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Biophysical characterization of anionic lipoplexes

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Abstract

Transfection efficiency of liposomal gene delivery vectors depends on an optimal balance in the electro-chemical and structural properties of the transfection-capable complexes. We have recently reported a novel anionic lipoplex DNA delivery system composed of a ternary complex of endogenous occurring non-toxic anionic lipids, physiological Ca^{2+} cations, and plasmid DNA encoding a gene of interest with high transfection efficiency and low toxicity. In this work, we investigate the electro-chemical and structural properties anionic lipoplexes and compare them with those of Ca^{2+}–DNA complexes. Biophysical characterization is used to explain the transfection efficiency of anionic lipoplexes in mammalian CHO-K1 cells. Circular dichroism and fluorescence spectroscopy showed that the plasmid DNA underwent conformational transition from native B-DNA to Z-DNA due to compaction and condensation upon Ca^{2+}-mediated complexation with anionic liposomes. Zeta potential measurements and gel electrophoresis studies demonstrated that Ca^{2+} interaction with plasmid DNA during the formation of lipoplexes also led to increased association of supercoiled plasmid DNA with the lipoplexes, leading to charge neutralization which is expected to facilitate transfection. However, even 10-fold higher concentrations of Ca^{2+} alone (in the absence of the anionic liposomes) were unable to induce these changes in plasmid DNA molecules. A model explaining the possible mechanism of anionic lipoplex formation and the correlation of high transfection efficiency to biophysical properties was proposed. These studies confirm the utility of biophysical studies to identify optimal formulation conditions to design efficient liposomal gene delivery vectors.

Keywords: Anionic lipoplex; Transfection; DNA delivery; Anionic liposome; DNA conformation

1. Introduction

Clinical success of gene therapy relies upon efficient introduction of plasmids containing transgenes into targeted cell populations [1,2]. Cellular uptake of naked plasmids is highly inefficient due to their large molecular size and short half-life [3]. Efficient delivery of plasmid DNA into cells has been achieved via viral as well as nonviral vectors [1,4]. Nonviral vectors are preferred since viruses may provoke immunogenic reactions, along with toxic complications due to possible reversion to pathogenic forms and undesirable genomic integration [1,4,5]. Nonviral vectors, such as cationic liposomes and polymers, form complexes with DNA that facilitate cellular uptake and delivery [6,7]. Nonviral vectors can be manufactured with a range of characteristics (i.e., size, charge, morphology, and composition) and can be tailor-made to deliver plasmid DNA with desired release and targeting properties [6,7]. In addition to their lack of immunogenicity, liposomes can improve the biological stability of plasmids by entrapping and shielding them from nuclease degradation [8,9]. However, cytotoxicity [10,11] and inactivation in the presence of serum [12,13] have restricted the potential applications of some cationic liposomal vectors. To reduce lipofection-associated cytotoxicity, we have recently developed an anionic lipoplex system composed of naturally occurring membrane lipids [8]. Anionic lipoplex formation was achieved by the formation of a ternary complex between plasmid DNA and anionic liposomes using divalent Ca^{2+} ion bridges [8].

The mechanism of formation of cationic lipoplexes, their biophysical properties, and ultimately the effect of these on transfection have been extensively investigated in previous studies [14–18]. Such biophysical characterizations have been very useful in optimizing cationic liposomal formula-

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tions and their transfection efficiency on the basis of their charge, lipid composition, and particle size. Although there have been few previous attempts [19,20] to use anionic lipids for DNA transfer, not much is known about anionic liposome entrapment and/or complexation with DNA molecules and the process of formation of anionic lipoplexes or complexes between anionic lipids and DNA using divalent Ca^{2+} cations.

In this work, the electrochemical properties of ternary complexes of anionic lipoplexes (Anionic liposome–Ca^{2+}–DNA) and binary Ca^{2+}–DNA complexes have been investigated and compared using such biophysical techniques as gel electrophoresis (size and charge), circular dichroism and fluorescence spectroscopy (DNA compaction and condensation), and zeta potential measurements (charge). These data are compared with previously reported transfection efficiencies of anionic lipoplexes [8]. Our studies reveal that the structural characteristics of anionic lipoplexes and Ca^{2+}–DNA complexes are very different and the effects of the individual complexation processes on the plasmid DNA secondary conformation are unique. Based on these data, a model is proposed that explains the biophysical basis of anionic lipoplex formation and their high transfection efficiencies compared with Ca^{2+}–DNA complexes.

2. Materials and methods

2.1. Materials

Lipids used in this work, i.e., 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, ethidium bromide, agarose, glycerol, bromophenol blue, xylene cyanol, and Tris-Borate-EDTA (0.5× TBE) buffer powder blend were purchased from Sigma Chemicals (St. Louis, MO). Plasmid containing a green fluorescence protein reporter gene (pCMV-GFP) was purchased from Invitrogen Corp. (Carlsbad, CA). Cell culture materials, media [Opti-MEM I Reduced Serum Medium, Luria Bertani, and F12K Nutrient Mixture (Kaighn's modification)], penicillin and streptomycin solution, trypsin, and fetal bovine serum were purchased from Invitrogen Corp. (Carlsbad, CA). Hank's balanced salt solution (HBSS), a reagent used in cell culture experimentation, was purchased from BioWhittaker (Walkersville, MD). CHO-K1 cell line was purchased from American Type Culture Collection (Manassas, VA).

3. Methods

3.1. Preparation of anionic liposomes

Liposomes were composed of DOPG/DOPE mixtures at mole percents of 17/83. Liposomes were prepared by constituting lipid films in 10 mM HEPES buffer, pH 7.4, as described previously by Patil et al. [8,21]. Briefly, the appropriate amounts of lipids were dissolved in chloroform and placed in glass tubes. Chloroform was allowed to evaporate under a steady stream of nitrogen gas, following which, the tubes were dried under vacuum to facilitate complete evaporation of the residual solvent. Lipids were deposited on the inner wall of the glass tubes in the form of thin films. Liposomes were prepared by reconstitution of the lipid films in HEPES buffer, pH 7.4, facilitated by intermittent heating and vortexing of the suspension. The resultant liposome suspension contained multilamellar vesicles (MLVs). Small unilamellar vesicles were prepared from the MLVs by sonication in a high intensity ultrasonic bath (Laboratory Supplies Company Inc., Hicksville, NY) for 15–30 min, until the suspension achieved clarity.

