reduction after long-term storage. A previous investigation reported that emulsions become unstable and have a bigger size after incorporating DOPE because it is not a good emulsifier (Kim et al., 2001). However, the DOPE-containing emulsion in this study exhibited high-stability and transfection efficiency as compared to the emulsion with DOTAP alone. This may have been due to the different oil phase utilized (squalene versus

squalane). Another possibility is the discrepancy between the preparations of emulsion, i.e., the energy of sonication, treatment period, and the sequence of the process. Tween 20 and Tween 80 maintained their zeta potentials to a certain degree for 49 days (Table 3). We hypothesized that the surface conjugation of PEG increases the hydrophilicity of the droplets, and lowers their surface free energy (Mao et al., 2001). However, this effect could not prevent the aggregation of droplets during long-term storage.

Although PEI caused unacceptable cell death during transfection (Table 2), the calibrated β-galactosidase amount was high in surviving cells. PEI promotes transfection by preventing degradation of DNA by lysosomal enzymes and by disrupting endosomes in the transfection process (Tang and Szoka, 1997; Lee et al., 2003). The disruption of endosomes may cause both cell death and high-gene expression. This is related to the buffering capacity of PEI and is well known as the "proton sponge" hypothesis (Gebhart and Kabanov, 2001). As a result as shown in Fig. 4, the acid–base titration curves of all PEIs and stearylamine, the cationic species, exhibited great buffer capacity over almost the entire pH range. The other co-emulsifiers tested did not show such potential buffering capacity. It may be demonstrated that the ability to bind protons resulting in the buffering properties is not a sufficient factor to ensure high-transgene expression.

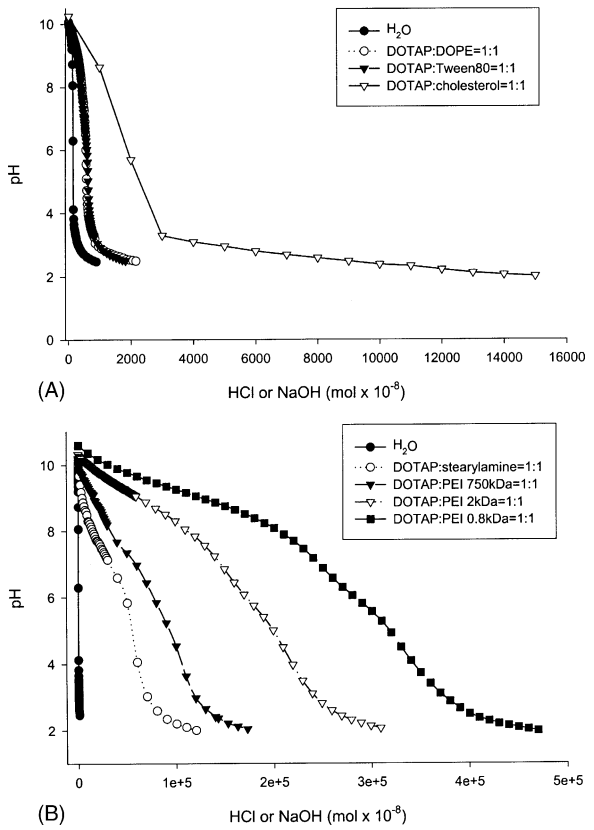

Fig. 4. The acid–base titration of lipid emulsions of DOTAP (1.5%) with various co-emulsifiers in a ratio of 1:1 (w/w).

