

Incorporation of oxyethylene units between hydrocarbon chain and pseudoglyceryl backbone in cationic lipid potentiates gene transfection efficiency in the presence of serum

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Abstract Four novel cationic lipids with different numbers of oxyethylene units at the linkage region between the pseudoglyceryl backbone and the hydrocarbon chains have been synthesized and used as mixtures with 1,2-dioleoyl-L- α -glycero-3-phosphatidyl ethanolamine (DOPE) for liposome-mediated gene transfection. Incorporation of different numbers of oxyethylene ($-\text{CH}_2\text{CH}_2\text{O}-$) units between long hydrocarbon chain at the C-1 and C-2 positions of the pseudoglyceryl skeleton improved the transfection efficiency considerably compared to the one in which the chains were connected via simple ether links. A pronounced improvement in the gene transfer efficiency was observed with the unsymmetrical cationic lipid **3** in which the long hydrocarbon at the C-1 position of the pseudoglyceryl segment is connected via two ($-\text{CH}_2\text{CH}_2\text{O}-$) units. Notably, the transfection ability of lipid **3** with DOPE in the presence of serum was significantly greater than LIPOFECTAMINE[®]. This suggests that introduction of oxyethylene units between long hydrocarbon chains at the C-1 and C-2 positions of the pseudoglyceryl skeleton provides a novel strategy to achieve efficient gene transfer, especially in conditions where the presence of serum is critical. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Cationic liposome; Cationic lipid; Oxyethylene linkage; DNA transfection

1. Introduction

Ever since its discovery [1], cationic lipid-mediated gene delivery remains at the forefront of present day scientific research owing to its possible applications in the field of gene therapy. Even though the virus-mediated gene transfer is more efficient compared to lipofection, in vivo applications involving viral delivery matrices are limited due to the inherent problems associated with it. As a consequence, cationic lipid formulations have emerged as useful DNA delivery systems due to their ease of large scale production, simplicity in preparation and administration, acceptable levels of toxicity, and low immunogenicity [2]. Combination methods taking advantages of both viral and non-viral methods are also

very promising [3,4]. To improve the efficiency of lipofection, it is important to fine-tune the various steps involved in transfection starting from the complexation of the DNA with a suitable cationic lipid to the final expression of the required gene [5,6].

The linkage between the polar headgroup and hydrophobic segments within a lipid molecule has been shown to influence its aggregation property [7]. The aggregation behavior may also play a role in mediating gene transfer events [8]. For instance, *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTMA), which contains an ether linkage between the trimethyl ammonium head group and the long alkyl chains is shown to have much greater in vivo transfection efficiency than the corresponding cationic lipid with an ester linkage (DOTAP) [9]. Phospholipid derivatives with polyoxyethylene ($-\text{CH}_2\text{CH}_2\text{O}-$)_{*n*} groups in their head group region have been shown to increase the particle stability of small unilamellar phosphatidylcholine liposomes in plasma by reducing its interaction with various macromolecules and cell surfaces [10]. Incorporation of such lipids into cationic liposomes led to enhanced stability of its complex with plasmid DNA [11]. However, nothing is known with regard to the transfection abilities of liposomes derived from cationic lipids that bear one or more oxyethylene ($-\text{CH}_2\text{CH}_2\text{O}-$) units at the chain backbone region. To address this issue with a new cationic lipid family, we have now synthesized a series of novel cationic lipids (**1–4**) in which the linkage between the glycerol backbone and the long hydrocarbon chain has been systematically modified with the insertion of oxyethylene units of various lengths. Since the introduction of such linkers should progressively alter the extent of lipid hydration at the interface region, we thought that it might be important to understand how these changes in lipid hydration and aggregation influence the plasmid DNA delivery efficiency inside the cell. It was observed that lipids bearing oxyethylene groups at the linkage region (lipids **1–4**) were generally more active than the lipid having just one ether linkage (DHTMA) in delivering DNA inside the cell in vitro. The lipid having two oxyethylene units at the C-1 chain linkage region (lipid **3**) was found to be the most efficient cytofectin among the lipids studied when used as a 1:1 formulation with helper lipid 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE). Most importantly, the transfection efficiency of these lipid formulations was particularly impressive even when serum was present during the DNA complexation.

