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Counterion Effects on Transfection Activity of Cationic Lipid Emulsion

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Abstract Cationic lipid emulsion systems consisting of 1,2-dioleoyl-sn-glycero-3-trimethylammonium-propane (DOTAP) and plasmid DNA with various counterions in the lipid headgroups were prepared. The transfection activity of the cationic lipid emulsion systems was then investigated *in vitro* and *in vivo*. The complex formation of plasmid DNA and lipid emulsion was affected by the counterions through charged headgroup repulsion and also by the salt concentration in the media. As such, the transfection activity of the DOTAP emulsion system can be controlled by changing the counterions.

Keywords: gene transfer, cationic lipid emulsion, counterion, DOTAP, COS-1 cell

INTRODUCTION

The treatment or prevention of disease by gene transfer (gene therapy) is a potential revolution in medicine. This is because gene therapies aim at treating or eliminating the cause of disease, whereas most current drugs only treat the symptoms [1]. Gene transfer into the body resulting in generalized or tissue-specific expression could facilitate precise *in vivo* manipulations of biological processes to cure diseases and induce immune responses against pathogens [2,3]. Various non-viral gene delivery systems have already developed for the purpose of gene therapy and cationic liposomes have been widely used in gene transfer both *in vitro* and *in vivo*. Various kinds of cationic lipids have been synthesized in the hope of identifying one, with a high transfection activity and low cytotoxicity [4-6].

Since Felgner introduced the first liposomal transfection agent [4], many scientists have searched to find a better and more efficient liposomal transfection agent [5]. One of the most important features in a successful liposomal gene carrier is the cationic lipid itself [6]. Some lipids have been proven as good agents in the absence of serum for *in vitro* transfection, yet failed in *in vivo* applications. Although the manner in which cationic lipids change the transfection activity has not been studied systematically, several attempts have been made to understand the underlying mechanism [7,8].

In previous work by the current authors, it was shown that the oil-in-water (o/w) cationic emulsion system is physically stable and can facilitate the transfer of genes successfully in the presence of up to 80% (v/v) serum in COS-1 and CV-1 cells [9]. This emulsion system was developed based on the principles of a liposomal transfection agent. As such, in both systems, cationic lipids govern the transfection efficiencies. Yet, while lipids form bilayer leaflets that separate the inner and outer aqueous phases in liposomes, in o/w emulsions they are used as the emulsifier, which distributes mainly at the interface between the inner oil and outer aqueous phases. Various emulsion formulations with different cationic lipids as emulsifiers, and additional helper lipids as co-emulsifiers, have already been tested as gene carriers. These stable emulsion systems were found to deliver genes more efficiently to endothelial cells in a mouse nasal cavity than commercialized liposomes [10].

Accordingly, the current study investigated various counterions of lipid headgroups to determine the counterion effect on the transfection activity of the cationic lipid emulsion system *in vitro* and *in vivo*. In the case of cationic liposomes, counterions have been shown to influence the transfection efficiency [11,12]. Since the interaction between DNA and the cationic carrier is mainly governed by an electrostatic interaction, counterions and their concentration can play a significant role in the complex formation and transfection. The tonicity of the media was also changed during the complex formation, plus the salt concentration in the complex media was also varied to determine its effect on the transfection activity of the cationic lipid emulsion system.

MATERIALS AND METHODS

Materials

Squalene was purchased from Sigma (St. Louis, MO, USA). 1,2-Dioleoyl-sn-glycero-3-trimethylammonium-propane (DOTAP) was purchased from Avanti Polar Lipids, (Alabaster, AL, USA) (AP) and Boerhinger

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Mannheim (Indianapolis, IN, USA) (BM) and used without further purification. Dulbecco's modified Eagle's medium (DMEM, high glucose) and the fetal bovine serum (FBS) were purchased from Gibco BRL/Life Technologies (New York, NY, USA). Chloride, methyl sulfate, bisulfate, and triflate were purchased from Aldrich (Sheboygan, WI, USA). All other chemicals and reagents were of tissue culture grade.

