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Anionic Liposomal Delivery System for DNA Transfection

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ABSTRACT

The present study investigates the use of novel anionic lipoplexes composed of physiological components for plasmid DNA delivery into mammalian cells in vitro. Liposomes were prepared from mixtures of endogenously occurring anionic and zwitterionic lipids, 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt) (DOPG) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), respectively, at a molar ratio of 17:83 (DOPG:DOPE). Anionic lipoplexes were formed by complexation between anionic liposomes and plasmid DNA molecules encoding green fluorescence protein (GFP) using Ca^{2+} ions. Transfection and toxicity were evaluated in CHO-K1 cells using flow cytometry and propidium iodide staining, respectively. Controls included Ca^{2+}-DNA complexes (without lipids), anionic liposomes (no Ca^{2+}), and a cationic liposomal formulation. Efficient delivery of plasmid DNA and subsequent GFP expression was achieved using anionic lipoplexes. Transfection efficiency increased with Ca^{2+} concentration up to 14 mM Ca^{2+}, where transfection efficiency was 7-fold higher than in untreated cells, with minimum toxicity. Further increase in Ca^{2+} decreased transfection. Transfection efficiency of anionic lipoplexes was similar to that of cationic liposomes (lipofectAmine), whereas their toxicity was significantly lower. Ca^{2+}-DNA complexes exhibited minimal and irregular transfection with relatively high cytotoxicity. A model was developed to explain the basis of anionic lipoplex uptake and transfection efficacy. Effective transfection is explained on the formation of nonbilayer hexagonal lipid phases. Efficient and relatively safe DNA transfection using anionic lipoplexes makes them an appealing alternative to be explored for gene delivery.

KEYWORDS: anionic liposomes, gene delivery, transfection, nonviral vector, lipoplex, flow cytometry.

INTRODUCTION

Gene medicine has emerged to be one of the most potent and promising strategies for the development of cures for inherited and acquired disorders with underlying genetic defects or malfunction.[1-3] More than 500 gene therapy protocols have been approved for a wide range of diseases that include cancer, cystic fibrosis, hemophilia, and neuromuscular disorders.[4,5] Successful gene therapy necessitates targeted transfection of plasmids containing transgenes of interest into cells.[6] Owing to the limited ability of naked DNA to enter cells and the susceptibility of DNA to enzymatic degradation, DNA transfection has largely been achieved using viral or nonviral delivery vectors.[7] Such delivery systems can protect the DNA from degradation and have been able to successfully transfer it both in vitro as well as in vivo.[6,8]

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Nonviral transfection systems such as cationic liposomes are generally preferred over viruses because they are nonimmunogenic, are relatively easy to assemble, and are amenable to scale-up for industrial production.[9,10] In addition, liposomal delivery vehicles offer versatility with a diverse range of morphologies, size, and release characteristics; can be used for tissue targeting; and provide protection of the plasmid DNA from attack by degradative nucleases. Since their introduction as potential delivery systems in 1987,[11] DNA-cationic lipid complexes, also known as cationic lipoplexes, have been used in numerous research protocols for DNA delivery in a range of cell types and are currently being investigated in several gene therapy clinical trials.[12]

However, although cationic liposomes are relatively efficient in delivering DNA into cells, they can be inactivated in the presence of serum, and there have been reports of instability upon storage.[13] In addition, cytotoxicity of cationic liposomes remains a concern irrespective of the preferred route of DNA transfer, and such effects have been shown to occur both in vitro[14] and in vivo.[15,16] Under in vitro and ex vivo conditions, massive cell death caused by toxic delivery systems can significantly reduce their overall potential for DNA delivery. Dose-dependent pulmonary toxicity of cationic lipids due to the production of reactive oxygen intermediates has been demonstrated in mice upon intratracheal instillation of a cationic liposome preparation.[15] Cationic lipids were demonstrated to be cytotoxic to phagocytic macrophages.[16] The commercially available cationic lipid-based transfection agent lipofectAmine has been reported to induce toxicity in various tissues such as pulmonary cells,[15] and in arterial cell walls in a porcine restenosis model for localized gene therapy.[17] It is evident that there is a need for efficient and well-tolerated delivery systems to exploit the benefits of gene medicine.

A few studies[18,19] that use anionic liposomal DNA delivery vectors as an alternative to cationic liposomes have been reported in recent years. Anionic liposomes have been previously used as models to simulate the cell surface to study non–receptor-mediated transport of DNA across cells.[20] For DNA delivery purposes, however, anionic liposomal formulations have predominantly been used to transfer oligonucleotides into hippocampal neurons[19] and bacterial cells.[18] However, these investigations are limited owing to (1) inefficient entrapment of DNA molecules within anionic liposomes,[19] and (2) lack of toxicity data.[18] Lack of further development of these systems may be attributed to the inefficient association between anionic lipids and DNA molecules resulting from the repulsive electrostatic interaction between these negatively charged species.[21,22]

In the present study, we have investigated the potential of endogenous anionic lipids for DNA delivery and expression. This study involves the design and evaluation of an anionic liposomal delivery vector consisting of a complex between lipids of physiological origins and plasmid DNA, mediated by divalent Ca^{2+}. It was demonstrated that anionic liposomes could successfully entrap high levels of DNA by complexation with divalent Ca^{2+} ions. These lipoplexes can deliver green fluorescence protein (GFP) plasmid into Chinese hamster ovary-K1 (CHO-K1) cells with low toxicity. In addition to the uptake of a model plasmid, we have also proposed a possible mechanism for anionic lipoplex internalization and efficacy. This model is proposed based on previously reported studies that have characterized the biophysical behavior of similar mixed-composition anionic liposomes and cationic lipid-based transfection.[21,23-32]

Materials and Methods

Materials

Lipids, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt) (DOPG), were purchased from Avanti Polar Lipids, Inc (Alabaster, AL). Calcium chloride, Luria Bertani medium, and all other reagents used for preparing buffers were purchased from Sigma Chemicals (St Louis, MO). Cell culture materials, media (Opti-MEM I Reduced Serum Medium and F12K Nutrient Mixture [Kaighn's modification]), pCMV-GFP plasmid, and lipofectAmine reagent were purchased from Invitrogen Corp (Carlsbad, CA). Hank's balanced salt solution (HBSS), used for cell culture purposes, was purchased from BioWhittaker (Walkersville, MD). CHO-K1 cell line was purchased from American Type Culture Collection (Manassas, VA).