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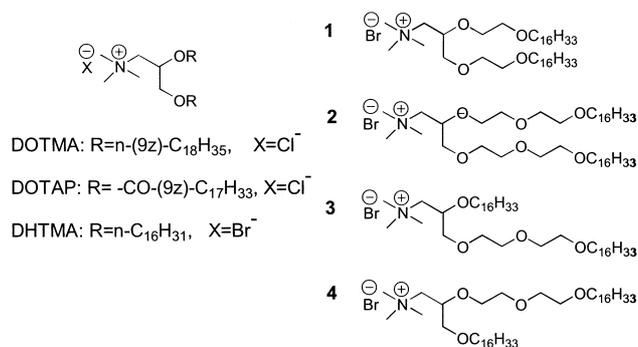


Fig. 1. Chemical structures of various cationic lipids used in the present study.

2. Materials and methods

2.1. Materials

DOPE was purchased from Avanti Polar Lipids. Commercially available transfection reagent LIPOFECTAMINE[®] was obtained from Gibco BRL (USA). The cationic lipids 1–4, shown in Fig. 1, have already been synthesized [12].

2.2. Plasmid DNA

Plasmid pEGFP-N1 (Clontech, USA), which encodes for an enhanced green fluorescence protein (GFP) under a CMV promoter, was amplified in *Escherichia coli* (DH5 α) and purified using Qiagen Maxi Prep Plasmid Purification protocol (Qiagen, Germany). Purity of the plasmid was checked by electrophoresis on 0.8% agarose gel and was found to be more than 80% supercoiled. Concentration of the DNA was estimated spectroscopically by measuring the absorption at 260 nm.

2.3. Liposome preparation

Individual cationic lipid or its mixture with DOPE in the desired mol ratio was dissolved in chloroform in autoclaved Wheaton glass vials. Thin films were made by evaporating the organic solvent under a steady stream of dry nitrogen. Last traces of organic solvent were removed by keeping these films under vacuum overnight. Freshly autoclaved Water (Milli-Q) was added to individual film such that final concentration of the cationic lipid was 1 mM. The mixtures were kept for hydration at 4°C for ~10 h and were repeatedly freeze–thawed (ice-cold water to 50°C) with intermittent vortexing to ensure hydration. Sonication of these suspensions in a bath sonicator above the phase transition temperature of the lipids for 10–15 min afforded closed cationic liposomes as evidenced from transmission electron microscopy (not shown). Liposomes were prepared and kept under strictly sterile conditions. Formulations were stable and if stored frozen, possessed long shelf life.

2.4. Cell culture

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, USA) supplemented with 10% fetal bovine serum (FBS) in T25 culture flasks (Nunc, Denmark) and were incubated at 37°C in a humidified atmosphere containing 5% CO₂. Cells were regularly passaged by trypsinization with 0.1% trypsin (EDTA 0.02%, dextrose 0.05%, and trypsin 0.1%) in PBS (pH 7.2).

2.5. Cytotoxicity

Toxicity of each cationic lipid formulation with or without DOPE toward HeLa cells in the presence of 10% FBS was determined using 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide reduction assay following literature procedures [13,14].