Plasmid DNA

The pCMV-beta encoding *Escherichia coli* (*E. coli*) *lacZ* (β-galactosidase) gene expression plasmid driven by the human cytomegalovirus immediate-early promoter was purchased from Clontech Laboratories (Palo Alto, CA, USA). The plasmid pCMV-Luc+ consisting of a cytosolic form of *Phontinus pyralis* luciferase cDNA, obtained from the plasmid pGL3 (Promega, Madison, WI, USA) using XbaI and HindIII, was subcloned into the plasmid pcDNA3.1 (Invitrogen) in laboratory of the current authors. The plasmids were amplified in the *E. coli* DH5-α strain and purified using a Qiagen mega-kit (Qiagen Inc., Chatsworth, CA, USA) according to the manufacturer's instruction. The DNA purity was determined by agarose gel electrophoresis and by measuring the optical density. DNA with OD_{260}/ $OD_{280} \geq 1.8$ was used.

Counterion Exchange of the Lipid

The counterion of DOTAP was exchanged through the ion-exchange column method [13]. Chloride substitution was achieved using Dowex strongly anionic exchange resin (8% crosslink (200-400 mesh), chloride form). The resin (1.0-1.5 g) was suspended in highly purified filtered water (10-20 mL), and loaded into a narrow bore glass column. The column was washed with water (5×, 5 mL) and methanol (10×, 5 mL), and then equilibrated with a $CH_3OHCH_2Cl_2$ (8:2 by volume) solution. The cationic lipid (50-70 mg) was solubilized in *ca.* 1 mL $CH_3OHCH_2Cl_2$ (8:2 by volume) and then gravity-eluted through the column. To substitute other counterions, the hydroxide resin was pretreated by washing with a 1 M solution of the desired counterion as its sodium salt. The loaded resin was then washed with water until the eluent pH stabilized at 7. Ion exchange chromatography was then performed using the $CH_3OHCH_2Cl_2$ equilibration sequence described above. Electrospray ionization mass spectrometry was used to verify the composition of the resulting salt forms.

Preparation of Lipid Carriers

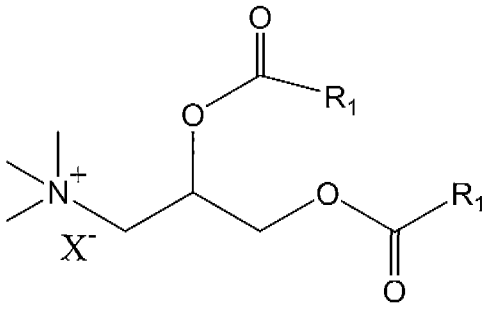
The liposomes and emulsions were prepared as described previously [9]. Briefly, 24 mg/mL of counterion exchanged DOTAP was weighed, dispersed in water, and sonicated for 2-4 minutes in an ice/water bath using a probe type sonicator (High intensity ultrasonic processor, 600W model, Sonics and Materials, Danbury, CT, USA). One hundred micrograms of squalene was then added to the lipid dispersion, and the mixture sonicated further in an ice/water bath for *ca.* 4 minutes to form the emulsion. To prepare the liposome carriers, the lipid dispersions were sonicated for another 4 minutes after the solution became clear. They were then kept at 4 °C for further experiments. The average particle size and surface potential of the lipid carriers (liposomes and emulsions) and DNA/carrier complexes were measured using the photon correlation spectroscopy (Malvern Zetasizer, Malvern Instruments Ltd., England), as described previously [9]. The emulsion and liposomes were diluted 300 and 3 times, respectively, with water or PBS for the measurement. The average particle size of the lipid emulsion system was 180 nm and the polydispersity was narrow (below 0.2).