3.2. Preparation of plasmid DNA

Plasmid containing pCMV-GFP was propagated in transformed *Escherichia coli* cells grown in standard Luria Bertani medium at pH 7.0 to a cell density of approximately $3–4 \times 10^9$ 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. Briefly, 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 spectroscopically determined to be 0.87 μg/μl using a UV-visible spectrophotometer, [SpectraMax Plus (Molecular Devices, Sunnyvale, CA)] using OD_{260}. The OD_{260}/OD_{280} ratio was determined to be 1.96, indicating that the plasmid preparation was sufficiently pure and could be used for transfection purposes. The ratio also validated the preparation and isolation technique.

3.3. Preparation of anionic lipoplexes (Anionic liposome–Ca^{2+}–DNA) and Ca^{2+}–DNA complexes

For a single experiment, 225 ng of DNA was mixed with anionic liposome suspension equivalent to 40 μg of lipid. Anionic lipoplex formation was achieved by addition of calcium chloride solution to obtain Ca^{2+} concentrations that typically ranged from ~7 mM to 132 mM. The anionic lipoplexes were incubated for 25 min at room temperature.

Ca^{2+}–DNA complexes were prepared by using identical amounts of plasmid DNA (225 ng) and Ca^{2+} cations (~7 mM to 132 mM) to those used in the preparation of anionic lipoplexes except without the presence of anionic liposomes.

3.4. Circular Dichroism (CD) studies

CD spectra were collected using a Jasco spectrometer, model 715 CD, (Jasco Inc, Easton, MD) at 20 °C, equipped

with a 10-mm path-length quartz cuvette (Starna Cells Inc, Atascadero, CA) in a thermostated holder. CD spectra were collected from 325 nm to 200 nm in 0.1 nm increments. Three scans were accumulated and averaged to improve the signal-to-noise ratio. After running a blank spectrum with buffer (10 mM HEPES, pH 7.4), 25 μl of plasmid DNA stock aqueous solution (0.89 μg/ml) was added to the cuvette. Ca^{2+} ions were added into the plasmid DNA containing cuvette by removing small aliquots of plasmid solution from the cuvette and replacing with equal volumes of the divalent cation stock. CD data were collected for the ion-containing solution and the process was continued to a final concentration of ~51.5 mM Ca^{2+}. CD data were also obtained for plasmid DNA in liposome suspensions (1 mg/ml lipid) until the concentration of Ca^{2+} reached a level where the signal became inaccurate and unreliable. CD studies necessitated low amounts of lipids to minimize associated light-scattering effects. All experiments were conducted in triplicate.

3.5. Fluorescence studies

Ethidium bromide fluorescence was measured using a Perkin-Elmer LS 50 Luminescence Spectrometer (Perkin-Elmer Corp., Shelton, CT). Briefly, the protocol involved the addition of 0.9 μg of DNA into 2 ml of ethidium bromide solution (400 ng/ml) and subsequent introduction of Ca^{2+} ions into the solution, by adding aliquots of 2 M calcium chloride solution. The fluorescence was monitored by direct as well as indirect (through energy transfer via DNA) excitation of ethidium bromide, at excitation wavelengths of 546 nm and 260 nm, respectively, and monitoring emission at 600 nm for both modes. Data were obtained using a 1-cm path-length cuvette with emission and excitation slit-widths of 10 nm and 15 nm, respectively. For fluorescence measurements conducted in the presence of liposomes, an aliquot of a suspension of SUVs (equivalent to 160 μg of lipids) was added to the ethidium bromide solution in the cuvette, followed by the addition of Ca^{2+}. All experiments were conducted in triplicate.

3.6. 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.

3.7. Agarose gel electrophoresis studies

Electrophoresis studies were conducted on 0.8 w/w agarose gels containing ethidium bromide in 0.5× TBE buffer using standard protocol [22]. Briefly, plasmid DNA, Ca^{2+}–DNA complexes, and anionic lipoplexes, prepared as described above, were mixed with loading buffer (glycerol 30% (v/v), bromophenol blue 0.25% (w/v), and xylene cyanol 0.25% (w/v)) and subjected to agarose gel electrophoresis for 2 h at 120 Volts. Typically, electrophoresis was conducted until the bromophenol blue dye front had migrated 50–75% down the gel, which took approximately 2 h. The electrophoresis gel was visualized and digitally photographed using a Kodak Image Station, model IS 440CF (Kodak, Rochester, NY).

3.8. Zeta potential studies

Zeta potential studies were carried out using a Zeta Plus, Zeta Potential Analyser, (Brookhaven Instruments Corporation, Holtsville, NY). Briefly, lipoplexes were prepared as described above and were introduced in a cuvette containing 1 mM NaCl solution. The surface charge was measured, using a diode laser beam. The final value for the surface charge was calculated to be a mean of 10 counting runs.

3.9. Transfection protocol

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. Cells were incubated until they were 75–80% confluent. Transfections were conducted as explained in detail previously [8]. Briefly, on the day of transfection, while the complexation between anionic lipoplexes or Ca^{2+}–DNA complexes was taking place, the medium containing serum was removed from the well plates, and replaced with transfection medium without serum (Opti-MEM I Reduced Serum Medium). Suspensions containing the anionic lipoplexes or Ca^{2+}–DNA complexes were overlaid on the rinsed cells and incubated for 2 h, after which the complex-containing solution was removed and replaced by the original complete growth medium. All experiments were conducted in triplicate.

3.10. Flow cytometry

Cells were assayed for GFP expression 24–48 h after exposure to the transfection agents as explained in detail previously [8]. Briefly, cultures were rinsed with HBSS and treated with trypsin (0.5% w/v in PBS) to detach the adherent cells from the well plates. After brief centrifugation, the cell pellets were re-suspended in 1 ml phosphate buffered saline, pH 7.4, and assayed for GFP expression.

GFP expression was analyzed using a FACS Calibur dual laser flow cytometer (Becton-Dickinson, San Jose, CA). GFP intensity was determined by gating cells at an excitation wavelength of 488 nm and emission wavelength of 508 nm using an argon ion laser, with a standard bandpass filter. 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 the formulations (% of transfected cells) were calculated using the following formula:

$$\text{Transfection Efficiency} = \frac{\text{Number of events with GFP Fluorescence} \times 100}{\text{Total number of events}}.$$

4. Results

4.1. Circular dichroism studies

4.1.1. Effect of Ca^{2+} on plasmid DNA conformation

CD spectra of the plasmid DNA demonstrated a positive peak at ~275 nm and a small negative peak at ~245 nm (Fig. 1). Addition of Ca^{2+} to the plasmid did not produce any change in the overall shape of the spectrum although the intensity was affected (Fig. 1). The positive and negative bands of Ca^{2+}–DNA complexes were similar to those of the plasmid DNA without Ca^{2+}. As the Ca^{2+} concentration increased, ellipticity at ~275 nm decreased. The maximum decrease at ~275 nm, which was ~50% of the initial ellipticity, occurred at ~25.9 mM of Ca^{2+}. Any further increase in Ca^{2+} concentration did not affect the spectrum.

