

# Nature of linkage between the cationic headgroup and cholesteryl skeleton controls gene transfection efficiency

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**Abstract** Three novel cationic cholesterol derivatives with different modes of linkage between the cationic headgroup and the cholesteryl backbone have been synthesized and used as mixtures with 1,2-dioleoyl-L- $\alpha$ -glycero-3-phosphatidyl ethanolamine (DOPE) for liposome-mediated gene transfection. A pronounced improvement in gene transfer efficiency was observed when the cationic center was appended to the cholesteryl backbone using an ether linkage as opposed to when the linkages were based on either ester or urethane groups. Amphiphiles with ether links such as cholest-5-en-3 $\beta$ -oxyethane-*N,N,N*-trimethyl ammonium bromide (**2**) and cholest-5-en-3 $\beta$ -oxyethane-*N,N*-dimethyl-*N*-2-hydroxyethyl ammonium bromide (**3**) showed transfection efficiencies considerably greater than commercially available gene transfer reagents. Notably, the transfection ability of **2** with DOPE in the presence of serum was significantly greater than Lipofectamine<sup>®</sup> and Lipofectin<sup>®</sup>. Interestingly, **3** did not require the helper lipid DOPE for transfection. This suggests that these newly described cholesterol-based amphiphiles should be very promising in liposome-mediated gene transfection. The advantage that the ether linkage possesses would be important in the design of newer, more efficient cholesterol-based delivery reagents.

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**Key words:** Cationic liposome; Cationic cholesterol amphiphile; Ether linkage; DNA transfection

## 1. Introduction

Experimental development of improved gene transfer methods is an essential prerequisite for gene therapy [1–3] to realize its early potential. We have been exploring the mechanisms of cationic lipid-mediated DNA transfer across eukaryotic cells [4,5]. Though currently less effective than viral gene transfer vectors, cationic liposomes are being increasingly considered for gene delivery due to their relative stability, greater carrier capacity and ease of large-scale production [6]. However, a comparison of the transfection abilities of reported cationic lipid systems toward a particular cell type is often difficult. This is because the molecular structure of the cationic lipids and the cell types that have been used to gauge transfection efficiencies are disparate. Despite such diversity both at the level of lipids and the cell types, a few correlations can be

drawn. For instance, *N*-(1,2,3-dioleoyloxy)propyl)-*N,N,N*-trimethyl ammonium chloride (DOTMA), which contains ether linkages between trimethyl ammonium headgroup and the long alkyl chains, is shown to have a much greater transfection efficiency than the corresponding cationic lipid, DOTAP, with ester linkages at identical locations [7]. Thus the functional group that connects the cationic headgroup and the hydrocarbon chains of the cationic lipid molecules plays an important role in their utilization as gene transfer agents.

In this connection, an alternative class of cholesterol-based molecules are also finding use as effective gene transfer agents. For instance, 3 $\beta$ [*N,N,N'*-dimethylaminoethane]-carbamoyl cholesterol (DC-Chol), a cationic cholesterol derivative, has been successfully used as coaggregate with 1,2-dioleoyl-L- $\alpha$ -glycero-3-phosphatidyl ethanolamine (DOPE) to prepare liposomes that transfect mammalian cells efficiently [8]. These DC-Chol liposomes have already been used in gene therapy applications in clinical settings also [9,10]. These early successes spurred recent interest in the development of novel cholesterol-based reagents [8,11,12,14–20]. However, the importance of the mode of linkage of the cationic headgroup to the steroid backbone has never been addressed. We have chosen three cholesterol-based cationic amphiphiles (Fig. 1) comprising a single positive charge attached to the steroid backbone through three different linkage types, namely an ester (**1**), ether (**2**) and urethane (**4**). The transfection efficacy of **4** has been reported by Nakanishi et al. [20] and was chosen for both assessing the importance of a urethane linker as well as a reference point to place our results in appropriate perspective with the emerging cholesterol-based transfection reagents. We have found as high as ~600% advantage in the transfection efficiency upon appending the charged headgroup to the steroid backbone through an ether linkage than with the corresponding ester or urethane derivatives. In order to see whether transfection efficiencies could be further improved by attaching a hydroxyethyl moiety as shown earlier [18,19], we also prepared **3**. Cholesterol amphiphiles **1–3** are easily synthesized in their pure forms with high yield and are efficient cytofectins. **2** is active as a 1:1 formulation with DOPE as a helper lipid. In contrast, **3** does not even require a helper lipid like DOPE and is almost as efficient a cytofectin as **2**. Both formulations are chemically stable and if stored frozen, possess long shelf life.