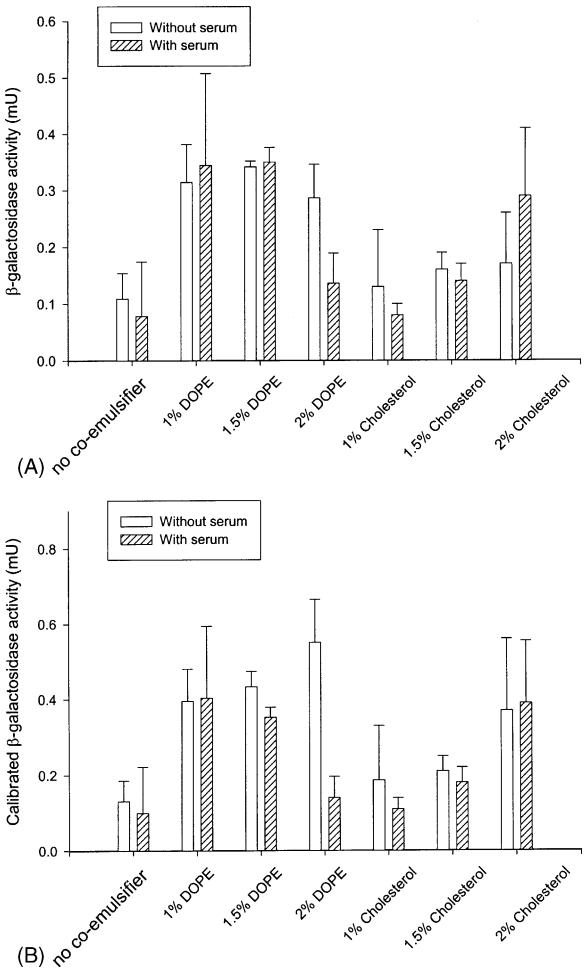

Fig. 5. In vitro transfection efficiency of lipid emulsions of DOTAP with DOPE or cholesterol in various ratios determined by β-galactosidase activity (A) and calibrated β-galactosidase activity. Each value represents the mean ± S.D. ($n = 4$). The calibrated β-galactosidase activity means β-galactosidase activity (mU) divided by cell viability (%).

Table 4
The cell viability and transfection efficiency of lipid emulsions on various cell lines

Cell line	Formulation	Cell viability (%)		Transfection (mU/well)		Calibrated transfection (mU/well)	
		Without serum	With serum	Without serum	With serum	Without serum	With serum
A549	DOTAP 1.5%	110.87 ± 6.55	105.04 ± 5.04	0.16 ± 0.04	0.10 ± 0.04	0.14 ± 0.04	0.09 ± 0.03
	DOTAP:DOPE = 1:1	101.83 ± 27.75	78.78 ± 27.38	0.21 ± 0.11	0.29 ± 0.08	0.21 ± 0.10	0.37 ± 0.10
	DOTAP:Tween 80 = 1:1	39.76 ± 18.38	97.42 ± 32.48	0.13 ± 0.07	0.15 ± 0.02	0.32 ± 0.16	0.15 ± 0.02
	DOTAP:cholesterol = 1:1	78.77 ± 13.24	83.69 ± 21.20	0.20 ± 0.04	0.23 ± 0.06	0.25 ± 0.06	0.24 ± 0.05
HaCaT	DOTAP 1.5%	27.08 ± 14.30	122.40 ± 39.67	0.23 ± 0.04	0.10 ± 0.02	0.86 ± 0.14	0.08 ± 0.02
	DOTAP:DOPE = 1:1	18.47 ± 13.02	97.43 ± 12.28	0.19 ± 0.08	0.15 ± 0.04	1.03 ± 0.44	0.15 ± 0.04
	DOTAP:Tween 80 = 1:1	32.26 ± 10.83	109.45 ± 17.75	0.12 ± 0.02	0.14 ± 0.01	0.39 ± 0.07	0.13 ± 0.01
	DOTAP:cholesterol = 1:1	39.60 ± 9.63	85.15 ± 19.23	0.25 ± 0.09	0.09 ± 0.04	0.63 ± 0.23	0.11 ± 0.06

Each value represents the mean ± S.D. ($n = 4$). The calibrated β-galactosidase activity means β-galactosidase activity (mU) divided by cell viability (%).

3.3. Effect of various DOPE or cholesterol proportions in lipid emulsions

Since DOPE and cholesterol showed good transfection efficiencies and stabilities for emulsions, DOPE or cholesterol and DOTAP in various ratios (1.5:1.5, 2:1, and 1:2) were used to form emulsions for further investigation. As shown in Table 1, an excess of DOPE at a ratio of DOTAP: DOPE of 1:2 was loosely associated with DNA to form complexes. The cell viability in the absence of serum was also acceptable ranges for this ratio (Table 2). However, the cell viability in the presence of serum, which mimics the in vivo condition, was tolerable for all DOPE-containing emulsions. Altering the DOPE proportion did not change the good stability of emulsions as demonstrated by droplet size and zeta potential (Table 3).

