

# The role of lipid charge density in the serum stability of cationic lipid/DNA complexes

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## Abstract

To evaluate the role of lipid charge density in the serum stability of DOTAP-Chol/DNA complexes (lipoplexes), lipid–DNA interactions, extent of aggregation, supercoil content, and in vitro transfection efficiency of lipoplexes were investigated. In general, higher serum concentration destabilized, and increasing molar charge ratio of DOTAP to negatively charged phosphates in the DNA (DOTAP<sup>+</sup>/DNA<sup>−</sup>) stabilized lipoplexes in serum as assessed by the criteria used in this study. The increase of cholesterol content led to increased serum stability, and DOTAP:Chol (mol/mol 1:4)/DNA lipoplex with DOTAP<sup>+</sup>/DNA<sup>−</sup> ratio 4 was the most serum stable formulation of all the formulations examined, and maintained lipid–DNA interactions, did not aggregate and exhibited high in vitro transfection efficiency in 50% (v/v) serum. The increased stability of this formulation could not be explained by the decreased charge density of the lipid component. Furthermore, no single parameter examined in the study could be used to consistently predict the in vitro transfection efficiency of lipoplexes in serum. Surprisingly, no correlation between the maintenance of supercoiled DNA content and in vitro transfection efficiency was found in the study.

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**Keywords:** Cationic lipid; Cholesterol; Serum stability; Charge density; Gene delivery

## 1. Introduction

Cationic liposomes have become widely used as nonviral gene transfer reagents for both in vivo and in vitro applications [1–3]. However, one drawback of liposome-mediated gene delivery is that the presence of serum greatly lowers transfection efficiency [4–10]. Thus, there is an intense effort to develop cationic lipid/DNA complexes (lipoplexes) that efficiently transfer genes in the presence of serum. The effort to improve the activity of cationic liposomes includes the synthesis of new cationic lipids [11–14], development of

new formulations of existing cationic lipids [9,15,16], and the addition of a third component such as a polycation or cationic peptide [17–20]. To date, most investigations of serum stability have been conducted in low serum concentrations (e.g., 10–30%) which do not accurately reflect the in vivo situation [9,12,13,15,19,20]. Among the barriers to transfection, serum has been reported to exert its inhibitory effect by binding serum proteins to cationic lipid/DNA complexes, which leads to structural reorganization, aggregation and/or dissociation of the complexes [8,10,21,22]. Aggregation results in rapid clearance and increased toxicity of lipoplexes, and reduced binding of lipoplexes to cells [6,9,23,24]. Dissociation can result in DNA degradation by serum nucleases, and decreased amounts of lipoplexes associated with the target cells. To overcome these barriers, it would be advantageous to design cationic lipid/DNA formulations that remain associated as a complex, retain their original particle size, protect DNA from nucleases, and efficiently transfect cells in the presence of physiological serum levels.

Previous studies have found that in vivo transfection efficiency is increased when cholesterol is included in the

*Abbreviations:* FRET, fluorescence resonance energy transfer; DOTAP, *N*-(1-(2,3-dioleoyloxy)propyl)-*N,N,N*-trimethylammonium chloride; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycerophosphocholine; NBD-PE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl); Chol, cholesterol; DOTAP<sup>+</sup>/DNA<sup>−</sup>, molar charge ratio of DOTAP to negatively charged phosphates in the DNA

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formulation as a helper lipid [18,21,22,25–27]. However, the mechanism responsible for this effect has yet to be fully elucidated. In an attempt to explain the greater stability upon incorporation of cholesterol, Crook et al. [22] suggested that serum stability may be determined by the charge density of the lipid component. In this context, we interpret “charge density” to refer to the number of positive charges distributed throughout the volume of the lipid component, i.e., the lipid charge density. To test the role of lipid charge density in serum stability, we maintained constant quantities of the cationic lipid DOTAP but varied the amounts of cholesterol within the formulation. Serum effects on physicochemical and biological characteristics of lipoplexes composed of 1, 2-dioleoyl-3-trimethylammonium propane (DOTAP):cholesterol (mol/mol 2:1, 1:1, 1:2, and 1:4) were assessed. To further investigate the effect of lipid charge density, the stability of different lipid formulations with equivalent charge densities [e.g. by incorporating POPC (1-palmitoyl-2-oleoyl-*sn*-glycerophosphocholine) or DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine)] was monitored in serum over 24 h. In monitoring serum “stability”, fluorescence resonance energy transfer (FRET) was used to assess the association between lipid and DNA in serum [28], and supercoiled DNA content was evaluated by agarose gel electrophoresis. Vector aggregation could not be reliably assessed by dynamic light scattering due to the presence of serum, so turbidity measurements were employed to monitor this aspect of lipoplex stability. These physicochemical properties were compared with transfection of mouse endothelial cells to more fully assess effects of serum on rates of gene delivery.