4.1.2. Effect of lipoplex formation on DNA structure

Sonicated anionic liposomes did not have any significant signal in the wavelength range used in this study and thus did not interfere with the CD signal of the DNA molecules. As expected, CD spectra of plasmid DNA in the presence of anionic liposomes (Fig. 2, trace a) were similar to those of the plasmid DNA alone (Fig. 1, trace a).

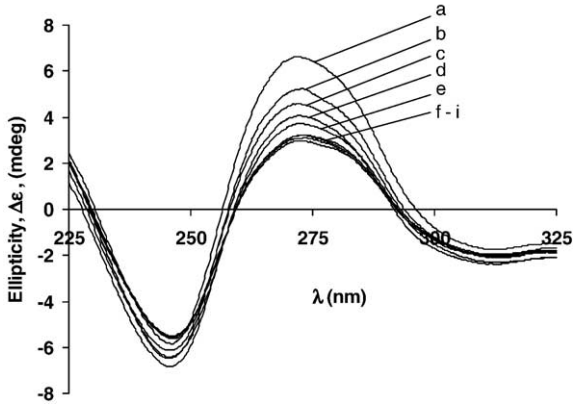

Fig. 1. CD spectra of plasmid DNA in the presence of Ca^{2+} in HEPES buffer, pH 7.4, at 20 °C. The curves represent Ca^{2+} concentrations of 0 (a), 0.2 (b), 0.6 (c), 1.6 (d), 6.6 (e), 25.9 (f), 27.9 (g), 31.8 (h), and 51.5 mM (i).

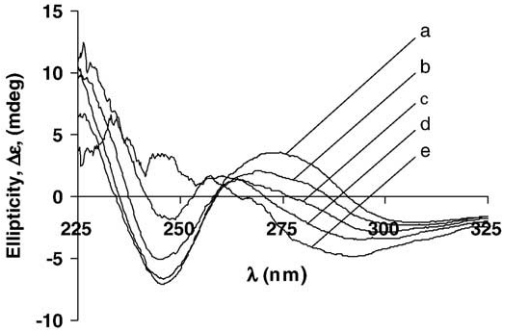

Fig. 2. CD spectra of plasmid DNA with DOPG/DOPE SUVs in the presence of Ca^{2+} in HEPES buffer, pH 7.4, at 20 °C. The curves represent Ca^{2+} concentrations of 0 (a), 0.2 (b), 0.6 (c), 1.6 (d), and 3.6 mM (e). Anionic SUVs were composed of a DOPG/DOPE mixture at a mole ratio of 17/83.

However, as Ca^{2+} was added, resulting in the formation of anionic lipoplexes, spectral changes were observed that were distinct from those observed in the presence of Ca^{2+} alone (Figs. 1 and 2). The spectra were analyzed for simultaneous changes at the following three wavelengths that have been previously used to identify specific changes and transitions in the secondary conformation of DNA molecules: (1) 275 nm for B-DNA conformation changes (Fig. 3); (2) 290 nm for Z-DNA conformation changes (Fig. 4); and (3) 245 nm for C-DNA conformation changes (Fig. 5).

It was observed that anionic lipoplex formation by addition of Ca^{2+} decreased and eventually eliminated the positive CD band at ~275 nm (Figs. 2 and 3). Simultaneously, during anionic lipoplex formation, a negative band began to appear at ~290 nm (Figs. 2 and 4). This negative peak at ~290 nm for DOPE/DOPG lipoplexes began to develop at ~1.6 mM Ca^{2+} and was distinctly observed at ~3.6 mM Ca^{2+}(Figs. 2 and 4). Along with the appearance of the negative peak at ~290 nm, the positive band of the plasmid DNA had a blue shift from ~275 nm to ~260 nm upon Ca^{2+}-mediated interaction with anionic liposomes (Fig. 2).

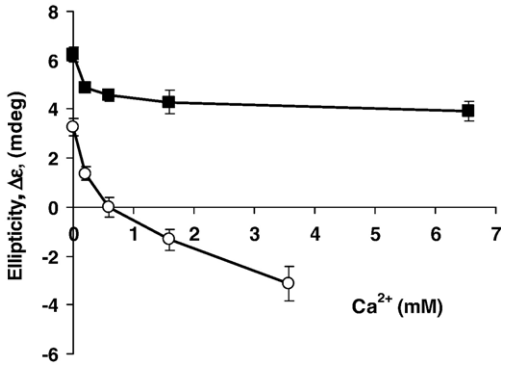

Fig. 3. Dependence of ellipticity ($\Delta\varepsilon$) of plasmid DNA on Ca^{2+} concentration at λ 275 nm in HEPES buffer, pH 7.4, at 20 °C. The curves represent plasmid DNA (■) and plasmid DNA and DOPG/DOPE (mole ratio 17/83) liposomes (○) (n = 3).

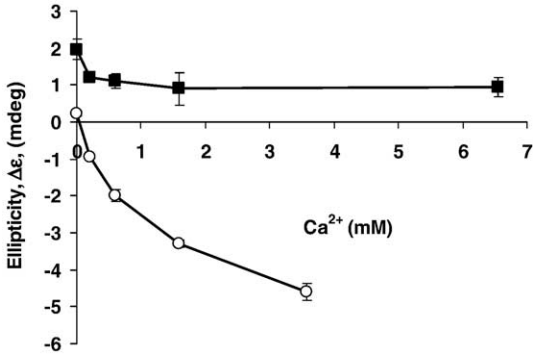

Fig. 4. Dependence of ellipticity ($\Delta\varepsilon$) of plasmid DNA on Ca^{2+} concentration at λ 290 nm, in HEPES buffer, pH 7.4, at 20 °C. The curves represent plasmid DNA (■), and plasmid DNA and DOPG/DOPE (mole ratio 17/83) liposomes (○) (n = 3).