As shown in Fig. 5, the transfection activities of emulsions with 1% and 1.5% DOPE were comparable (Student's *t*-test $p > 0.05$). The similar trapping volumes of DNA and physicochemical characteristics of both emulsions may predict this result of transfection. Lower β-galactosidase expression from the emulsion with 2% DOPE was observed in the presence of serum. This may have been due to the looser interaction between DNA and the emulsion. The components in serum may decompose the dissociated DNA. Therefore, a sufficient concentration of DOTAP of greater than 1.5% was needed for lipid emulsions to maintain good transfection ability.

Similar to the trapping efficiency of DOPE, the higher proportion of cholesterol (2%) resulted in a lower interaction with DNA (Table 1). However, this low interaction did not reduce the transfection activity of the emulsions (Fig. 5). This may indicate that the trapping efficiency between lipid emulsions and DNA does not necessary affect DNA expression via the emulsions. Another observation is that the transfection activity in the presence of serum increased following the increase in cholesterol proportions. This result somewhat differs from that of DOPE, indicating the different mechanisms of transfection between these two lipids. As depicted in Table 3, the emulsions with 2% DOPE or cholesterol significantly (Student's *t*-test, $p < 0.05$) reduced the droplet size as compared to the emulsions with lower concentrations. The profiles of size and zeta potential after 49 days of storage also assured the good stability of the emulsions with cholesterol (Table 3). The safety of cholesterol was acceptable according to the MTT assay, except for the emulsion with 2% cholesterol in the absence of serum. This might not affect the application of this emulsion in in vivo conditions.

3.4. Transfection of lipid emulsion by different cell lines

β-Galactosidase expression was evaluated following transfection of lipid emulsions in various cell lines to assess cell-type dependence of the emulsions. Two cell lines, A549 and HaCaT, were used for examination. A549 is a cell line of human lung epithelial cells, while HaCaT is a cell line of human keratinocytes in the skin. A previous investigation showed that liposomes

with DOTAP effectively passed through the epidermis of the skin (Alexander and Akhurst, 1995). As shown in Table 4, all emulsions tested showed high-cell viability in the presence of serum. However, a lower viability was detected in the absence of serum especially for HaCaT. This may have been because of the small size of HaCaT cells, which could not undergo incubation in lipid emulsions over a long period. Since the addition of serum mimics the in vivo situation, it is worth examining the transfection efficiencies of lipid emulsions of these two cell lines. The inclusion of DOPE, Tween 80 or cholesterol increased (Student's *t*-test, $p < 0.05$) the transfection of both A549 and HaCaT in the presence of serum (Table 4). DOPE and cholestarol showed greater transfection activity than Tween 80 in the A549 cell line, which is the same trend as the result for COS-1. However, this phenomenon was not observed for HaCaT. Furthermore, discrepancies in transfection in the presence of serum among the four emulsions tested was not large for HaCaT.

4. Conclusions

The transfection efficiency and safety of lipid emulsions with various co-emulsifiers were evaluated in the absence and presence of serum. Different physicochemical properties, including size, zeta potential, buffering capacity, and stability, were also examined. Non-ionic surfactants such as Tween 80 produced low-cell death in the presence of serum. However, their low-transfection activity and physical stability may reduce their practical use. The addition of cationic stearylamine and PEI produced significant cell death, although the remaining cells were able to express high transfection of β-galactosidase. Two lipids, DOPE and cholesterol, were preferable for emulsion-mediated DNA transfer due to their high-transfection activities and safety. The stability was also enhanced after inclusion of DOPE or cholesterol into emulsions with DOTAP.

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References

Alexander, M.Y., Akhurst, R.J., 1995. Liposome-mediated gene transfer and expression via the skin. Hum. Mol. Ther. 4, 2279–2285.

Bonadio, J., 2000. Tissue engineering via local gene delivery: updated and future prospects for enhancing the technology. Adv. Drug Deliv. Rev. 44, 185–194.

Chung, H., Kim, T.W., Kwon, M., Kwon, I.C., Jeong, S.Y., 2001. Oil components modulate physical characteristics and function of the natural oil emulsions as drug or gene delivery system. J. Control. Release 71, 339–350.

Dodds, E., Dunckley, M.G., Naujoks, K., Michaelis, U., Dickson, G., 1998. Lipofection of cultured mouse muscle cells: a direct comparison of lipofectamine and DOSPER. Gene Ther. 5, 542–551.