Methods

Liposome Preparation

Liposomes were composed of a DOPG/DOPE mixture at a mol ratio of 17/83. Liposomes were prepared by reconstituting shell-dried lipid films in HEPES buffer, pH 7.4, as described previously.[32] In brief, a stock solution of DOPG dissolved in chloroform was added to glass tubes. Chloroform was allowed to evaporate under a steady stream of nitrogen gas, after which the tubes were kept under vacuum to effect complete evaporation of the residual solvent. DOPG was deposited on the inner wall of the glass tubes in the form of a thin film. The actual amount of DOPG was verified gravimetrically. A stock solution of DOPE in chloroform was then added to the same tubes containing the DOPG film. The tubes were vortexed to dissolve the DOPG film in the DOPE stock solution and thus ensure homogeneous distribution of both the lipids in the tubes. The mixture of lipids was dried as explained above. The molar ratios were adjusted according to the volumes of the stock solutions. Liposomes were prepared by reconstitution of the lipid film in 10 mM HEPES buffer, pH 7.4, at 45°C, facilitated by intermittent vortexing of the suspension. The liposome suspension generated via reconstitution contained multilamellar vesicles (MLV). Small unilamellar vesicles were prepared from the MLV by sonication in an ultrasonic bath (Laboratory Supplies Co, Hicksville, NY) for 15 to 30 minutes, until the suspension achieved clarity.

Preparation of Plasmid DNA

Plasmid containing pCMV-GFP was propagated in transformed *Escherichia coli* cells. *E coli* cells were grown in standard Luria Bertani medium at pH 7.0 to a cell density of ~3 to 4×10^9 cells/mL. Cells were harvested by centrifugation and the plasmid DNA was extracted and purified using a Qiagen Plasmid Maxi Kit (Qiagen, Santa Clarita, CA) as per the manufacturer's recommended protocols. In brief, the plasmid purification procedure involved alkaline hydrolysis of the cells and isolation of the plasmid by binding to an anion-exchange resin of proprietary composition. The concentration of the purified plasmid preparation was 0.87 µg/µL determined using a UV-visible spectrophotometer (SpectraMax Plus, Molecular Devices, Sunnyvale, CA) using OD_{260} (optical density at wavelength 260 nm). The OD_{260}/OD_{280} ratio was 1.964, indicating that the plasmid preparation was sufficiently pure and could be used for transfection purposes. Standard agarose gel electrophoresis on a 0.8% wt/wt agarose gel was conducted to investigate the plasmid structural integrity and revealed 2 major bands. The high-mobility band was attributed to the most compact or supercoiled form of plasmid DNA. The other band with low mobility indicated the overall nonsupercoil content in the plasmid preparation.

Formation of DNA-Anionic Liposome Complexes

For a single transfection experiment, 225 ng of DNA was diluted into a liposome suspension equivalent to 40 μg lipid in a centrifuge tube. Anionic lipoplex formation was achieved by addition of calcium chloride solution. Ca^{2+} ions were introduced into the formulation using stock solution of a 2 M calcium chloride solution that had been previously filter sterilized through a 0.2-μm filter. The amount of plasmid DNA was identical in all experiments irrespective of the Ca^{2+} concentrations (~7 mM to 132 mM) introduced into each complexation tube. The final volume of the lipoplex suspension applied to the cells was 300 μL. The complexes were incubated for 25 minutes at room temperature.

Particle Size Determination Studies

Anionic lipoplexes were prepared as described above and were dispersed in nanopure water. Particle size was determined using dynamic light scattering (Submicron Particle Sizer, Autodilute model 370, Particle Sizing Systems, Santa Barbara, CA). All measurements were conducted at 25°C in triplicate.

Transfection of CHO-K1 Cells

CHO-K1 cell line was cultured in complete growth medium F12K Nutrient Mixture (Kaighn's modification) containing penicillin and streptomycin supplemented with 10% fetal bovine serum, at 37°C in a 5% CO_2 atmosphere incubator. One day before transfection, 2.5×10^4 cells were seeded per well of a 24-well plate in 500 μL of complete growth medium, and incubated at 37°C in a 5% CO_2 environment. Cells were incubated until they were 75% to 80% confluent, which generally took 18 to 24 hours. On the day of transfection, the complete growth medium was removed from the well plates and replaced with 150 μL of transfection medium without serum (Opti-MEM I Reduced Serum Medium). Suspension of anionic lipoplexes, prepared as described above, was overlaid on the rinsed cells. Cells were incubated for 2 hours, after which the complex-containing suspension was removed and replaced by the original complete growth medium. For transfection experiments conducted in the presence of serum, the cells were incubated and transfected in original complete growth medium, omitting rinsing of the cells with HBSS.

For comparison of transfection efficiencies, the following controls were conducted: (1) untreated cells, (2) cells transfected with DNA, (3) cells transfected with DNA and empty anionic liposomes, (4) cells transfected with DNA and Ca^{2+}, and (5) cells transfected with cationic liposomes (lipofectAmine). Ca^{2+}-DNA complexes were prepared identically to those of anionic lipoplexes described above but without the anionic liposomes. The amount of DNA, the Ca^{2+} concentrations, and the volumes of suspension used for the transfection experiments were identical to those used for anionic lipoplexes. LipofectAmine transfections were performed as per the manufacturers' instructions using 1 to 3 μL of lipofectAmine and identical amount of DNA (225 ng).