Ca^{2+} alone did not affect the ellipticity at ~245 nm (Figs. 1 and 5). However, during the formation of anionic lipoplexes ellipticity at ~245 nm diminished as Ca^{2+} concentration increased and was indistinguishable among the noise in the data at concentrations over ~1.6 mM Ca^{2+}(Figs. 2 and 5). The final values of the ellipticities at 245 nm during lipoplex formation were positive as opposed to the negative values obtained for the plasmid DNA conformation either the in native form or in the presence of equivalent Ca^{2+} concentration.

4.2. Fluorescence studies

4.2.1. Effect of Ca^{2+} on ethidium bromide fluorescence

Ethidium bromide fluorescence intensity in the absence of Ca^{2+} ions was higher for the indirect (through energy transfer via DNA at 260 nm) mode of excitation compared with direct excitation (546 nm) (Figs. 6 and 7). Fluorescence intensities at both wavelengths were sensitive to the addition of Ca^{2+} (Figs. 6 and 7). The intensity of the ethidium bromide fluorescence decreased with increase in Ca^{2+} concentration in the plasmid solution. Addition of as little as ~1 mM Ca^{2+} to the plasmid solution produced significant reductions of 50% and 40% in the ethidium bromide fluorescence intensities in both the direct as well

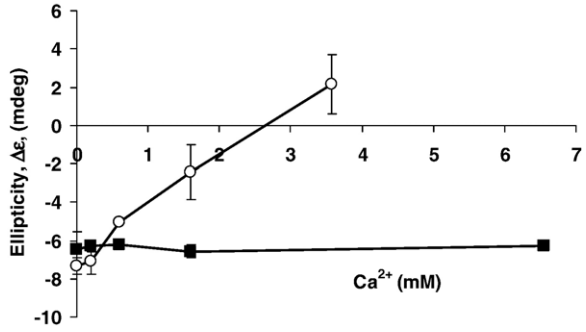

Fig. 5. Dependence of ellipticity ($\Delta\varepsilon$) of plasmid DNA on Ca^{2+} concentration at λ 245 nm in HEPES buffer, pH 7.4, at 20 °C. The curves represent plasmid DNA (■) and plasmid DNA and DOPG/DOPE (mole ratio 17/83) liposomes (○) (n = 3).

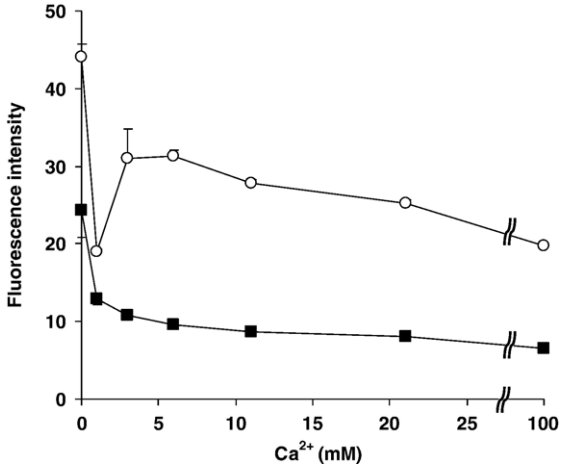

Fig. 6. Effect of Ca^{2+} on the fluorescence intensity of ethidium bromide in HEPES buffer, pH 7.4, at 20 °C. Data were obtained by direct excitation of ethidium bromide at λ 546 nm and using emission at λ 600 nm. The curves represent plasmid DNA (■), and plasmid DNA and DOPG/DOPE (mole ratio 17/83) liposomes (○) (n = 3).

as the indirect modes of excitation, respectively (Figs. 6 and 7). Further addition of Ca^{2+} to the plasmid solution produced a less marked effect on ethidium bromide fluorescence compared with the ~1 mM concentration and the fluorescence intensities reached continued to remain low (Figs. 6 and 7).

4.2.2. Effect of lipoplex formation on ethidium bromide fluorescence

During anionic lipoplex formation, there was a significant and characteristic effect on the fluorescence intensity of ethidium bromide that was dependant on the concentration of Ca^{2+} ions (Figs. 6 and 7). It was observed that addition of low concentrations of Ca^{2+} (up to ~1 mM) decreased the fluorescence intensity, but higher Ca^{2+} ion concentrations

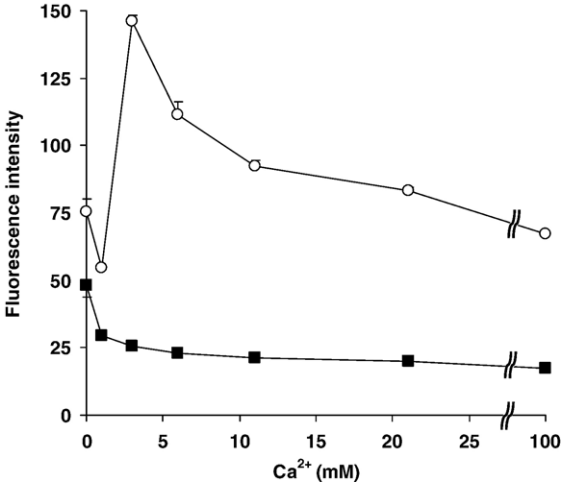

Fig. 7. Effect of Ca^{2+} on the fluorescence intensity of ethidium bromide in HEPES buffer, pH 7.4, at 20 °C. Data were obtained by indirect (through energy transfer via DNA) excitation of ethidium bromide at λ 260 nm and using emission at λ 600 nm. The curves represent plasmid DNA (■) and plasmid DNA and DOPG/DOPE (mole ratio 17/83) liposomes (○) (n = 3).

led to an increase in the fluorescence intensity (Figs. 6 and 7). The restoration of fluorescence intensities was different in the indirect (through energy transfer via DNA) and the direct mode of ethidium bromide excitation (Figs. 6 and 7). For equivalent concentration of Ca^{2+}, the fluorescence intensity of direct excitation (546 nm) never exceeded the original, Ca^{2+}-free, intensity (Fig. 6), whereas for indirect (through energy transfer via DNA) excitation (260 nm), the fluorescence intensity doubled than that of the original (Fig. 7). Any further addition of Ca^{2+} to the anionic lipoplex solution led to steady reduction in ethidium bromide fluorescence intensities in both the direct as well as the indirect (through energy transfer via DNA) modes of excitation.