2.6. Transfection procedure

All transfection experiments were carried out in antibiotic-free media. In a typical experiment, 96-well plates were seeded with 9000 cells/well in antibiotic-free DMEM 24 h before transfection such that they were ~70% confluent at the time of transfection. For transfection in the absence of serum, lipid formulation and DNA were serially diluted separately in DMEM containing no serum to have the required working stocks. DNA was used at a concentration of

0.1 μ g/well. The lipid and DNA were complexed in a volume of 20 μ l by incubating them together at room temperature for about 30 min. The lipid concentrations were varied so as to obtain the required lipid/DNA charge ratios of 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0. Charge ratios here represent the ratio of charge on cationic lipid (in mol) to nucleotide base molarity and were calculated by considering the average nucleotide mass of 330. After 30 min of complexation, 50 μ l of medium was added to the complexes (final DNA concentration = 4.33 μ M). Old medium was removed from the wells and lipid–DNA complexes in 70 μ l media were added to the cells. The plates were then incubated for 5 h at 37°C in a humidified atmosphere containing 5% CO₂. At the end of the incubation period, 130 μ l of DMEM containing 15.4% FBS was added per well such that final serum concentration in the medium was 10%. Plates were further incubated for a period of 43 h before checking for the reporter gene expression. GFP expression was estimated by fluorescent microscopy and was quantified by flow cytometry analysis. Control transfections were performed in each case by using commercially available transfection reagent LIPOFECTAMINE[®] with the same concentration of DNA using the standard conditions specified by the manufacturer. All the experiments were done in duplicates and the results presented are the average of at least four such independent experiments.

For transfections in which complexation was performed in the absence of serum but incubation in presence of serum, lipid and DNA were separately diluted in serum-free media as already mentioned and the complexation was done in serum-free media (20 μ l) for 30 min. The complex was then diluted to 70 μ l with DMEM containing 14% FBS so as to achieve a final serum concentration of 10%. The cells were then incubated with this complex for 5 h. At the end of the incubation period, 130 μ l of DMEM containing 10% FBS was added and further incubated for 43 h as described earlier.

For transfections in which the complexation as well as the incubation was done in the presence of serum, the experiments were performed as described earlier using medium containing 10% FBS for all the steps involved in transfection.

2.6.1. Gel electrophoresis. To examine the complexation of DNA with cationic lipid suspensions in the presence or absence of DOPE at different lipid–DNA ratios, we prepared lipid–DNA complexes at different lipid–DNA charge ratios in an identical manner as was done with the transfection experiments. After 30 min of incubation, these complexes were electrophoretically run on a 0.8% agarose gel. The uncomplexed DNA moved out of the well but the DNA that was complexed with lipid remained inside the well [15].

2.7. Flow cytometry

The reporter gene expression was examined by fluorescence microscopy at regular intervals and was quantified 48 h post-transfection by flow cytometry. Percentage of transfected cells was obtained by determining the statistics of cells fluorescing above the control level wherein non-transfected cells were used as the control. Approximately 10000 cells were analyzed to achieve the statistical data, which have been presented as the average of at least four independent measurements. For flow cytometry analysis, ~48 h post-transfection, old medium was removed from the wells; cells were washed with PBS and trypsinized by adding 25 μ l of 0.1% trypsin. To each well 200 μ l of PBS containing 20% FBS were added. Duplicate cultures were pooled and analyzed by flow cytometry immediately using Becton and Dickinson flow cytometer equipped with a fixed laser source at 488 nm.

3. Results

3.1. Transfection efficiency of pEGFP-N1 plasmid DNA

Closed liposomes could be conveniently prepared from each lipid (1–4) and with DOPE by first subjecting the films of the individual lipid or their mixtures to hydration, then repeated freeze–thaw cycles followed by sonication in aqueous buffer. All the cationic lipid (1–4)-based formulations having an oxyethylene group at the linkage were found to be capable of delivering DNA inside the cell. In contrast, the control lipid (DHTMA) possessing a single ether linkage between the pseudoglycerol backbone and each hydrocarbon chain did not