## 2. Materials and methods

### 2.1. Materials

DOPE was purchased from Avanti Polar Lipids. Commercially

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available transfection reagents Lipofectin® and Lipofectamine® were obtained from Gibco BRL (USA). The cationic cholesteryl amphiphiles 1–3 shown in Fig. 1 have already been synthesized and will be described elsewhere (Bhattacharya, S. and Krishnan-Ghosh, Y., unpublished work). The luciferase assay kit was obtained from Promega (MA, WI, USA).

## 2.2. Liposome preparation

A mixture of cationic amphiphile and DOPE in the desired mol ratio was taken in  $\text{CHCl}_3$  in autoclaved Wheaton glass vials and the solvent was evaporated under vacuum from the solutions to form respective thin films. This was re-suspended in freshly autoclaved Milli-Q water. The final lipid concentration was 0.2 mg/ml for all the cationic cholesterol-derived formulations. The mixture was left for hydration at room temperature for ~6 h and then vortex-mixed for 5 min followed by bath sonication for 15 min. The formulations were filtered under sterile conditions through a 0.22  $\mu\text{m}$  filter (Sartorius) and stored at 4°C. The diameters of the closed, cationic liposomes were generally between ~80 and 400 nm as checked by transmission electron microscopy of the individual aliquots of suspensions from mixtures of respective cationic cholesterol derivative with DOPE.

## 2.3. Plasmid DNA

Plasmid pGL3-Control vector (Promega, MA, WI, USA) containing the luciferase reporter gene under the control of the SV40 promoter vector was amplified in *Escherichia coli* (DH5 $\alpha$ ) and purified using the Qiagen Midi Prep Plasmid Purification protocol (Qiagen, Germany).

## 2.4. Transfection procedure

A general procedure for a typical transfection was as follows. COS-7 cells were seeded in 24-well tissue culture plates (Nunc) the day before transfection in order to be approximately 80% confluent the next day. Cells were cultured in DMEM:F12 supplemented with 10% fetal calf serum (FCS). Cells were maintained at 37°C in a humidified 5%  $\text{CO}_2$ /95% air containing atmosphere. Plasmid DNA (0.3  $\mu\text{g}$ ) and the desired cationic cholesteryl amphiphile (4  $\mu\text{g}$ ) were each diluted to 100  $\mu\text{l}$  individually of DMEM without FCS. After  $\approx$  5 min, the two solutions were combined, gently mixed and the resulting solution was allowed to incubate for 15 min at room temperature to allow complex formation. The transfection mixture was then added (0.2 ml/well) to the cells which had been washed with serum free medium. After 8 h incubation at 37°C, the transfection mixture was removed and 0.4 ml of DMEM:F12 containing 10% FCS was added to each well. The cells were harvested 2 days post-transfection for monitoring of the transient expression of the luciferase gene. Control transfections were performed by using commercially available transfection reagents. The lipopolyamine Lipofectamine® (DOSPA/DOPE) was used at a concentration of 4  $\mu\text{g}$ /well. For transfection with the commercial grade Lipofectin®, (DOTMA/DOPE)–DNA complexes were obtained using the standard conditions specified by the manufacturer. Transfections in the presence of serum were carried out in an identical manner with the transfection mixture being overlaid on wells containing an equal volume of 20% FCS such that the wells contained the lipid–DNA complex in 10% FCS. All experiments were performed in duplicates.