4.2.3. Particle sizing

The average particle size of uncomplexed anionic liposomes was 114 ± 25 nm. Addition of plasmid DNA to liposome suspension, in the absence of Ca^{2+} ions did not produce any change in the particle size. Anionic lipoplexes formed on the addition of low levels of Ca^{2+} ions (7–68 mM Ca^{2+}) had an average particle size of 465 ± 46 nm, whereas those obtained on the addition of higher levels of Ca^{2+} ions (>100 mM Ca^{2+}) had a larger average particle size of 745 ± 147 nm. In formulations containing high levels of Ca^{2+} ions (>100 mM Ca^{2+}), some turbidity was observed.

4.2.4. Gel electrophoresis

Gel electrophoresis of plasmid DNA, Ca^{2+}–DNA complexes, and anionic lipoplexes revealed the effect of complexation on DNA structure and were useful in distinguishing the interactions of Ca^{2+} with plasmid DNA in the presence or absence of anionic liposomes. Standard agarose gel electrophoresis, conducted to investigate the plasmid structural integrity, revealed two major bands for the untreated plasmid DNA (Fig. 8A, lane b). The high mobility band was attributed to the most compact or supercoiled form of plasmid DNA. The other band with low mobility was so small that it was considered to be the overall non-supercoil content in the plasmid preparation.

Addition of Ca^{2+} to the plasmid DNA solution, in the absence of anionic liposomes, did not affect the mobility of the plasmid DNA (Fig. 8A, lanes c–h). High Ca^{2+} levels produced some band smearing and retained very minute amount of DNA in the wells (Fig. 8A, lanes f–h). On the contrary, the formation of anionic lipoplexes (Fig. 8B, lanes d–j) led to a complete loss of the DNA bands since most of the complexed DNA remained in the wells. Presence of DNA in the wells containing samples of anionic lipoplexes also confirmed the presence of DNA external to the liposomes. Due to the negative charges of plasmid DNA and anionic liposomes and subsequent lack of interaction (confirmed by the CD, fluorescence spectra, and particle size measurements), plasmid DNA mobility was expected to remain unaffected in the presence of only anionic liposomes without Ca^{2+} cations. However, plasmid DNA was retained

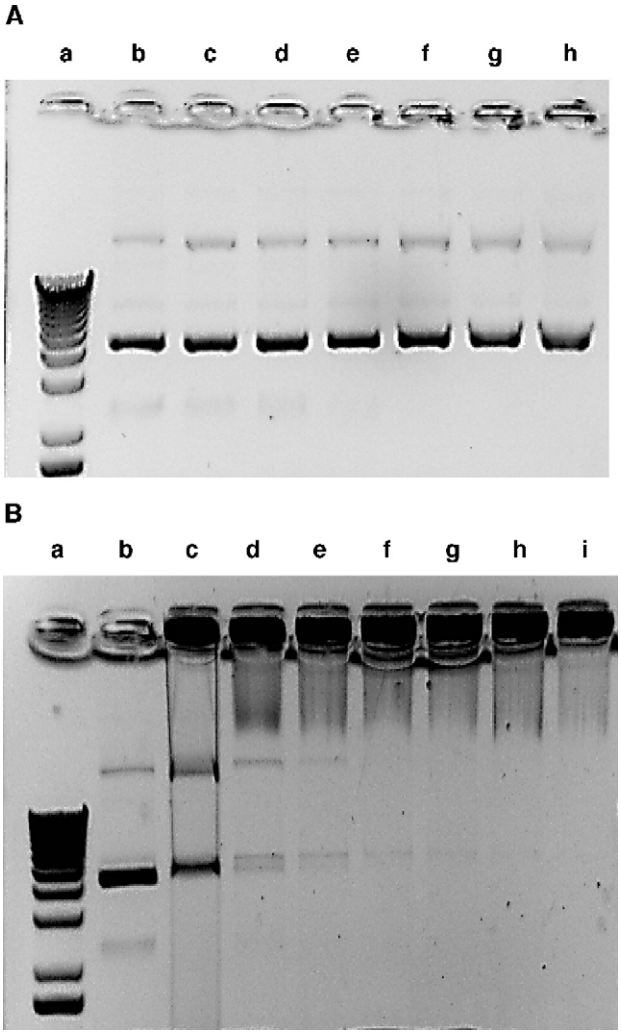

Fig. 8. Effect of Ca^{2+} on the electrophoretic mobility of Ca^{2+}–DNA complexes (A) and anionic lipoplexes (Anionic liposome–Ca^{2+}–DNA) DOPG/DOPE (mole ratio 17/83) (B). For (A), the lanes represent control DNA ladder (a); plasmid DNA (b); and Ca^{2+}–DNA complexes at Ca^{2+} concentrations of 7.1 (c), 14.1 (d), 34.9 (e), 64.6 (f), 101.1 (g), and 132.6 mM (h). For (B), the lanes represent control DNA ladder (a); plasmid DNA (b); plasmid DNA and empty anionic liposomes (c); anionic lipoplexes at Ca^{2+} concentrations of 7.1 (d), 14.1 (e), 34.9 (f), 64.6 (g), 101.1 (h), and 132.6 (i). Contrast is adjusted for to show the diminishing supercoiled plasmid band at ~4 kb.

in the wells even in the absence of Ca^{2+} cations (Fig. 8B, lane c). This retention of plasmid DNA in the absence of Ca^{2+} can be explained by the presence of other cations (Na^+) due to the use of Na_2EDTA in the preparation of electrophoresis buffer (0.5× TBE). Although weak compared with that of divalent Ca^{2+}, electrostatic interaction due to the monovalent Na^+ could facilitate some complexation of plasmid DNA molecules with anionic liposomes and thus contribute to hinder their electrophoretic mobility.

4.2.5. Zeta potential studies

As expected, anionic liposomes possessed negative zeta potential values (Fig. 9). Addition of DNA to the liposomes, in the absence of Ca^{2+}, did not alter the charge, which was

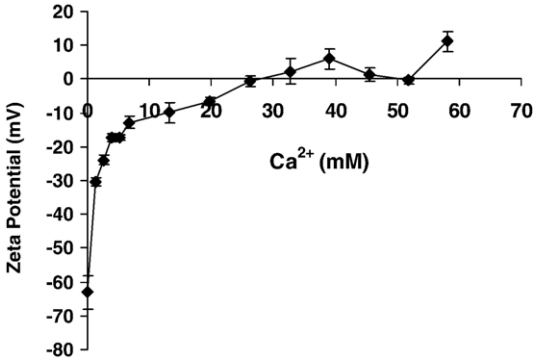

Fig. 9. Effect of Ca^{2+} on the zeta potential of anionic lipoplexes in 1 mM NaCl solution. The curve represents plasmid DNA and DOPG/DOPE (mole ratio 17/83) liposomes (♦) (n = 10).

also confirmed using gel electrophoresis. The formation of lipoplexes, via the addition of Ca^{2+} to the liposome–DNA suspension, reduced the negative surface charge of the lipoplexes, and ultimately the lipoplexes became positive at high levels of Ca^{2+}(≥60 mM Ca^{2+} concentration) (Fig. 9).