Flow Cytometry

Cells were assayed for GFP expression 48 hours after exposure to the transfection agents. In brief, cells in the well plates were rinsed with HBSS, treated using 100 μL trypsin EDTA, and incubated for 10 minutes (37°C) in a 5% CO_2 incubator to detach the adherent cells from the well plates. The cell suspension was then diluted to 1 mL using phosphate buffered saline (PBS), pH 7.4, containing 50 μL of the complete growth medium. The cells were then assayed for expression of GFP.

GFP expression was analyzed using a FACS Calibur dual laser flow cytometer (Becton-Dickinson, San Jose, CA). In addition to green fluorescence detection, forward angle light scattering (FSC) and 90° light scattering (side, SSC) measurements were conducted. GFP intensity (FL1) was determined by gating cells at an excitation wavelength of 488 nm using an argon ion laser with a standard bandpass filter. The presence of GFP was detected by emission at a wavelength of 508 nm. For each cell sample, 10 000 events were collected using the high-speed mode (200-300 cells/s). Cells were also visually inspected microscopically for the presence of fluorescence using a Leica DM IL fluorescence microscope (Leica, Wetzlar, Germany). Transfection efficiencies of various formulations were reported as multiples of those of untreated cells.

Toxicity Assay

The toxicity assay for the cells was based on the ability of dead cells to be stained by propidium iodide (PI). PI was used to assess cytotoxicity because of its ability to rapidly penetrate apoptotic cells followed by its binding to cellular DNA producing a characteristic fluorescence that can be detected using flow cytometry.[33] Preliminary experiments were conducted to ensure dead cells were not lost in the washing process during the trypsin-induced detachment within the time frame of toxicity evaluation of the formulation. Cell counting estimated an insignificant amount of dead necrotic cells in the washings. Prior to flow cytometric analysis, 20 μL of PI solution (100 μg/mL) was added to the cells. The excitation and emission wavelengths for PI fluorescence (FL2) were 536 nm and 617 nm, respectively. Toxicities of various formulations were reported as multiples of those of untreated cells.

Statistical Analysis

All transfection experiments were conducted in triplicate, and the results are shown as means ± SD. Statistical significance of results was analyzed using GraphPad Prism Version

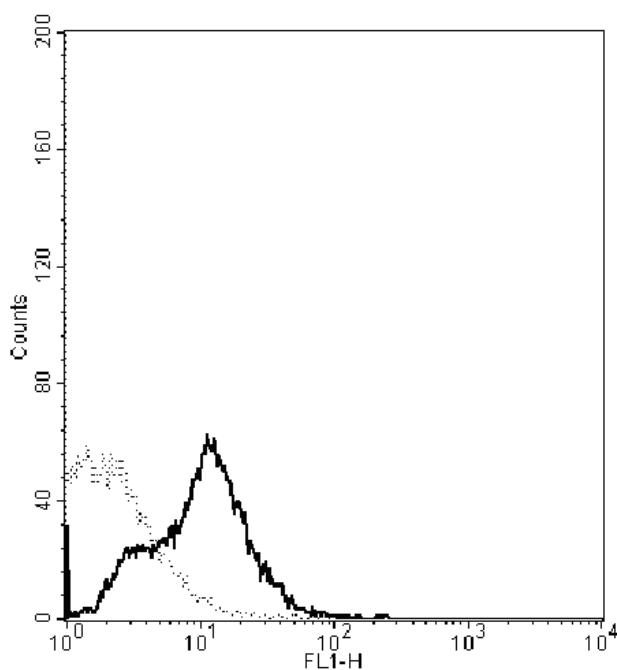

Figure 1. Representative histogram plot of flow cytometry data of CHO-K1 cells suspended in PBS, pH 7.4. The bold line represents flow cytometry pattern for transfected cells, and the dotted line represents a flow cytometry pattern for nontransfected cells. Transfections were conducted at 37°C in a 5% CO_2 environment.

4.0 for Windows (GraphPad Software, San Diego CA). Multiple comparisons were assessed using 1-way analysis of variance (ANOVA) followed by post hoc analysis using Dunnet test. Student *t* test was used to compare differences between 2 groups. Results were considered significant when probability values were < .05.

RESULTS AND DISCUSSION

Design of Anionic Lipoplexes

In this study, we have demonstrated the use of a novel anionic liposomal delivery system for transfection of mammalian CHO-K1 cells. Ca^{2+} serves as a complexing agent between plasmid DNA and the anionic liposomes. The anionic lipids used, DOPE and DOPG, are native to many biological membranes and therefore we anticipated low toxicity associated with this vector. The safety of other phosphoglycerol anionic lipids has been previously demonstrated when administered to epithelial lung tissue.[15] A model plasmid containing a transgene for GFP was used as the reporter plasmid to assay the transfection efficacy of these complexes.

Particle Sizing

The average particle size of uncomplexed anionic liposomes was 114.1 ± 25.3 nm. Anionic lipoplexes formed on the addition of Ca^{2+} ions (7-68 mM Ca^{2+}) had an average particle size of 464.7 ± 45.6 nm. Anionic lipoplexes obtained on the addition of higher levels of Ca^{2+} ions (>100 mM Ca^{2+}) had a larger particle size of 745.4 ± 146.9 nm. Some turbidity was observed in formulations containing high levels of Ca^{2+} ions (>100 mM Ca^{2+}). These larger liposomes were less effective in transfection as shown below.