show any transfection capabilities. Transfection abilities of the neat lipid suspensions (1–4) were rather moderate when compared with the commercially available transfecting agent LIPOFECTAMINE® (not shown). Naturally occurring lipids such as DOPE and cholesterol have been shown to increase the efficiency of transfection in lipid-mediated gene transfer applications [1,5,16,17]. It may be noted that the positive control LIPOFECTAMINE® reagent also contains DOPE in its formulation. In order to find out the most effective formulation in the present instance, transfections were also performed with varying amounts of helper lipids as mixed liposomal formulation. While cholesterol had minimal effects in enhancing the transfection efficiency with these lipids, the inclusion of DOPE dramatically increased the efficiency of gene transfer. At 1:1 DOPE/cationic lipid mol ratio, best transfection efficiencies were observed and such mixtures were used in all further experiments. No free DNA was observed on the agarose gel at a charge ratio of ~ 1.2 (cationic lipid/DNA), suggesting a saturation of DNA complexation by cationic lipids. Maximal transfections were usually seen at this or at slightly higher charge ratios in which the complete complexation occurred and the transfection efficiency followed a bell-shaped dependency on lipid concentration as reported earlier with other formulations (Fig. 4) [5].

One of the serious limitations of the cationic lipid-mediated gene delivery, especially involving in vivo trials, is that the transfection is inefficient particularly in the presence of serum proteins. For instance LIPOFECTAMINE®, an excellent commercially available reagent for achieving in vitro transfection is virtually ineffective in the presence of serum. Transfections in the absence of serum even for in vitro experiments are also cumbersome due to increased toxicity of synthetic cationic lipid formulations. To have better potential especially in gene therapy, it is important to have reagents that are capable of delivering and expressing an external gene inside a cell in the presence of serum. To have a clear understanding of the effect of serum in the presently described cationic lipid-mediated gene delivery, transfections were performed under the following three different conditions: (a) complexation (30 min) and incubation (5 h) in the total absence of serum (–fbs –fbs conditions); (b) complexation (30 min) in the absence of serum but incubation (5 h) in the presence of 10% FBS containing DMEM (–fbs +fbs conditions); and (c) both complexation and incubation in the presence of DMEM containing 10% FBS (+fbs +fbs conditions).

3.1.1. Complexation and incubation in the total absence of serum. Comparison of the optimal transfection efficiencies in the absence of serum revealed that lipid 3, which possessed two oxyethylene units at the linkage region of the C-1 chain was the most effective one among the lipids studied (Fig. 2). The number of cells getting transfected using this lipid formulation was comparable to LIPOFECTAMINE® under identical transfection conditions.

3.1.2. Complexation in the absence of serum and incubation in the presence of DMEM containing 10% FBS. When serum was present during the incubation period of 5 h, the transfection efficiency of LIPOFECTAMINE® was reduced to $\sim 20\%$ of its efficiency in the absence of serum. Notably however, no significant loss in transfection efficiency was observed in the case of presently described lipid (1–4)-based formulations under this condition. A substantially higher population of cells was found to be transfected when these synthetic lipid

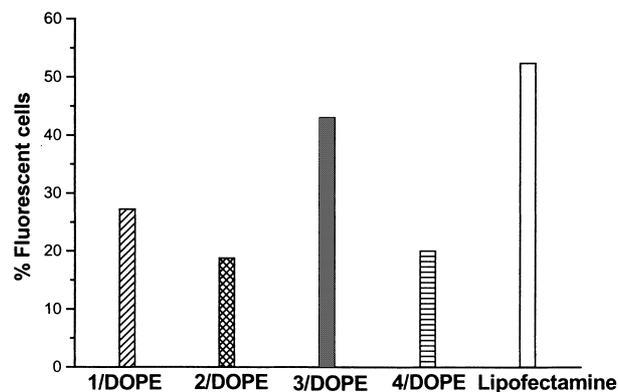


Fig. 2. Optimal transfection efficiencies the lipid (1–4)/DOPE formulations (1:1) for pEGFP-N1 plasmid DNA in the total absence of serum in comparison with LIPOFECTAMINE®.

formulations were used for transfection when compared with LIPOFECTAMINE® (Fig. 3 and 4). The formulation, lipid 3+DOPE (1:1 mol ratio) was found to be the most effective transfecting agent. With this formulation, the number of cells transfected was at least four times more than that of the LIPOFECTAMINE®.