4.3. Transfection studies

As seen in Fig. 10, Ca^{2+}–DNA complexes produced negligible transfection at low levels of Ca^{2+}, whereas high levels produced highly variable transfection as reported in detail previously [8]. Control conditions of only anionic liposomes and plasmid DNA (no Ca^{2+}) and naked plasmid DNA resulted in negligible transfection. However, the ternary complexes of anionic lipoplexes (plasmid DNA, Ca^{2+}, and anionic liposomes) produced high and reproducible transfection efficiencies that were of the same order as that of cationic liposomal formulation (Lipofectamine™, Invitrogen Corporation, Carlsbad, CA) reported previously [8]. It was observed that at lower levels of Ca^{2+}(< 68.6 mM Ca^{2+}) DNA transfection efficiency using anionic lipoplexes was several folds higher than that of naked DNA as well as that of Ca^{2+}–DNA complexes. The transfection efficiency of the anionic lipoplexes increased with increase in Ca^{2+} concentration up to ~14 mM Ca^{2+} and then reduced as the Ca^{2+} concentration increased. At higher levels of Ca^{2+}(≥68.6 mM Ca^{2+} concentration), the lipoplexes as well as Ca^{2+}–DNA complexes had similar transfection efficiencies, however, transfection by Ca^{2+}–DNA complexes was inconsistent and highly variable, a feature that is highly undesirable for therapeutic gene delivery.

5. Discussion

Systematic biophysical evaluation of the complexation process revealed that Ca^{2+}-mediated interaction of plasmid DNA with anionic lipids during the formation of the ternary anionic lipoplexes (liposome–Ca^{2+}–DNA) was significantly different from that which occurred during the formation of the binary Ca^{2+}–DNA complexes. This difference in the complexation behavior of DNA during the generation of anionic lipoplexes is considered to be one of the factors responsible, at least in part, for their high transfection efficiency compared with Ca^{2+} alone. It is speculated that the optimum Ca^{2+} concentration for maximum transfection results from optimized biophysical properties such as charge and maximum condensation and compaction of the DNA molecule in the lipoplex. The overall influence of Ca^{2+} concentration on the biophysical properties and transfection efficiencies is summarized in Table 1.

5.1. Conformational transitions in DNA upon anionic lipoplex formation

Circular dichroism studies identified conformational transitions that took place during anionic lipoplex formation. Specific wavelengths that are signature characteristics of distinct DNA conformations were monitored to identify conformational transitions. Plasmid DNA had a characteristic native right-handed B-DNA conformation identified by a positive band at ~275 nm (Fig. 1) in its CD spectrum. Though formation of Ca^{2+}–DNA complexes produced minute loss of B-DNA conformation (as evidenced by a reduction in the positive CD band at 275 nm), the lack of any change in the overall shape of the signal suggested that even higher levels of Ca^{2+} did not produce conformational transitions. Addition of anionic liposomes to plasmid DNA in the absence of divalent Ca^{2+} ions, as expected, did not affect its CD spectrum, thereby confirming the lack of interaction between DNA and anionic liposomes. Particle size measurements, fluorescence spectra, and agarose gel electrophoresis studies also confirmed the inability of negatively charged DNA molecules to interact with anionic liposomes.

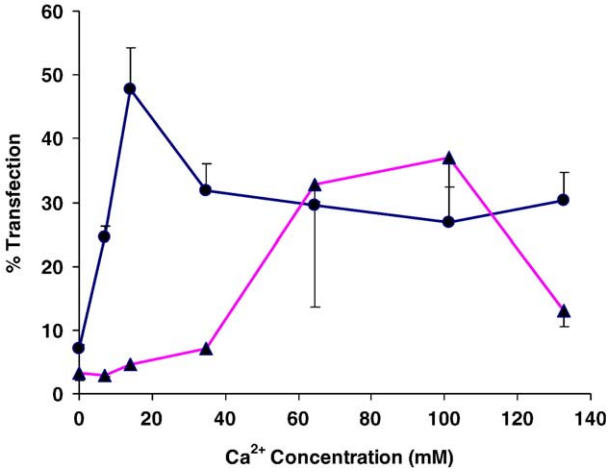

Fig. 10. Effect of Ca^{2+} on the transfection efficiencies of anionic lipoplexes (Anionic liposome–Ca^{2+}–DNA) DOPG/DOPE (mole ratio 17/83) (▲) and Ca^{2+}–DNA complexes (●) (n = 3).

Table 1
Summary of biophysical properties of Ca^{2+}–DNA complexes and anionic lipoplexes

Analytical technique	Ca^{2+}–DNA complexes	Anionic lipoplexes
Circular dichroism	No conformational change	Conformational transition from B-DNA to Z-DNA
Fluorescence spectroscopy using ethidium bromide	Linear decrease in accessibility of ethidium bromide to the DNA molecules	Conformational change followed by linear decrease in accessibility of ethidium bromide to the DNA molecules
Gel electrophoresis	No change in the fraction of supercoiled DNA	Reduction in the fraction of supercoiled DNA and high loading of DNA on anionic liposomes
Zeta potential	Not applicable	Reduction in the anionic charge of the complexes
Transfection efficiency	Low at low Ca^{2+} concentrations Irreproducible and moderate at high Ca^{2+} concentrations	High at low Ca^{2+} concentrations Moderate and reproducible at high Ca^{2+} concentrations

On the other hand, dramatic spectral changes were observed during anionic lipoplex formation that were distinct from changes observed in the presence of Ca^{2+} alone (Figs. 1 and 2). A decrease in and eventual elimination of the positive band at 275 nm during lipoplex formation suggested loss of B-DNA conformation. The simultaneous appearance of other bands in the spectral signal also suggested conformational transitions from the original native B-DNA. The appearance of a strong negative band at ~290 nm and a positive band at ~260 nm, both characteristic of left-handed Z-DNA demonstrated that anionic lipoplex formation facilitated the conformational transition of native B-DNA into the highly condensed and left-handed Z-DNA. The formation of Z-DNA, previously implicated in the condensation and supercoiling of chromosomal genomic DNA [23], may also contribute to the high transfection efficiency of anionic lipoplexes, whereas lack of conformational changes and DNA compaction could be responsible, at least in part, for the relatively low and unpredictable transfection of Ca^{2+}–DNA complexes.