## 2.5. Luciferase assay

Luciferase activities were assayed 48 h post-transfection using a modified procedure of De Wet et al. [22] and according to the manufacturer's instructions. After removal of the culture medium, the cells were washed with cold phosphate buffer–saline and lysed by incubation with 60  $\mu\text{l}$  cell lysis buffer (25 mM Tris–HCl and 25 mM phosphate buffers, pH 7.8, 2 mM dithiothreitol, 2 mM CGTA, 10% glycerol, 1% Triton X-100). The lysate was clarified from insoluble material by centrifugation (for 5 s at 12000 $\times$ g). The supernatant was transferred to a new tube and the pelleted cell debris was discarded. An aliquot (2  $\mu\text{l}$ ) of the cell extract at room temperature was mixed with 20  $\mu\text{l}$  of luciferase assay substrate (Promega, WI, USA). Samples were placed in a photon counting luminometer (Turner Designs Model, TD 20/20 Luminometer) and the integration value of light emission was measured for 10 s. Protein concentrations were measured by using Bradford's method with bovine serum albumin as a standard [23]. Data for luciferase activity are expressed as relative light units (RLU)/ $\mu\text{g}$  of cell protein.

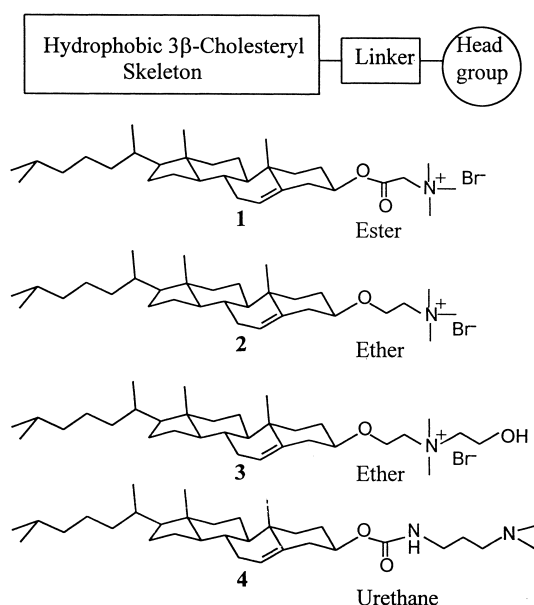


Fig. 1. Stereochemical representation of the chemical structure of the cationic cholesterol-based amphiphiles 1–4. 4 contains a dimethylamino group which gets protonated at physiological pH and thus behaves as a cationic headgroup, potentiating transfection [20].

## 3. Results

### 3.1. Transfection ability of pGL3 plasmid DNA

Stable, closed liposomes (80–400 nm) could be easily prepared from each of 1–4 and DOPE by first subjecting them to repeated freeze–thaw cycles followed by sonication in aqueous media. These liposomes were able to mediate the transfection of pGL3-Control DNA. In order to see the most effective formulations, transfections with identical concentrations of DNA (0.3  $\mu\text{g}$ ) and lipid (4  $\mu\text{g}$ ) varying the mol ratios of 1–4 in DOPE were performed (Fig. 2A–D). 1 was most effective as a 1:2 of 1/DOPE mol ratio composition (Fig. 2A) and 2 was active in a 1:1 mol ratio (Fig. 2B). However, 3 was found to be most active without DOPE (Fig. 2C). When the optimal transfection efficacies for a particular lipid were compared, no extra advantage in the transfection efficiency on replacement of the urethane group with an ester linkage was seen. Interestingly, replacement of the urethane linkage with an ether linkage led to a 6-fold increase in transfection efficiency. 2 with DOPE showed a transfection efficiency comparable to Lipofectamine under the same conditions. According to previous reports [20,21] of potentiation of transfection by appending a hydroxyethyl moiety, 3 was hence tested. 3 behaved in a manner completely different from the other amphiphiles showing increasing efficiency with decreasing mol percent of DOPE (Fig. 2C). It showed the highest transfection efficiency when it was used as a single component system. Under identical conditions, 3 was almost 3-fold more efficient than Lipofectin.