Representative Flow Cytometry Data and Interpretation

Flow cytometry performed on CHO-K1 cells posttransfection demonstrated control cells and those treated with the delivery systems grew homogeneously with uniform complexity. The dotted line in Figure 1 is a typical histogram representation of flow cytometry data obtained from untreated CHO-K1 cells, whereas the solid line is representative of cells that had significant GFP expression such as those transfected with anionic lipoplexes or lipofectAmine. Untreated CHO-K1 cells possessed some innate fluorescence that is typically observed and attributed to the presence of riboflavin or flavoproteins in the cells. A shift in the fluorescence intensities of the cells was observed following successful transfection and expression of GFP. Fluorescence intensity of the majority of cells transfected using anionic lipoplexes was at least 10 times greater than that of the control cells. Cells with these or higher fluorescence intensities were used in determining the transfection efficiencies of the delivery systems.

Transfection Efficiency of Anionic Lipoplexes

Transfections were performed under identical conditions to determine the transfection efficiencies of anionic lipoplexes, their components, and lipofectAmine, using untreated cells as controls. As expected, DNA by itself and a mixture of DNA and anionic liposomes (without Ca^{2+}) were incapable of transfection (Figure 2). Under identical concentrations of DNA (225 ng), Ca^{2+} (14 mM), and anionic liposomes (40 µg lipids), the transfection efficiencies of Ca^{2+}-complexed DNA was negligible, resulting in fluorescence comparable with that of untreated cells in control experiments (Figure 2). In contrast, cells treated with DNA in anionic lipoplexes demonstrated high transfection efficiency, ~7-fold greater than using Ca^{2+} alone ($P < .01$) (Figure 2). Transfection efficiency of anionic lipoplexes was similar to that of the positive control lipofectAmine, which produced a ~6-fold increase in transfection (Figure 2).

Effect of Ca^{2+} on the Transfection Efficiency of Anionic Lipoplexes

In cell populations with an initial seeding density of 2.5×10^4 cells per well, the delivery system was capable of plasmid DNA delivery into cells with high transfection efficiency (Figure 3). Increase in the concentration of Ca^{2+} improved the

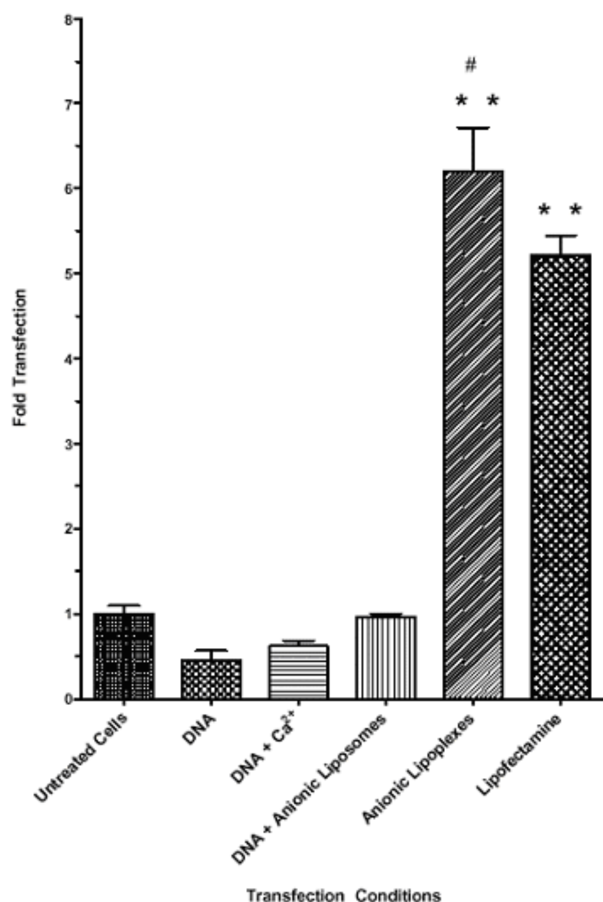

Figure 2. Comparison of transfection efficiencies of naked DNA, Ca^{2+}-DNA complexes, DNA and anionic liposomes, anionic lipoplexes, and lipofectAmine along with a control of untreated cells, in identical cell populations. Transfections were conducted in serum-free medium at 37°C in a 5% CO_2 environment. For each transfection vehicle, identical amounts of DNA (225 ng) were used along with 14 mM Ca^{2+}, and anionic liposomes (equivalent to 40 μg lipid), as specified. Anionic liposomes were composed of a DOPG/DOPE mixture at a mol ratio of 17:83. LipofectAmine (1 μL) was used as per manufacturer's instructions. Values represent the average of triplicates ± SD. Statistical analysis for comparing various delivery systems with control was performed using 1-way ANOVA followed by post hoc analysis using Dunnet test, * * $P < .01$. Statistical analysis for comparing anionic lipoplexes vs Ca^{2+}-DNA complexes was performed using Student *t* test, # $P = .0035$.

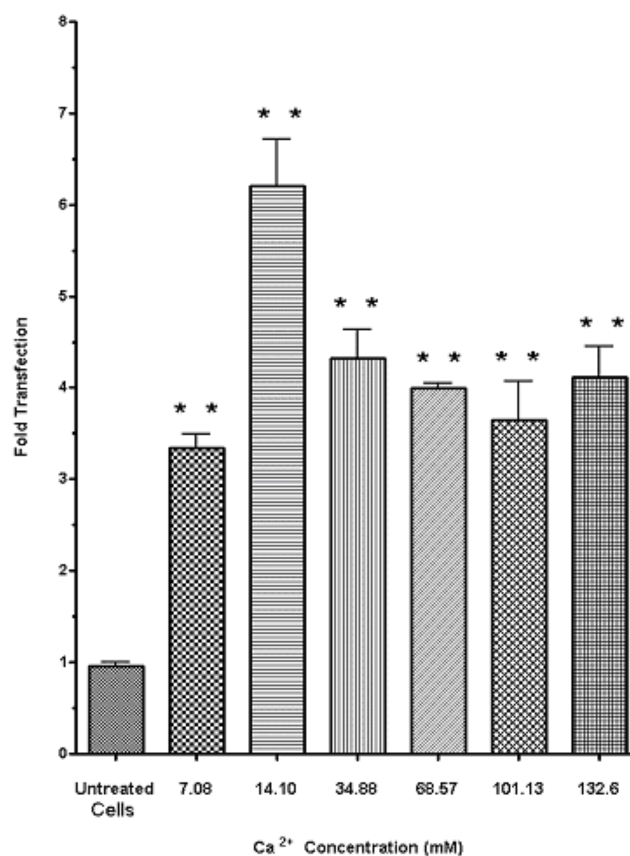

Figure 3. Effect of Ca^{2+} concentration on transfection efficiencies of anionic lipoplexes. Transfections were conducted in serum-free medium at 37°C in a 5% CO_2 environment. Values represent the average of triplicates ± SD. Statistical analysis for comparing various Ca^{2+} concentrations with control was performed using 1-way ANOVA followed by post hoc analysis using Dunnet test, * * $P < .01$.