In vitro Gene Transfer

COS-1, a derivative of simian kidney cell line, was cultured in DMEM supplemented with 10% FBS at 37°C in a humidified 5% carbon dioxide incubator. The cells were seeded at 2×10^4 cells per well in 96-well plates 12 hours before transfection. The cells were ca. 70-80% confluent at the time of transfection. For a single well, 500 ng of pCMV-beta or 50 ng of pCMV-Luc+ were mixed with an appropriate amount of carriers (weight ratio between carrier and DNA, C/D=4) to form a complex in 40 μL of serum-free DMEM. After washing the COS-1 cells with serum-free DMEM, 160 μL of serum-free DMEM was added. To test effect of serum, 160 μL of FBS was added instead of 160 μL of serum free DMEM. After one hour of incubation, the cells were washed with serum-free DMEM to remove the remaining carrier/DNA complexes. The cells were then fed again with DMEM containing 10%(v/v) FBS and cultured for 24 h after transfection. The transfected cells were assayed for β-galactosidase activity using a photometric assay as previously described [6] and for luciferase activity using a kit purchased from Promega (Madison, WI, USA) with a luminometer (Turner Designs Luminometer Model TD-20/20, Promega).

In vivo Gene Transfer and Luciferase Assay

To prepare the carrier/DNA complexes, a DNA solution containing 20 μg of pCMV-Luc+ and 80 μg of the carrier, each diluted with 100 μL of PBS (0.75 fold dilution with deionized water), respectively, was mixed by inversion. The complex solution was incubated at room temperature for 20 minutes and administered to female Balb/c mice weighing approximately 20-25 g (6-8 weeks old) via the intranasal route. The mice were sacrificed after 24 h, and the organs, such as the lungs and nasal cavity were removed and homogenized using a high-speed homogenizer (T-25-Ultra-Turrax, Janke & Kunkel GmbH & Co KG, Germany) in a lysis buffer (0.1 M Tris-HCl, 2 mM EDTA, 0.1% Triton X-100, pH 7.8, 5 μL/mg for each collected organ). After two freeze/thaw cycles, the homogenized organ lysates were centrifuged at 4°C for 10 minutes at 12,000 rpm in an Eppendorf



Kosmotropic X⁻	Chaotropic X⁻
HSO_4^{-1}	$CH_3C(O)O^-$
$CF_3SO_3^{-1}$	Cl^-
$H_2PO_4^-$	Br^-
SO_4^{-2}	I^-

Fig. 1. Structure of DOTAP; R_1 = Oleoyl (18:1). The variable X⁻ represents the selected counterions tabulated.

centrifuge. A portion of the supernatants was assayed for the protein concentration using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The luciferase activity in the lysates was quantified using a Promega kit (Madison, WI, USA) with a luminometer (Turner Designs Luminometer Model TD-20/20, Promega).

RESULTS AND DISCUSSION

In vitro Transfection with Various Counterions

The effect of counterions on the *in vitro* transfection of the DOTAP emulsion system was investigated in this study. The typical structure of DOTAP with various counterions is shown in Fig. 1. Fig. 2 shows the transfection activity of the DOTAP emulsion with various counterions, chloride, bromide, methyl sulfate, bisulfate, and triflate. The counterions of the lipid headgroups affected the binding of the DNA and carrier system. Bisulfate and triflate, with three resonance forms, promoted significant water structuring. The extent of the water organization has previously been shown to be responsible for lipid headgroup dehydration [14]. Thermodynamically, lipid headgroup dehydration promotes greater lipid packing, leading to lipid particles that are destabilized through charged headgroup repulsions. While increased electrostatic repulsions give rise to metastable particles whose free energies are reduced upon DNA-induced lipid reorganization. The methyl sulfate counterion, although including four resonance structures, promoted the lowest levels of transfection activity, presumably due to the stronger electrostatic interaction of sulfate dianion that supersedes the predicted Hofmeister series of neutral salts. The free base form of DOTAP was examined *in vitro* and showed activities comparable to free DNA. The halogens were