## 2. Materials and methods

### 2.1. Materials

Plasmid DNA (5 kb, >90% supercoiled) encoding green fluorescent protein under control of the cytomegalovirus promoter (pGreen lantern-1; Gibco-BRL) was propagated in *E. coli* and purified by Aldevron Custom Plasmid Purification (Fargo, ND). It was used for experiments investigating physicochemical characteristics. Luciferase plasmid DNA (5.9 kb) was a generous gift from Valantis Inc. (Burlingame, CA). *N*-(1-(2,3-Dioleoyloxy)propyl)-*N,N,N*-trimethylammonium chloride (DOTAP), 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE), cholesterol, 1-palmitoyl-2-oleoyl-*sn*-glycerophosphocholine (POPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-PE) were purchased from Avanti Polar Lipids (Alabaster, AL). Cy3 Label IT kits used for covalent labeling of plasmid DNA were acquired from Mirus (Madison, WI). The luciferase assay kit was obtained from Promega (Madison, WI). Mouse serum was purchased from PanBio (Columbia, MD). It was filtered with 0.2- $\mu$ m low protein binding cellulose

acetate filter from Fisher Scientific (Pittsburgh, PA) before use. All chemicals were of reagent grade or higher quality.

### 2.2. Preparation of labeled DNA

DNA labeling with Cy3 was carried out as previously described [28]. Briefly, DNA and labeling reagent were mixed in HEPES buffer (25 mM HEPES, 1 mM EDTA, pH 7.5) and incubated for 1 h at 37 °C. Unreacted labeling reagent was removed by NaCl/ethanol precipitation. The labeled DNA was redissolved in 2.5 mM HEPES buffer and quantitated by UV absorbance at 260 nm. The fluorescence of Cy3-DNA was measured and compared to a standard curve to determine the extent of Cy3-labeling on DNA. Utilizing this procedure, a Cy3 label concentration of 2.6% (w/w) of plasmid DNA was obtained, and used in our experiments.

### 2.3. Preparation of liposomes

DOTAP, combined with cholesterol, DOPE, or POPC in different mole ratios, was mixed in chloroform. The lipid mixture was dried under a stream of nitrogen gas and placed under vacuum (100 mTorr) for 2 h to remove residual chloroform, and dried lipids were subsequently resuspended in autoclaved, distilled water. Cationic liposomes were prepared the day before the experiment, stored overnight at 4 °C, and sonicated immediately before use. For the labeled lipid preparations, different lipid compositions in chloroform were mixed with NBD-PE, to achieve a final concentration of 2.6 mol% labeled lipid as previously described [28].

### 2.4. Preparation of lipoplexes

Lipoplexes with different DOTAP<sup>+</sup>/DNA<sup>−</sup> charge ratios (0.5, 1, 2 and 4) were prepared by mixing different volumes of our stock lipid suspension with DNA. Lipoplexes were incubated for 1 h at room temperature; our previous work has demonstrated that this incubation period is necessary for these lipoplexes to form stable complexes [28]. The lipoplex suspension was subsequently diluted in buffer (2.5 mM Tris-HCl, pH 7.5) with different amounts of serum to achieve final serum concentrations of 0%, 10%, 30%, and 50% (v/v). It should be noted that there is some discrepancy in the literature regarding how serum concentrations are reported. While we have chosen to report serum levels as volume-to-volume percentages ranging from 0% to 50%, some previous investigators consider a 1:1 (v/v) mixture of serum and buffer to be 100% serum. This twofold discrepancy likely stems from the fact that blood is approximately half serum by volume, and therefore a solution prepared by diluting serum with an equal volume of buffer represents “full strength” serum. Although we appreciate this argument, we feel that volume-to-volume percentages are the most accurate way to report

serum concentrations, and thus 50% (v/v) serum represents a concentration that approximates physiological serum concentrations.