transfection efficiency of anionic lipoplexes at least 4 times and reached a maximum of ~7-fold using 14 mM Ca^{2+} in the formulation. Any further increase in Ca^{2+} reduced the transfection efficiency but was ~4-fold higher than that of controls. The differences in the transfection efficiencies of anionic lipoplexes prepared with varying amounts of Ca^{2+} can be explained based on the mechanism of lipoplex formation (Figure 3). Since anionic lipoplexes form by interaction of anionic residues on the surface of liposomes with the phosphates in the plasmid DNA backbone, mediated by divalent Ca^{2+} bridges, increase in Ca^{2+} can increase the amount of DNA complexed to the liposomes and is predicted to increase transfection efficiencies, as was observed for Ca^{2+} concentrations up to 14 mM (Figure 3). However, stoichiometric calculations predict that there are a limited number of sites available for interaction between DNA and liposomes, thereby limiting the DNA that can be complexed to the liposome. Although the Ca^{2+} is in excess even at the optimal transfection conditions, this excess Ca^{2+} may interact with the lipoplex externally and/or associate with the DNA. These ions may associate with the lipoplex surface and be responsible for its interaction with the cell surface, thus facilitating cellular uptake.

As we showed in a previous study,[34] very high levels of Ca^{2+} may result in interliposome interactions leading to aggregation and increase in particle size. This increase in size and potential change in the morphology and electronic charge of the lipoplexes may be some of the contributing factors that

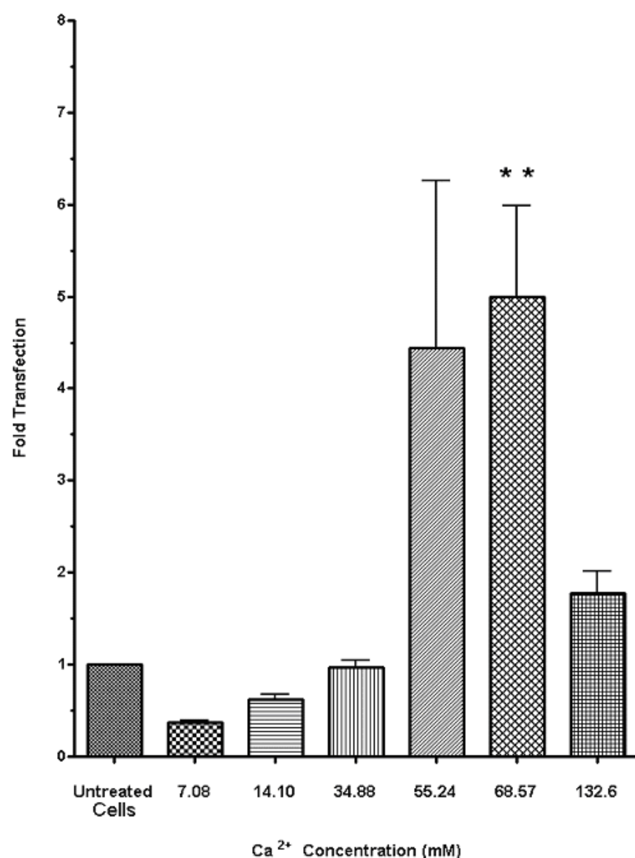

Figure 4. Effect of Ca^{2+} concentration on transfection efficiencies of DNA in the absence of anionic liposomes. Transfections were conducted in serum-free medium at 37°C in a 5% CO_2 environment. Values represent the average of triplicates ± SD. Statistical analysis for comparing various Ca^{2+} concentrations with control was performed using 1-way ANOVA followed by post hoc analysis using Dunnet test, * * $P < .05$.

can explain the reduced transfection efficiency of anionic lipoplexes prepared with high levels of Ca^{2+}, as was observed (Figure 3). This hypothesis was corroborated by measurement of lipoplex particle size at varying Ca^{2+} concentrations. We observed that anionic lipoplexes formed with low levels of Ca^{2+} with average size of 464.7 ± 45.6 nm had high transfection efficiencies, whereas those formed with higher levels of Ca^{2+} with average particle sizes of 745.4 ± 146.9 nm had lower levels of transfection. These studies are in agreement with several recently reported investigations that have shown the ability of a wide range of particles (100 nm-2 μm) to be capable of transfection.[35-37]

As an important control, the transfection efficiency of DNA-Ca^{2+} complexes (without the lipids) was evaluated under identical conditions and concentrations of Ca^{2+} to those used in the anionic lipoplexes since Ca^{2+} alone is capable of transfection (Figure 4). In control experiments without anionic lipids, it was observed that low levels of Ca^{2+} (7.08, 14.10, and 55.24 mM) were incapable of transfection. However, at high levels of Ca^{2+}, CHO-K1 cells were transfected, albeit very inconsistently and unreliably (Figure 4). This transfection effect was optimal at ~55 to 68 mM Ca^{2+}, where transfection levels were ~5-fold. Any further increase in Ca^{2+} concentration resulted in negligible transfection (Figure 4). The Ca^{2+}-mediated transfection of DNA molecules is speculated to be similar to the well-known calcium phosphate method of introducing eukaryotic DNA into mammalian cells. Ca^{2+}-DNA complexes are DNA agglomerates that are formed in a nonuniform, uncontrolled, and unpredictable manner. The lack of formulator control on the production of these complexes yields particles with such inconsistent electrochemical properties as floculate size and charge, which in turn lead to irreproducible and highly variable transfection and toxicity.