### 3.2. Effects of serum

We then checked the effects of serum on the gene transfection by the presently described cationic liposomes. We first probed the efficiency of transfection of 1:1 2/DOPE in the presence of serum. The concentrations of Lipofectin and Lipofectamine for transfection were maintained at those rec-

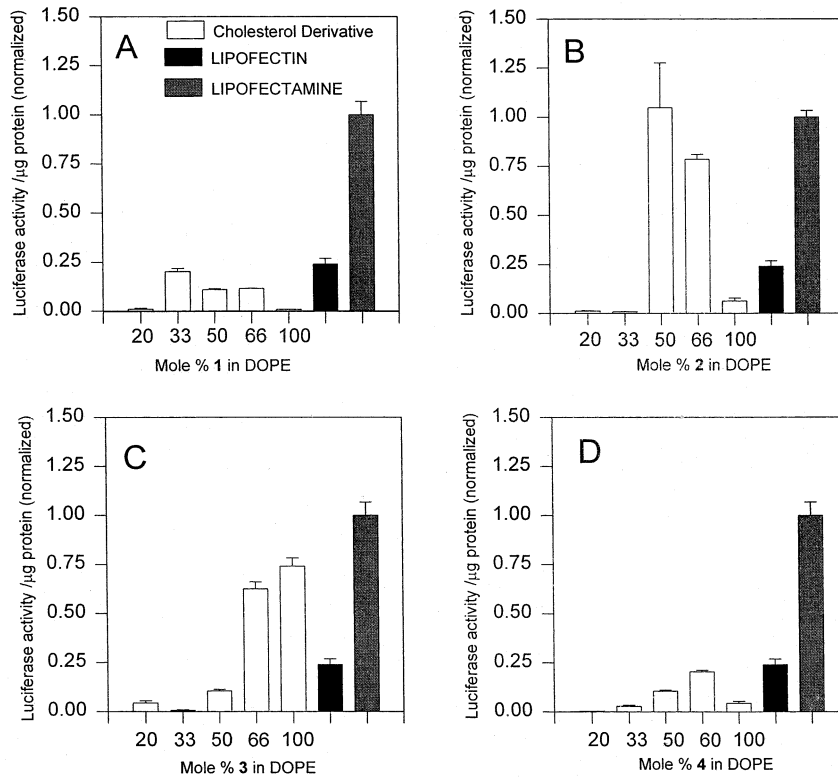


Fig. 2. Transfection efficiencies of pGL3-Control DNA of cholesterol derivatives 1–4 with various compositions in DOPE. 1 (A), 2 (B), 3 (C), 4 (D). Concentration of DNA = 0.3 µg/well and that of the given lipid formulation was 4.0 µg/well. Data are expressed as RLU/µg total cell protein content normalized to Lipofectamine under identical conditions.

ommended by the manufacturers. Transfections were carried out in the presence and absence of 10% FCS (Fig. 3). Notably, the efficiency of Lipofectin was not much affected by the presence or absence of serum. However, Lipofectamine in the presence of serum was inhibited to about 5% of its efficiency without serum. In comparison, the efficiency of 3 in the presence of serum was only halved. Thus, in the presence of serum, 3 is about ~11 times more efficient in conferring gene

transfer than Lipofectamine and Lipofectin under these conditions.

These findings suggest that the compounds 2 and 3 induce gene transfer by a mechanism that is probably different from the one mediated by Lipofectamine. Lipofectamine is composed of a lipid derivative of spermine (DOSPA) and at physiological pH, this remains polycationic. The large concentration of positive charges offered by this lipid on the membrane surface facilitates both DNA binding and fusion with the eukaryotic cell surface leading to an efficient transfection. However, in the presence of serum, the multi-cationic character of Lipofectamine makes it compete with serum against DNA leading to a significant suppression in transfection. On the other hand, 2, which is based on a rigid steroid skeleton, contains only one positive charge per amphiphile molecule and therefore is not affected as drastically by serum.

3.3. Optimization of DNA–lipid ratio for transfection

In order to optimize the conditions of transfection for the most effective formulations of 2 and 3 in COS-7 cells, transfections were carried out with different concentrations of DNA and lipid. Four different concentrations of DNA from 0.05 to 2 µg were taken and for each DNA concentration, the lipid concentration was varied from 0.5 to 10 µg for 2 and 0.5–16 µg for 3 (data not shown). Transfection efficiencies increased with increasing concentrations of DNA until 1 µg/well and decreased thereafter (Fig. 4, inset). The optimum DNA concentration for transfection for both 2 and 3 was 1.0 µg/well of DNA. This bell-shaped curve has previously been reported for other cationic lipid formulations as well and is presumably related to the over-saturation of the cat-