### 2.5. FRET assay

FRET was employed to monitor the extent of dissociation of DNA from lipids that occurs in the presence of serum. FRET was determined by monitoring the decrease in fluorescence of NBD-PE (donor) in the presence of the Cy3-DNA (acceptor) on a SpectraMax microplate fluorescence reader (Molecular Devices, Sunnyvale, CA) as previously described [28]. Data were reported as efficiency of FRET, which is calculated according to the equation:

$$E = 1 - F_{DA}/F_D$$

where  $F_{DA}$  and  $F_D$  are the fluorescence intensity of the NBD-PE (excitation at 463 nm and emission at 528 nm with 4-nm excitation and emission slits) in the presence of Cy3-DNA and unlabeled DNA, respectively. The final DNA concentration in the lipoplexes was held constant at 1.67  $\mu\text{g}/$

ml (5  $\mu\text{M}$  DNA phosphate) in all the experiments. The samples were incubated at 37 °C and analyzed at 2 min, 5 min, 30 min, 1 h, 4 h, 6 h and 24 h. The FRET efficiency of time zero lipoplexes in Tris buffer served as a reference. The relative FRET efficiency was determined by dividing the sample FRET efficiency by the FRET efficiency of reference samples.

### 2.6. Turbidity determination

The absorbance of complexes in the absence and presence of different serum concentrations (0–50% v/v) was measured at 500 nm with a corresponding amount of serum alone as a reference. Lipoplexes were formed with 2 mg/ml liposomes and 668  $\mu\text{g}/\text{ml}$  DNA solutions, and the final DNA concentration was 8.35  $\mu\text{g}/\text{ml}$ . The samples were incubated at 37 °C and analyzed at 2 min, 5 min, 30 min, 1 h, 4 h, 6 h and 24 h. Relative turbidity was determined by dividing the sample turbidity by the turbidity of time zero lipoplexes incubated in Tris buffer. Therefore, a relative turbidity value of 1 indicates that the turbidity from lipoplexes incubated in