Effect of Serum on the Transfection Efficiency of Anionic Lipoplexes

As has been commonly observed with artificial DNA delivery vectors and cationic liposomes, the presence of serum reduced the transfection efficiency of anionic lipoplexes (Figure 5). Maximum transfection efficiency of formulations containing ~14 mM Ca^{2+} was reduced from ~6-fold to ~3-fold on addition of 10% fetal bovine serum to the medium (see Figure 3 for comparison). It was observed that although Ca^{2+}-DNA complexes with high levels of Ca^{2+} were capable of transfection, the process was extremely low in the presence of serum and was associated with toxicity. The role of serum as a modulator of the efficacy of cationic delivery systems is extremely complex and not yet completely understood.[38] As implicated previously for cationic liposomes, the presence of a large fraction of DOPE in the anionic lipoplexes might have contributed to their serum inactivation.[35,37,38] Further in vivo studies, which typically require larger amounts of DNA, have to be conducted to ascertain if serum-based inactivation indeed affects the performance of the delivery system. However, this lipoplex-mediated DNA delivery system can be used for ex vivo conditions where DNA transfer can take place in the absence of serum.

Comparison of Cytotoxicities of Various Delivery Systems

Cells that had experienced transfection either by calcium chloride, anionic lipoplexes, or by cationic lipofectAmine demonstrated higher populations of dead cells, compared with other controls, suggesting that cells that underwent transfection were vulnerable to cell death (Figure 6). Low populations of nonviable cells in control untreated cultures may be due to normal cell death during cell culture maintenance (Figure 6). As expected, anionic liposomes along with plasmid DNA showed no cytotoxicity, which was attributed primarily to their inability to interact with cell membranes

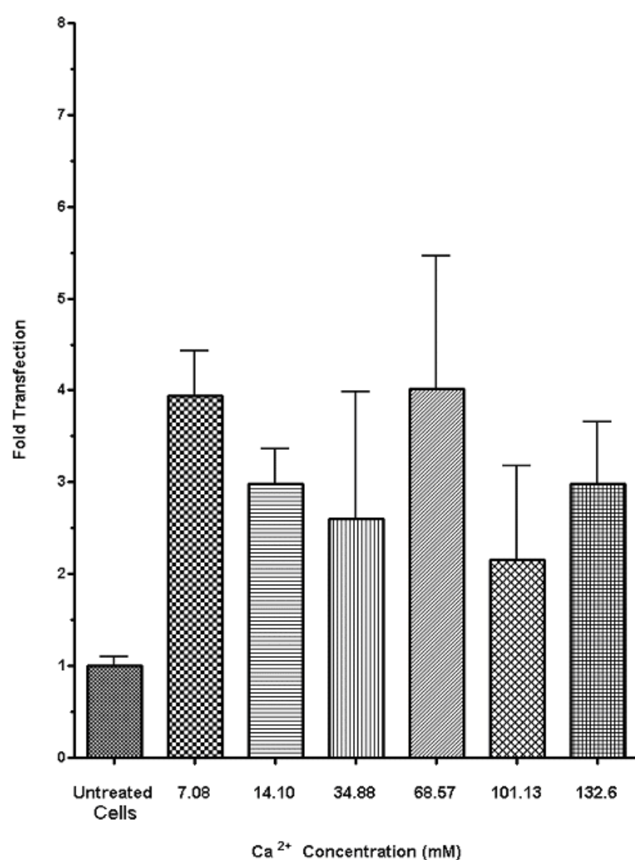

Figure 5. Effect of serum on the transfection efficiencies of anionic lipoplexes. Transfections were conducted at 37°C in a 5% CO_2 environment in the presence of 10% fetal bovine serum in the medium. Values represent the average of triplicates ± SD.

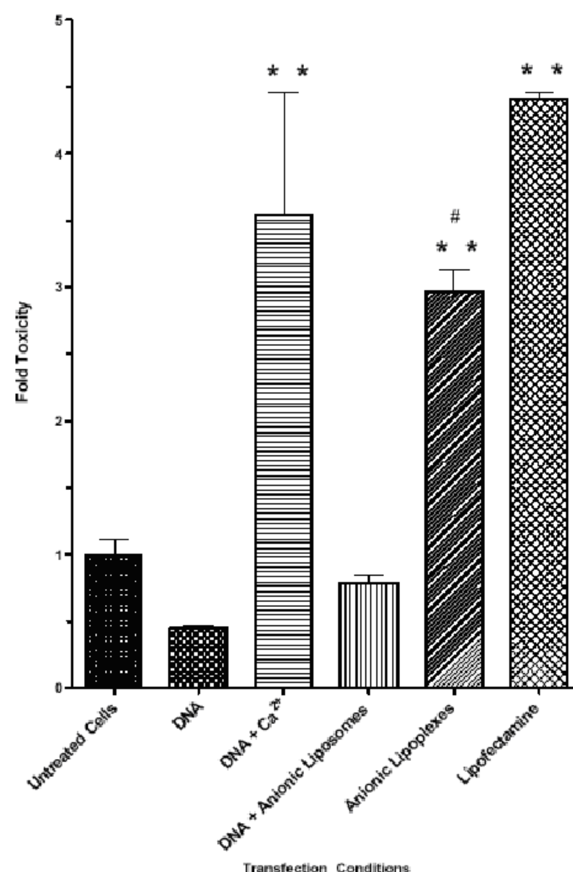

Figure 6. Comparison of cytotoxicities of naked DNA, Ca^{2+}-DNA complexes, DNA and anionic liposomes, anionic lipoplexes, and lipofectAmine along with a control of untreated cells, in identical cell populations. Transfections were conducted with identical amounts of DNA and Ca^{2+} in 2.5×10^4 cells, in serum-free medium at 37°C in a 5% CO_2 environment. Anionic liposomes were composed of a DOPG/DOPE mixture at a mol ratio of 17/83. LipofectAmine (1 μL) was used as per manufacturer's instructions. Values represent the average of triplicates ± SD. Statistical analysis for comparing various delivery systems with control was performed using 1-way ANOVA followed by post hoc analysis using Dunnet test, * * $P < .01$. Statistical analysis for comparing anionic lipoplexes vs lipofectAmine was performed using Student *t* test, # $P = .0073$.