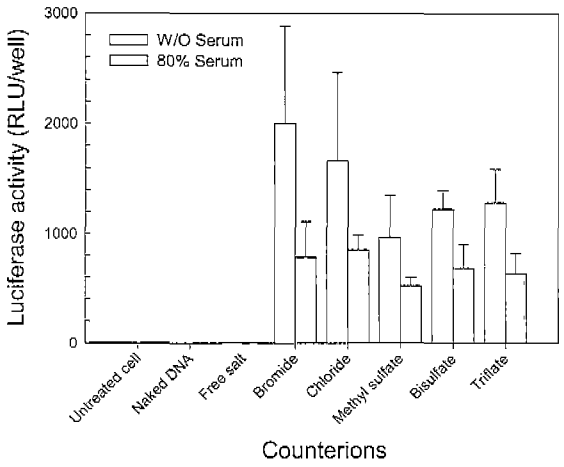


Fig. 2. *In vitro* transfection activity of DOTAP emulsion with various counterions, chloride, bromide, methyl sulfate, bisulfate, and triflate in COS-1 cell line (The error bars mark the standard deviation when n=5).

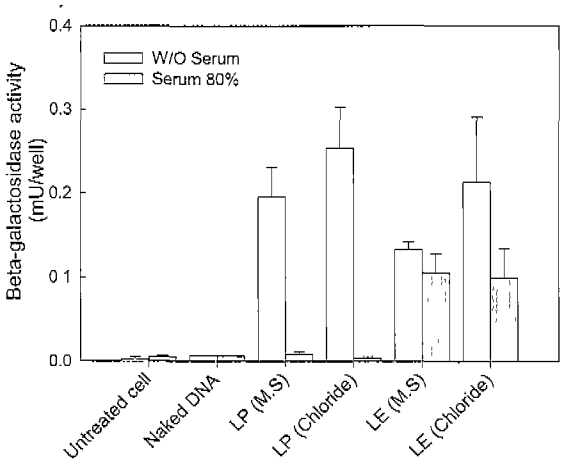


Fig. 3. *In vitro* transfection activity of DOTAP liposome and emulsion with different counterions, methyl sulfate and chloride in COS-1 cell line (The error bars mark the standard deviation when n=5).

more closely associated with the alkyl ammonium headgroup (charge shielding), in the order of chloride to bromide. An increase in charge shielding leads to water exclusion and closer interchain packing. Indeed, the transfection activity averages were found to increase from chloride to bromide [13]. Fig. 3 shows a comparison of the transfection activity of liposome and emulsion made with DOTAP purchased from two different companies with different counterions, methyl sulfate (BM) and chloride (AP). The emulsion system transfected more efficiently than the liposome system in the presence of serum. Since the ultimate goal of gene delivery is curing diseases, the gene delivery carrier should transfect efficiently with serum *in vitro* to be useful for *in vivo* application. The proposed emulsion system ap-

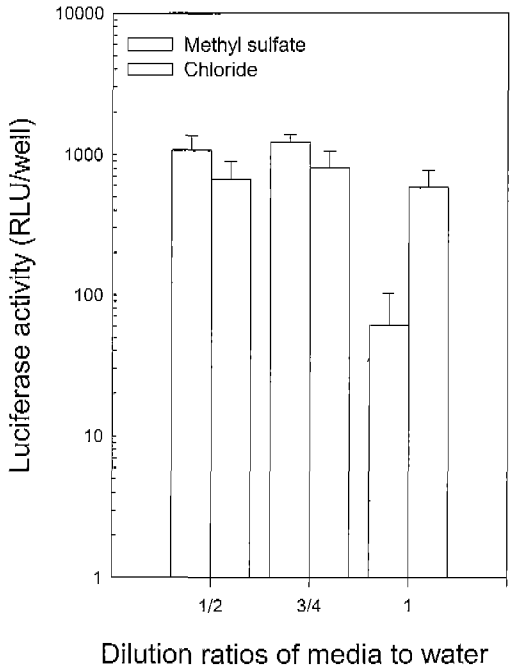


Fig. 4. *In vitro* transfection activity of DOTAP emulsion in various concentrations of PBS media with serum in COS-1 cell line (The error bars mark the standard deviation when n=5).