In addition, the very large negative band at ~245 nm, characteristic of condensed DNA structures such as C-DNA and Ψ-DNA (typically observed during DNA condensation with cationic liposomes [21,24]), did not appear during the formation of either of anionic lipoplexes (Figs. 2, 3, and 5). Instead, during the formation of DOPE/DOPG lipoplexes, the band at ~245 nm diminished and become positive as the Ca^{2+} concentration increased (Figs. 2, 3, and 5). These observations suggest that the polymorphic transitions in the secondary structure of DNA during the formation of anionic lipoplexes are significantly distinct from those induced during cationic lipoplex formation.

We have previously demonstrated that Ca^{2+}-mediated interaction of single stranded oligonucleotides with anionic liposomes led to unique conformational changes that were dependent on the oligonucleotide sequence [25]. However, as observed in this work, double-stranded plasmid DNA underwent a B- to Z-DNA transition upon Ca^{2+}-mediated interaction with anionic liposomes. These results suggest that conformational transitions upon divalent cation-mediated complexation with anionic lipids might be dependent not only on the sequence, but also on the size of the DNA molecule. Furthermore, since Z-DNA formation is commonly observed in poly[d(G-C)]$_2$, sequences in the presence of charged ions and these results suggest that Ca^{2+}-mediated interaction between anionic liposomes and plasmid DNA may also selectively involve the guanine and cytosine residues [26–28].

The identified conformational transitions are remarkable biophysical findings with respect to the following two aspects: (1) Ca^{2+} concentrations as low as 3.6 mM induced B to Z conformational transition in the DNA molecules, by their complexation to anionic liposomes, only during the formation of anionic lipoplexes, whereas identical amounts of Ca^{2+} or even approximately a 20-fold increase in Ca^{2+} were incapable of independently producing these conformational transitions in the absence of these liposomes; (2) changes in DNA condensation and conformational transitions upon lipoplex formation appear to be dependent on the charge of the lipids involved in the lipoplex. For example, cationic lipids appear to induce a structural transition from native B-form to C-form DNA in cationic lipoplexes [15,17,21,24,29]. In some cases of extreme dehydration, C-form DNA has been shown to further collapse into an even more condensed and compact chiral Ψ-DNA phase or have properties similar to it [24]. On the other hand, anionic lipids appear to promote native B-form to Z-form structural transition in anionic lipoplexes.

5.2. Ethidium bromide fluorescence

Ethidium bromide fluorescence has been used to investigate the nature and extent of binding of the liposomes to the DNA-therapeutic of interest [30–36]. Ethidium bromide exhibits weak intrinsic fluorescence in aqueous solution compared with that of the dye intercalated DNA bases [30]. Transfer of protons from the excited state of the molecule to its aqueous environment is significantly inefficient and delayed when ethidium bromide is intercalated with hydrophobic DNA bases relative to when the dye is free in water; thus, leading to a considerably enhanced fluorescence intensity of the intercalated dye. Both direct [31–34] as well as indirect (through energy transfer via DNA) [35,36] modes of generating ethidium bromide fluorescence have been successfully used in several studies to investigate the

efficacy of complexation of liposomes with DNA, although very few studies [30] use both simultaneously.

The ability of ethidium bromide to intercalate with the DNA molecules is directly proportional to the availability of DNA bases in the molecule [30]. As observed during formation of Ca^{2+}–DNA complexes, quenching of ethidium bromide fluorescence in the presence of Ca^{2+} suggested Ca^{2+}–DNA complexation, condensation of the DNA helix, as well as simultaneous displacement of ethidium bromide from the intercalation sites in the DNA molecules due to the high binding affinity of Ca^{2+} with DNA. Additionally, DNA condensation is expected to lower the binding affinity of ethidium bromide for DNA molecules.

Changes in ethidium bromide fluorescence intensities during anionic lipoplex formation were different from those observed during formation of Ca^{2+}–DNA complexes thus revealing the significant difference in Ca^{2+} ion-mediated interaction of DNA with liposomes compared with that of just Ca^{2+}–DNA complexes. Ca^{2+}-induced displacement of ethidium bromide from the DNA backbone, similar to that observed during the formation of Ca^{2+}–DNA complexes, can explain the instantaneous and characteristic initial decrease in the fluorescence intensity of ethidium bromide upon addition of Ca^{2+} to anionic liposomes during lipoplex formation. Since Ca^{2+} in the presence of anionic liposomes as well as DNA molecules is capable of interaction with both species either exclusively (lipids or DNA) or simultaneously (lipids and DNA), the extent of decrease in the ethidium bromide fluorescence intensity is much stronger during formation of Ca^{2+}–DNA complexes compared with that observed during anionic lipoplexes. The characteristic restoration of the fluorescence intensities following the initial reduction suggests change in the conformation of the DNA molecule that permitted greater accessibility of ethidium bromide for intercalation with the DNA molecule. It is also possible that the fluorescence quantum yield of ethidium bromide increases in the new DNA conformation. This distinctive pattern of change in the fluorescence intensities may also indicate Ca^{2+}-mediated spontaneous rearrangement of DNA molecules around anionic lipids during anionic lipoplex formation. At higher concentrations of Ca^{2+}, plasmid DNA underwent rearrangement upon Ca^{2+}-mediated complexation with anionic liposomes, yielding a DNA conformation that resulted in decreased intercalation of ethidium bromide. Following rearrangement, any excess of Ca^{2+} ions were capable of condensing and compacting the liposome-complexed DNA evident by a steady decrease in ethidium bromide fluorescence intensity. It should be noted though that DNA upon rearrangement in anionic lipoplexes was not as condensed and compact as that observed during the formation of Ca^{2+}–DNA complexes.