(Figure 6). On the contrary, cationic lipids have been shown to be cytotoxic even when administered on their own.[15]

The toxicity of anionic lipoplexes and Ca^{2+}-DNA complexes directly correlated to the transfection efficiency of the formulations (Figures 3, 4, 7, and 8) and was dependent on the concentration of Ca^{2+} in the formulation. For anionic lipoplexes, there was a peak in toxicity at ~14 mM Ca^{2+} (~3-fold), but toxicity decreased to less than 2-fold at Ca^{2+} concentrations ≥ ~35 mM (Figure 7). Ca^{2+}-DNA complexes produced high toxicity with very high variability. Ca^{2+}-DNA complexes formulated with low levels of Ca^{2+} (≤34.88 mM) exhibited negligible toxicity, whereas higher concentrations of Ca^{2+}, (such as ~55 and 68 mM) demonstrated significant cell toxicity (3- to 4-fold higher than controls), with high variability (Figure 8). Toxic effects of Ca^{2+}-DNA complexes prepared with high levels of Ca^{2+} may be attributed to the presence of Ca^{2+}, based on the high levels of toxicity observed when CHO-K1 cells were independently treated with calcium chloride. Other studies have also demonstrated that Ca^{2+} can cause phospholipid reorganization, which may be responsible for membrane destabilization.[30,31] Although the cationic lipoplex formulation, lipofectAmine, had comparable transfection efficiency with that of anionic lipoplexes, toxicity was 40% higher for lipofectAmine compared with the anionic lipoplexes. It should also be noted that for identical cell populations and DNA content, anionic lipoplexes, even at ~10-fold higher doses, had 40% lower toxicity than cationic liposomes. It is evident that complexes made from anionic lipids are safer and better tolerated compared with lipofectAmine and Ca^{2+}-DNA complexes.

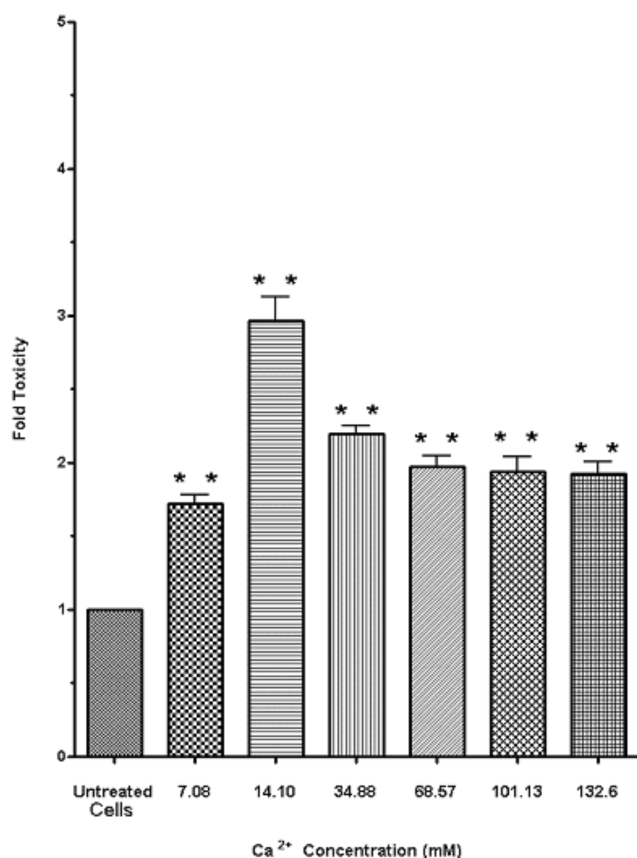

Figure 7. Effect of Ca^{2+} concentration on toxicities of anionic lipoplexes. Transfections were conducted in serum-free medium at 37°C in a 5% CO_2 environment. Values represent the average of triplicates ± SD. Statistical analysis for comparing Ca^{2+} concentrations to control was performed using 1-way ANOVA followed by post hoc analysis using Dunnet test, * * $P < .01$ and * $P < .05$.