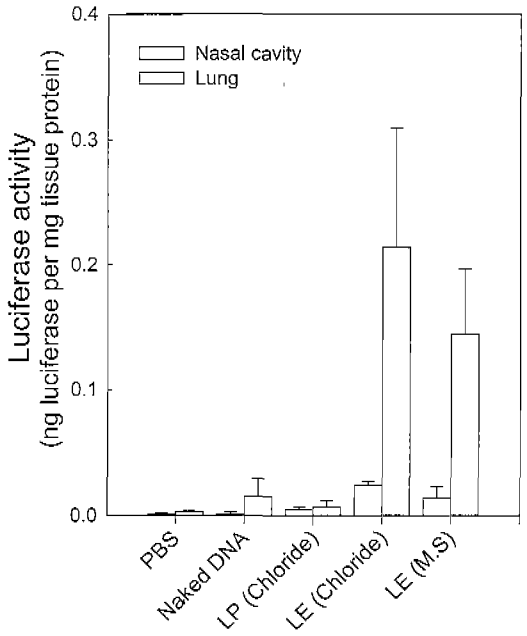


Fig. 5. *In vivo* transfection activity of DOTAP emulsion with different counterions via the intranasal administration (The error bars mark the standard deviation when n=5).

peared to overcome any destabilization of the complex induced by the serum proteins during the *in vivo* experiment. The DOTAP chloride (AP) carrier systems transfect more efficiently than DOTAP methyl sulfate (BM) carrier systems in the absence of serum. However, the transfection efficiency was similar for the two systems in the presence of serum. Fig. 4 shows the transfection activity of the DOTAP emulsion with different counterions in various concentrations of PBS media with serum. The salt concentration in the media also affected the transfection activity of the DOTAP emulsion. The counterion effect on the DOTAP emulsion was apparent in the 1× PBS solution. The counterion effect almost disappeared in the PBS solution diluted with deionized water. Also, the *in vitro* transfection efficiency was higher under hypotonic conditions with or without serum.

In vivo Transfection via Intranasal Administration

To evaluate the *in vivo* transfection activities of the DOTAP emulsions with the counterion effect, 20 μg of pCMV-Luc+ was made into complexes with emulsions in a PBS solution and administered to BALB/c mice via the intranasal route. Counterpart liposomes were used for comparison. Twenty-four hours after the injection of the complex, the luciferase activity in different organs was analyzed. No signs of toxicity were noticed under the experimental conditions used. The levels of gene expression were determined in tissues from the lung and nasal cavity (Fig. 5). In all lipid formulations, the expression level in the lung lysates was 5-6 fold higher than that in the nasal cavity lysates. They can be useful for the treatment of respiratory disease such as cystic fibrosis. The relative transfection efficiencies in different organs can be varied by changing the components of emulsions (data not shown). In the lung lysates, the emulsions showed at least 10 times more luciferase activity than in the counterpart liposomes. The DOTAP emulsion with chloride salts yielded higher transfection activities in the lung lysates than the emulsion with methyl sulfate salts. This was consistent with the results from the *in vitro* experiment.

CONCLUSION

This study used an identical cationic lipid, DOTAP to formulate emulsions and liposomes. The counterion of the lipid headgroup was exchanged through a column packed with specific counterions. The complex formation of the DNA and lipid emulsion was affected by the counterions through charged headgroup repulsion induced by lipid headgroup dehydration and a charge shielding effect. The transfection activity of the cationic lipid emulsion was high in bromide and lowest in the methyl sulfate attached DOTAP emulsion. The transfection activity was controlled by changing the counterions of the lipid headgroups. The transfection activity of the DOTAP emulsion system was also affected by the salt concentration of the complex media since it interfered in the complex formation of the DNA

and cationic lipid emulsion.

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