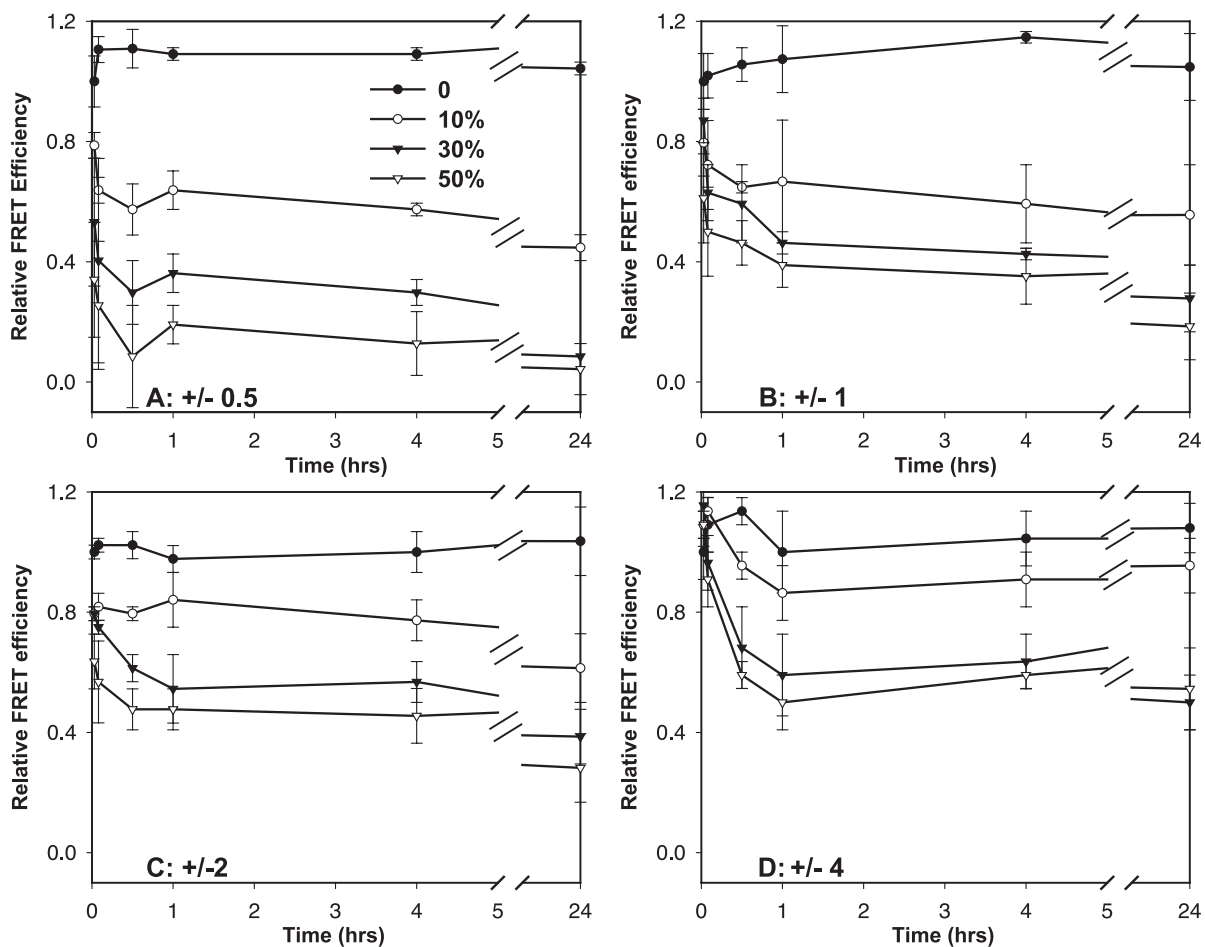


Fig. 1. The effect of serum concentration (0–50%) on the relative FRET efficiency of DOTAP:Chol (mol/mol 1:1)/DNA lipoplexes at different DOTAP<sup>+</sup>/DNA<sup>−</sup> charge ratios. Panel A, charge ratio 0.5; panel B, charge ratio 1; panel C, charge ratio 2; and panel D, charge ratio 4. Closed circle, no serum; open circle, 10% serum; closed triangle, 30% serum; open triangle, 50% serum. Symbols and error bars represent the mean  $\pm$  1 S.D. of triplicate samples.

serum is equivalent to the turbidity arising from the identical lipoplex formulation incubated in buffer.

### 2.7. Agarose gel electrophoresis and quantification of DNA integrity

The vectors possessing different lipid compositions were incubated at 37 °C, and aliquots containing 800-ng DNA were collected at 5 min, 30 min, 1 h, 4 h, and 24 h. Samples were subjected to a standard phenol–chloroform extraction procedure to remove lipids and serum proteins [29]. The amounts of supercoiled plasmid were detected on a 0.8% agarose gel to monitor DNA degradation due to nucleases in serum. DNA was visualized under UV light by ethidium bromide staining [29]. The fluorescent staining was then quantified by fluorometric image analysis with a Fluor-S MultiImager and Quantity One® software (Biorad, Hercules, CA).

### 2.8. In vitro transfection assay

Murine brain endothelial cells (middle T antigen transformed; bEnd.3) were obtained from American Type Culture Collection (Rockville, MD). Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 units/ml penicillin G, and 50 µg/ml streptomycin sulfate, and are propagated by reseeding at  $1-3 \times 10^5$  cells/100-mm dish every 2–3 days. For in vitro transfection, cultures were freshly seeded at 2500 cells/well in 96-well plates 24 h before transfection. Lipoplexes (20 µl) containing 0.2-µg DNA were incubated at room temperature for 1 h and then preincubated in the presence of different concentrations of mouse serum for 5 min, 30 min, 1 h, 4 h and 24 h before being applied to wells containing freshly washed cells. The cells were incubated with lipoplexes for