Use of ethidium bromide fluorescence as a probe to investigate DNA structure upon binding to DNA molecules also brings to light the role of Ca^{2+} as a mediator that facilitates complexation between DNA molecules and at the same time contributes to DNA condensation and compaction. The use of both direct as well as indirect (through energy transfer via DNA) modes of excitation of ethidium bromide to investigate the effect of DNA complexation to anionic liposomes presents a comprehensive analysis of this phenomenon since the parallel use of both techniques can not only detect changes in DNA conformation independently of each other, but can also be used to complement and confirm results indicating DNA condensation and compaction. Additionally, as shown by others before [30], the indirect mode of excitation is a more sensitive probe to clarify minute changes in DNA conformation compared with that of the direct mode of excitation. Although the mechanism of conformational change cannot be deduced from ethidium bromide fluorescence alone, these results confirm DNA conformational changes observed using CD measurements. For example, during anionic lipoplex formations, Ca^{2+} concentrations of ~1.6 mM led to the conformational transition from B-DNA to Z-DNA as was revealed by CD, whereas fluorescence spectroscopy demonstrated a characteristic increase in fluorescence at these concentrations.

5.3. Zeta potential, electrophoretic mobility, and particle size measurements

Zeta potential measurements showed reduction in the negative overall surface charge of anionic lipoplexes thereby confirming the role of Ca^{2+} as a mediator between the anionic DNA and the liposomes. This reversal and reduction of the overall negative surface charge of the anionic lipoplexes should promote interaction of the lipoplexes with cell membranes, thereby facilitating cellular uptake. Complexation also led to a significant increase in the particle size of the lipoplexes. Gel electrophoresis studies showed that charge reduction (which was inferred by measuring zeta potential), observed during anionic lipoplex formation, was accompanied by a decrease in the uncomplexed supercoiled DNA fraction in these formulations. However, the fraction of supercoiled DNA in formulations containing Ca^{2+}–DNA complexes remained unaffected. Although the running buffer (0.5× TBE) used in the electrophoresis experiments contains EDTA, it is not expected to disrupt anionic lipoplexes or Ca^{2+}–DNA complexes by chelating Ca^{2+} ions because Ca^{2+} levels in the formulations are significantly higher (7 to 132 mM) than that of EDTA (2 mM). Furthermore, these studies are consistent with changes in DNA accessibility to ethidium bromide intercalation when complexed to anionic lipoplexes as determined using fluorescence spectroscopy.

5.4. Elucidation of transfection efficiency of anionic lipoplexes and Ca^{2+}–DNA complexes

The mechanism of anionic lipoplex formation and its enhanced transfection efficiency compared with Ca^{2+}–DNA complexes composed of identical Ca^{2+} concentrations can

be speculated on the evidence generated by these biophysical studies. Transfection efficiency of anionic lipoplexes is dependent on such characteristic biophysical and electrochemical properties as particle size, compaction, and condensation of the plasmid DNA in the complex, and electrostatic charge. An intricate balance of these properties appears to contribute to the distinctive transfection patterns of anionic lipoplexes and Ca^{2+}–DNA complexes. Transfection studies demonstrated that although Ca^{2+}–DNA complexes were capable of inducing some transfection, anionic lipoplexes were clearly efficient and reliable. The reproducible and high transfection efficiencies of these anionic lipoplexes make them very suitable for therapeutic gene delivery.

An increase in Ca^{2+} concentration in the anionic lipoplex formulation led to DNA charge neutralization (zeta potential studies) with simultaneous DNA compaction and condensation (circular dichroism and fluorescence spectroscopy) along with greater association of the supercoiled plasmid DNA with the anionic liposome surface (gel electrophoresis). Thus, greater complexation of supercoiled plasmid DNA along with a stronger association with the liposomes using divalent Ca^{2+} bridges resulted in an improvement in the transfection efficiencies of anionic lipoplexes. Conformational transition of the plasmid DNA molecules at specific Ca^{2+} concentration may also involve redistribution of the lipids and plasmid DNA that may contribute to the improved transfection efficiency.

Transfection efficiencies of anionic lipoplexes increased until an optimum Ca^{2+} concentration was present in the formulation, beyond which a reduction was observed. Formulation of anionic lipoplexes at high Ca^{2+} levels may increase the amount of DNA associated with the lipoplex and reduce their negative charge (zeta potential), both properties may improve cellular association and should lead to improved transfection. However, anionic lipoplexes with high levels of Ca^{2+} were also associated with an increased particle size, which may prevent them from being internalized by the cells, which in turn may explain an overall reduction in the transfection efficiency. High Ca^{2+} levels, as demonstrated previously, can destabilize DNA conformations [37], perturb liposomal bilayers [38–40], and disrupt the lipoplex structures [38–40], thereby leading to reduction in the transfection efficiencies.

In contrast to anionic lipoplexes, low levels of Ca^{2+} ions could not affect the charge of the DNA molecule or compact and condense the plasmid DNA leading to poor transfection efficiency of Ca^{2+}–DNA complexes. However, at high levels of Ca^{2+}, very little charge neutralization was obtained along with some retention of plasmid DNA in the loading wells (gel electrophoresis), that could explain some highly variable transfection of Ca^{2+}–DNA complexes at these levels. The fact that even high levels of Ca^{2+} alone did not lead to the disappearance of the DNA bands or compaction and condensation of the plasmid DNA molecules suggested that DNA complexation with Ca^{2+} in the presence of anionic lipids was much stronger than that of Ca^{2+} alone. High transfection obtained using these anionic lipoplexes also indirectly demonstrates their efficacy in protecting the plasmid DNA from degradation compared with Ca^{2+}–DNA complexes.

6. Conclusions

The combination of bio-physical characterization techniques used in this study was able to identify DNA structural changes upon Ca^{2+}-mediated complexation with anionic liposomes and can explain the transfection efficiency of anionic lipoplexes. The structural changes induced during anionic lipoplex formation were different from those typically produced by either cationic liposomes or Ca^{2+} alone, thereby suggesting that DNA cellular uptake, intracellular trafficking, and transfection of anionic lipoplexes may involve different pathways compared with other DNA delivery systems. Complexation of plasmid DNA with anionic lipid carriers resulted in DNA conformational transitions from the native B-form into the condensed Z-form and also reduced the liposomal negative surface charge density. Transfection efficiency of anionic lipoplexes can be affected by the secondary conformation of plasmid DNA in the lipoplex. Such biophysical studies were useful in identifying formulation conditions and electrochemical properties for optimal transfection. The biophysical studies reported here would also be useful in identifying potential formulations and processing conditions that may affect the structural integrity of plasmid DNA. Although the relative importance of any of these properties to independently facilitate transfection over others is impossible to assess, a systematic biophysical evaluation has helped to identify and optimize the ideal set of desirable biophysical attributes that may affect DNA transfection.

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