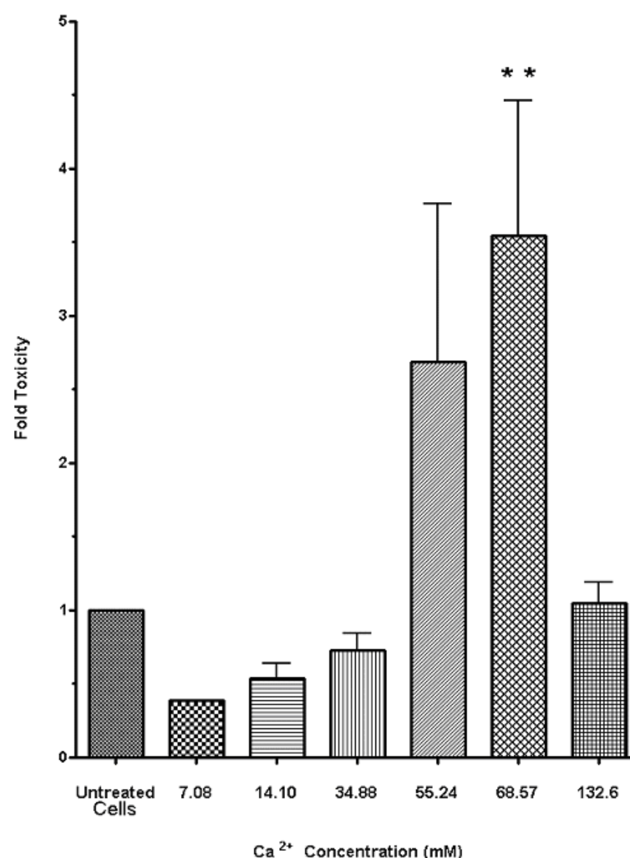

Figure 8. Effect of Ca^{2+} concentration on toxicities of Ca^{2+}-DNA complexes in the absence of anionic liposomes. Transfections were conducted in serum-free medium at 37°C in a 5% CO_2 environment. Values represent the average of triplicates ± SD. Statistical analysis for comparing Ca^{2+} concentrations to control was performed using 1-way ANOVA followed by post hoc analysis using Dunnet test, * * $P < .05$.

Model for Mechanism of Anionic Lipoplex-mediated DNA Transfection

The mechanism of anionic lipoplex-mediated transfection may be speculated based on (1) our previous investigations on the biophysical interaction of anionic liposomes with oligonucleotides as well as plasmid DNA in the presence of divalent cations (S.D.P., D.G.R., D.J.B., unpublished data, 2004),[21,32] (2) the phase behavior of mixed composition liposomes of unsaturated zwitterionic DOPE and anionic DOPG lipids in the presence of Ca^{2+},[26, 29-31] and (3) previously established models of cationic lipid-based transfection.[23-25,27,28]

We have previously demonstrated that interaction of plasmid DNA, Ca^{2+}, and anionic liposomes during the formation of ternary anionic lipoplexes is dramatically different from that of the binary complexes of plasmid DNA and Ca^{2+} alone (S.D.P., D.G.R., D.J.B., unpublished data, 2004). The formation of anionic lipoplexes induced a conformational transition from the native B-DNA conformation of the plasmid into the compact and condensed Z-DNA; Ca^{2+} alone, even at concentrations 1000-fold higher, could not facilitate this transformation. The electrophoretic mobility of anionic lipoplexes was retarded due to higher particle size, whereas Ca^{2+}-DNA complexes had no effect on the mobility of DNA.

Cellular internalization of cationic lipoplexes and mechanism of DNA release from cationic lipoplexes can be explained based on the model put forth by the Szoka and coworkers.[25,27,28] This model suggests that cellular uptake of cationic lipoplexes first results in their accumulation in endosomes followed by intracellular DNA release due to destabilization of the endosomal membrane. Interaction of endosomal membrane anionic lipids with the cationic lipoplexes through formation of ion pairs, lateral diffusion, and lipid mixing are some of the mechanisms speculated to facilitate the DNA and lipoplex release.

X-ray diffraction studies have also indicated that cationic lipoplexes are successful in transfection because of the formation of the H_{II}^c (hexagonal) phase instead of the $L\alpha$ (lamellar) phase due to the overall molecular inverted-cone shape of the lipid and the small, less-hydrated DOPE head group in the lipoplexes.[23,25] In addition, fluorescent cationic DOPE-containing lipoplexes in the H_{II}^c phase have been demonstrated to undergo rapid fusion and release DNA intracellularly.[24]

On the contrary, in the case of mixed composition anionic liposomes with sufficient levels of DOPG, similar to those used in this study, metastable $L\alpha$-phase bilayers have been shown to spontaneously occur instead of the transfection-favoring nonbilayer reversed hexagonal H_{II} configuration.[25] In addition, strong electrostatic repulsion between the liposomes and plasmid DNA normally prevents any association of the 2 components. However, during anionic lipoplex formation, addition of Ca^{2+} not only stimulates binding of DNA to the liposome (S.D.P., D.G.R., D.J.B., unpublished data, 2004), but also has been shown to promote and stabilize the formation of the H_{II} phase, which, as in the case of cationic liposomes, is hypothesized to facilitate cellular uptake.[26]

In the case of anionic lipoplexes, upon endosomal compartmentalization, the formation of lipid ion pairs that promote release of the lipoplex is impossible because lipid populations in both the endosomal membrane lipids and those in the anionic lipoplex have negative charges. Previous studies have demonstrated that Ca^{2+} interaction may cause lipid scrambling,[29] and intercellular Ca^{2+} ions may induce redistribution of the phospholipids in erythrocyte membranes.[30,31] Based on these reports, it is speculated that such mechanisms as lipid scrambling and redistribution caused due Ca^{2+} ions dissociated from the anionic lipoplexes may play a role in endosome destabilization and release of DNA from anionic lipoplexes. Additional biophysical experimentation needs to be conducted to conclusively demonstrate such phenomena.

CONCLUSION

Efficient delivery of plasmid DNA was achieved in mammalian cell cultures using anionic lipoplexes. Anionic liposomes by themselves, as expected, did not transfect the mammalian cells owing to the lack of interaction between similarly charged DNA and lipids. However anionic lipoplexes were capable of DNA delivery. Although Ca^{2+} alone is capable of facilitating DNA transfection, Ca^{2+} interaction with DNA results in precipitation and, consequently, transfection is highly variable. Toxicity of naturally occurring anionic lipoplexes is demonstrated to be lower than the cationic liposome lipofectAmine and Ca^{2+}-DNA complexes. A mechanism of anionic lipoplex transfection is proposed on the basis of the formation of nonbilayer hexagonal phases in the lipoplex that facilitate cellular internalization. Anionic lipoplexes described here might be useful in ex vivo and in vitro applications for DNA transfer particularly in cell lines sensitive to the toxic effects of cationic lipids.

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