3.1.3. Effect of complexation and incubation in the presence 10% FBS. The transfection ability of LIPOFECTAMINE® was almost completely lost when serum was present in the medium during DNA–lipid complexation. Practically no fluorescent cells due to expressed GFP could be detected. With the present set of cationic lipid formulations, however, no considerable loss in transfection efficiencies was observed in the presence of serum irrespective of whether it was present during complexation step or not (Fig. 5). Independent experiment to probe DNA–lipid complexation using gel electrophoresis suggested that lipid–DNA complexation was not affected significantly with these cationic lipid/DOPE formulations even if serum was present during DNA–lipid complexation.

3.2. Cytotoxicity

With all the cationic lipid-based formulations (Fig. 1), the cell viability was found to be more than 80% in the range of concentration used for transfection experiments. ID_{50} (concentration that caused 50% cell death) values of the liposomal suspensions of lipids 1–4 were ~ 190 , 75, 175 and 195 μM respectively. For DHTMA, the ID_{50} value was higher than the range studied ($> 500 \mu\text{M}$). LIPOFECTAMINE® under the same conditions as used for transfection showed a cell viability of $\sim 75\%$. All the ID_{50} values were much higher than the range of concentration in which a typical transfection experiment was performed (i.e. $\sim 5 \mu\text{M}$). Incorporation of the helper lipid did not alter the cytotoxicity of the lipid formulations significantly.

4. Discussion

In this paper we introduce some new cationic lipid-based DNA transfection reagents. These lipids possess oxyethylene linkages between the pseudoglycerol backbone and long alkyl chains of the lipid. These lipids form liposomes, which remained intact for several months. The lipids bearing non-scissile ether-based links are hydrolytically insensitive. Moreover, the alkyl chains in these lipids are saturated, making them

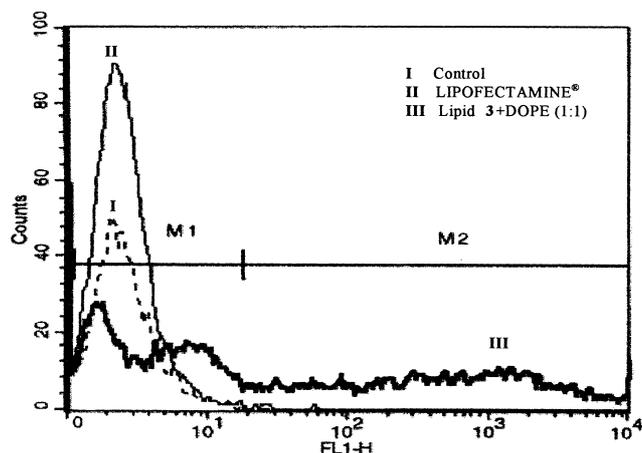


Fig. 3. Flow cytometric scans of the transfection experiments using lipid 3/DOPE (1:1 mol ratio) formulation and LIPOFECTAMINE® when 10% FBS is present during the incubation step. Untransfected cells, used as the control are also shown. M1 and M2 mark the population of cells having fluorescence within the control level and above the control level respectively.

oxidatively and photochemically stable, a feature which often renders most commercially available formulations based on lipids bearing unsaturated hydrocarbon chains unsatisfactory. The introduction of a variable number of oxyethylene units at the linkage region significantly modified the aggregation properties of the resulting lipid suspensions [18]. It was also manifested in their ability to deliver the DNA inside the mammalian cell and its ability to induce efficient expression. When the number of oxyethylene linkages were increased, a substantial increase in toxicity was observed with a concomitant decrease in transfection efficiency. Interestingly, the transfection efficiencies were higher when there was a mismatch in the hydrophobic tail region of the lipid. The most effective lipid was found to be the one in which the oxyethylene group was present only on C-1 chain of the lipid (lipid 3). Due to mismatch in the chain lengths and chain backbone linkage region, this lipid would assemble in a rather 'disorganized' manner in