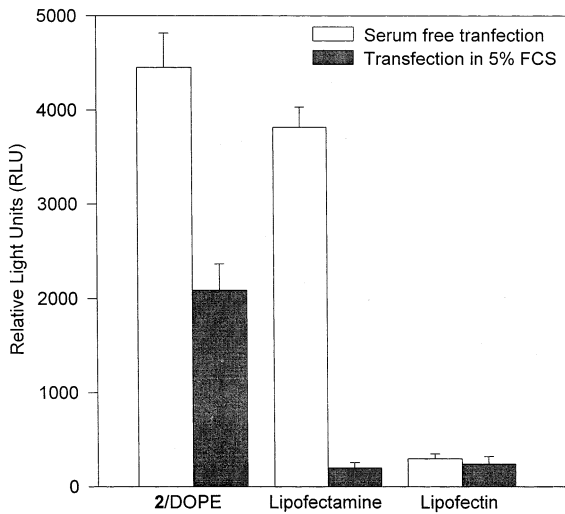


Fig. 3. Transfection efficiencies in the presence and absence of serum for 2/DOPE, Lipofectamine and Lipofectin. Concentration of DNA = 0.3 µg/well, concentration of 2 = Lipofectamine = Lipofectin = 4.0 µg/well. Data expressed as RLU for 2 µl of cell lysate.

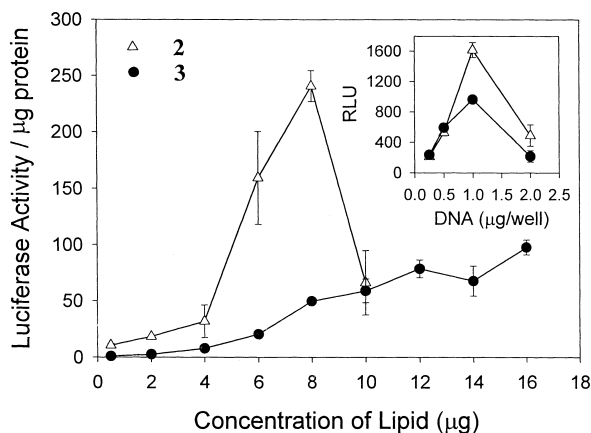


Fig. 4. Transfection efficiency as a function of lipid concentration for **2** and **3** for an optimum DNA concentration of 1.0  $\mu\text{g}/\text{well}$ . Data are expressed as RLU/ $\mu\text{g}$  of total cellular protein. Experiments were performed twice and in duplicate. Inset shows optimal transfection efficiencies in RLU obtained as a function of DNA concentration for **2** and **3**.

ionic charges by the excess DNA [24]. However, the activity profiles of both formulations as a function of lipid concentration showed different characteristics (Fig. 4). **2** exhibited a bell-shaped dependence with lipid concentration. For all DNA concentrations, optimum transfection mediated by **2** was at a DNA–lipid ratio of 1:8. In contrast, **3** showed a trend that saturated at a 1:10 DNA–lipid ratio.

#### 4. Discussion

Of the numerous cationic lipid systems that have been developed, most of the widely used polyamine cationic lipid formulations, though quite effective, are associated with their respective disadvantages in that their syntheses are multi-step [11,25] or are composed of regio-isomers [12] and obtained in relatively low yield [13]. In this paper, we introduce two new DNA transfection reagents with ether linkages between the cationic headgroup and the cholesterol backbone, which can be easily synthesized in high yields using simple one- or two-step procedures. The presence of an ether linkage makes these compounds hydrolytically stable and their aqueous suspensions were found to have long shelf lives. The use of an ether linkage led to dramatic increase in transfection efficiencies of these systems. The new reagent, **2**, is considerably more efficient than the commercially available formulations of Lipofectamine and Lipofectin, especially in the presence of serum. Moreover, **2** and **3** are less toxic to cells. In addition, **3** is able to transfect efficiently without a helper lipid like DOPE which increases its usefulness significantly. Therefore, the present investigation provides useful insights to effective molecular design of new cationic cholesterol-based amphiphiles and should be valuable for achieving gene transfer in other applications.

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