Escriou, V., Ciolina, C., La Croix, F., Byk, G., Scherman, D., Wils, P., 1998. Cationic lipid-mediated gene transfer: effect of serum on cellular uptake and intracellular fate of lipopolyamine/DNA complexes. Biochim. Biophys. Acta 1368, 276–288.

Fasbender, A.J., Zabner, J., Welsh, M.J., 1995. Optimization of cationic lipid-mediated gene transfer to airway epithelia. Am. J. Phys. 269, L45–L51.

Filion, M.C., Phillips, N.C., 1998. Major limitations in the use of cationic liposomes for DNA delivery. Int. J. Pharm. 162, 159–170.

Gebhart, C.L., Kabanov, A.V., 2001. Evaluation of polyplexes as gene transfer agents. J. Control. Release 73, 401–416.

Hofland, H.E.J., Shephard, L., Sullivan, S.M., 1996. Formation of stable cationic lipid/DNA complexes for gene transfer. Proc. Natl. Acad. Sci. U.S.A. 93, 7305–7309.

Ishiwata, H., Suzuki, N., Ando, S., Kikuchi, H., Kitagawa, T., 2000. Characteristics and biodistribution of cationic liposomes and their DNA complexes. J. Control. Release 69, 139–148.

Kim, T.W., Chung, H., Kwon, I.C., Sung, H.C., Jeong, S.Y., 2001. Optimization of lipid composition in cationic emulsion as in vitro and in vivo transfection agents. Pharm. Res. 18, 54–60.

Kim, T.W., Kim, Y.J., Chung, H., Kwon, I.C., Sung, H.C., Jeong, S.Y., 2002. The role of non-ionic surfactants on cationic lipid mediated gene transfer. J. Control. Release 82, 455–465.

Kurisawa, M., Yokoyama, M., Okano, T., 2000. Gene expression control by temperature with thermo-responsive polymeric gene carriers. J. Control. Release 69, 127–137.

Lee, M., Rentz, J., Han, S.O., Bull, D.A., Kim, S.W., 2003. Water-soluble lipopolymer as an efficient carrier for gene delivery to myocardium. Gene Ther. 10, 585–593.

Liu, F., Yang, J., Huang, L., Liu, D., 1996. Effect of non-ionic surfactants on the formation of DNA/emulsion complexes and emulsion-mediated gene transfer. Pharm. Res. 13, 1642–1646.

Mao, H.Q., Roy, K., Troung-Le, V.L., Janes, K.A., Lin, K.Y., Wang, Y., August, J.T., Leong, K.W., 2001. Chitosan-DNA nanoparticles as gene carriers: synthesis, characterization and transfection efficiency. J. Control. Release 70, 399–421.

Mislick, K.A., Baldeschwieler, J.D., 1996. Evidence for the role of proteoglycanes in cation-mediated gene transfer. Proc. Natl. Acad. Sci. U.S.A. 93, 12349–12354.

Ren, T., Zhang, G., Song, Y.K., Liu, D., 1999. Synthesis and characterization of aromatic ring-based cationic lipids for gene delivery in vitro and in vivo. J. Drug Target. 7, 285–292.

Rungsardthong, U., Deshpande, M., Bailey, L., Vamvakaki, M., Armes, S.P., Carnett, M.C., Stolnik, S., 2001. Copolymers of amine methacrylate with poly (ethylene glycol) as vectors for gene therapy. J. Control. Release 73, 359–380.

Tang, M.X., Szoka, E.C., 1997. The influence of polymer structure on the interactions of cationic polymers with DNA and morphology of the resulting complexes. Gene Ther. 4, 823–832.

Wong, F.M.P., Macadam, S.A., Kim, A., Oja, C., Ransay, E.C., Bally, M.B., 2002. A lipid-based delivery system for antisense oligonucleotides derived from a hydrophobic complex. J. Drug Target. 10, 615–623.

Yi, S.W., Yune, T.Y., Kim, T.W., Chung, H., Choi, Y.W., Kwon, I.C., Lee, E.B., Jeong, S.Y., 2000. A cationic lipid emulsion/DNA complex as a physically stable and serum-resistant gene delivery system. Pharm. Res. 17, 314–320.

Zelphati, O., Nguyen, C., Ferrari, M., Feigner, J., Tsai, R., Feigner, P.L., 1998. Stable and monodisperse lipoplex formulations for gene delivery. Gene Ther. 5, 1272–1282.