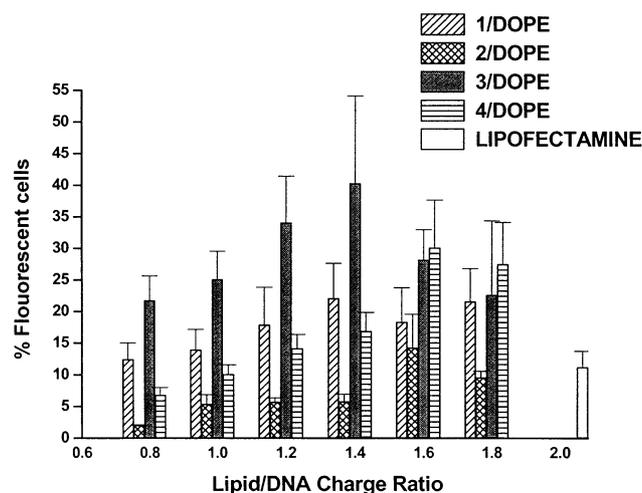


Fig. 4. Transfection efficiencies of the lipid (1–4)/DOPE formulations (1:1) at various lipid/DNA charge ratios with 10% FBS during incubation step. Data expressed as the percentage of fluorescent cells as obtained from flow cytometry analysis.

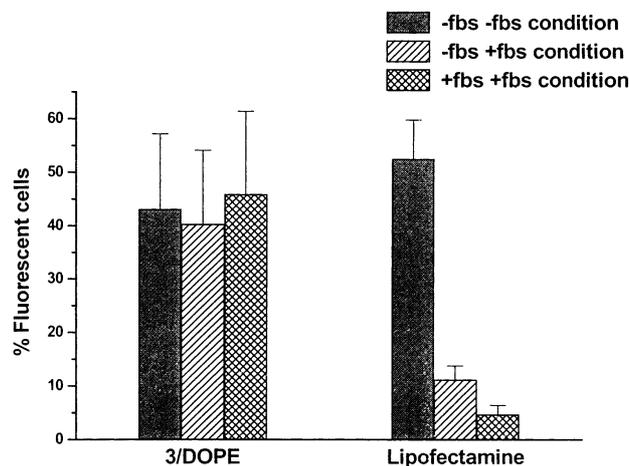


Fig. 5. The effect of serum on the transfection efficiencies of the lipid 3/DOPE (1:1) formulation in comparison with that of LIPOFECTAMINE®. Data expressed as the percentage of fluorescent cells as obtained from flow cytometry analysis.

its membranous aggregate. Recently, it was demonstrated that such local disorders in the cell membrane might aid for the efficient delivery of the DNA inside the cell [19]. The transfection studies further showed that in the case of this class of lipids, the number of cells getting transfected were very high and was often even higher than that of commercially available reagent LIPOFECTAMINE®. These lipids also showed better transfection efficiency in the presence of serum. DOPE as a helper lipid at a mol% of 50 was found to be the best for transfection, while inclusion of cholesterol did not potentiate transfection. Lipid 3 with DOPE at 1:1 mol ratio was found to be the most efficient formulation for DNA delivery among the lipids studied. It was able to transfect comparable number of cells with that of the commercial reagent LIPOFECTAMINE® in the absence of serum. In the presence of serum this lipid formulation was much better than the commercial reagent. Transfection efficiencies of the lipid formulation were not affected even when serum was present at the complexation step. This suggests that the DNA complexes of these lipid formulations are not affected by serum. Work is also underway to examine the generality of the above observations in other cell lines.

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