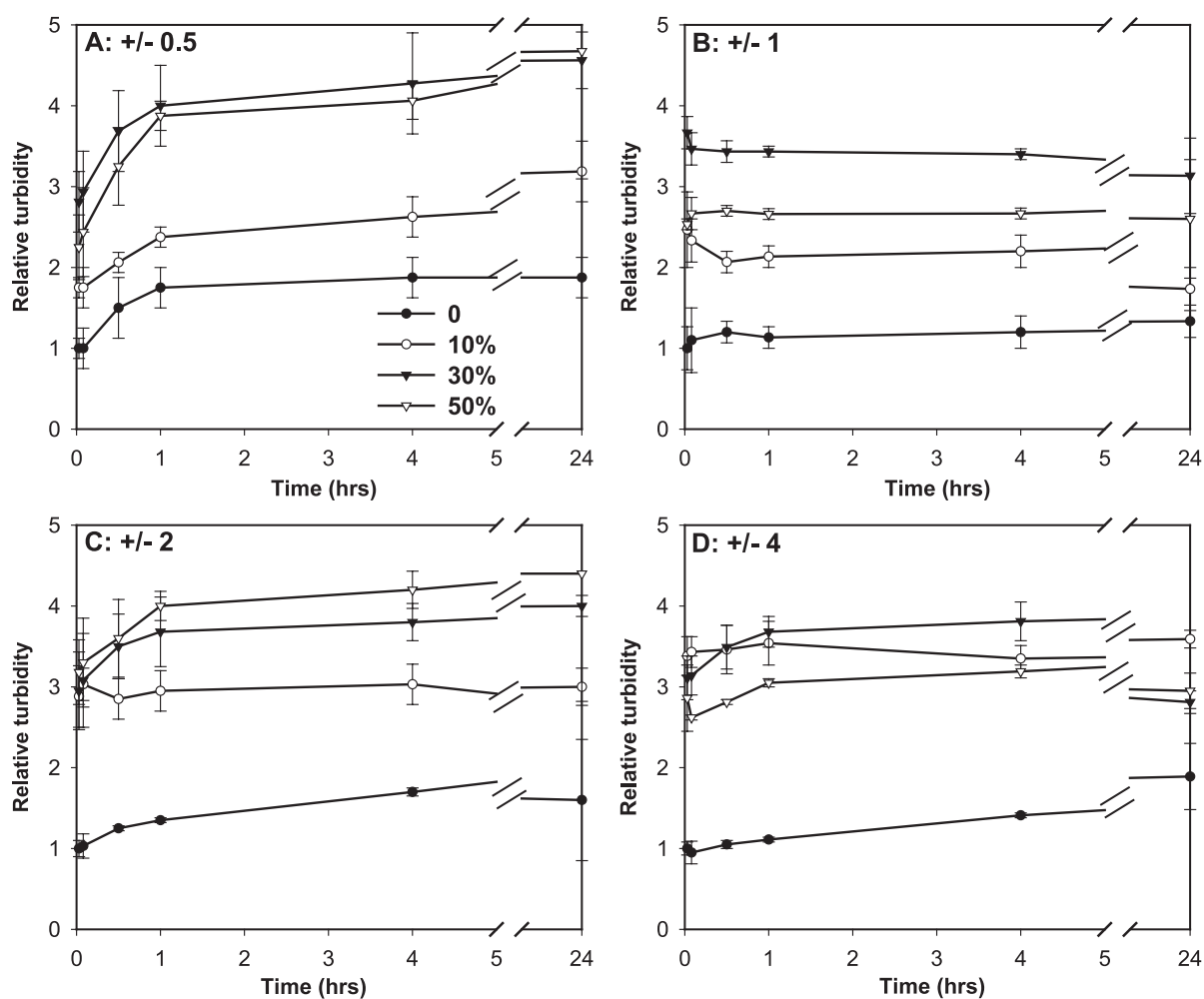


Fig. 2. The effect of serum concentration (0–50%) on the relative turbidity of DOTAP:Chol (mol/mol 1:1)/DNA lipoplexes at different DOTAP<sup>+</sup>/DNA<sup>-</sup> charge ratios. Panel A, charge ratio 0.5; panel B, charge ratio 1; panel C, charge ratio 2; and panel D, charge ratio 4. Closed circle, no serum; open circle, 10% serum; closed triangle, 30% serum; open triangle, 50% serum. Symbols and error bars represent the mean ± 1 S.D. of triplicate samples.

4 h before the medium was replaced with 100- $\mu$ l DMEM containing FBS and antibiotics. Forty hours after transfection, the culture medium was discarded, and the cells were washed twice with 100- $\mu$ l phosphate buffered saline (PBS) and then lysed with 80  $\mu$ l of lysis buffer (Promega). Twenty microliters of cell lysis solution was used to assay for luciferase expression using the luciferase assay kit (Promega), according to the manufacturer's protocol. The signal was quantified using a Monolight 2010 Luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI). Protein contents were determined with a Bio-Rad protein assay kit (Hercules, CA) according to the manufacturer's instructions. The absorbance was measured at 550 nm using a THERMOMax microplate reader (Molecular Devices).

### 2.9. Dynamic light scattering and zeta potential analysis

Samples containing 4- $\mu$ g plasmid were diluted to a final volume of 500  $\mu$ l with 2.5 mM Tris-HCl pH 7.5. Diluted samples were transferred to a cuvette for dynamic light

scattering analysis on a Nicomp 380 Zeta Potential/Particle Sizer (Particle Sizing Systems, Santa Barbara, CA). Channel width was set automatically based on the rate of fluctuation of scattered light intensity and  $1 \times 10^6$  counts were accumulated at 25 °C from triplicate samples representing each formulation. Volume-weighted Gaussian size distribution was fit to the autocorrelation functions and particle size values obtained as previously described [30]. Samples were then diluted to a final KCl concentration of 1 mM, and subjected to an electric field in a Nicomp 380 Zeta Potential/Particle Sizer for zeta potential determination.

## 3. Results

### 3.1. Effect of serum concentration and DOTAP<sup>+</sup>/DNA<sup>-</sup> ratio on lipid–DNA interactions within lipoplexes

To investigate the effect of serum on lipid–DNA interactions, FRET efficiency was monitored using DOTAP:

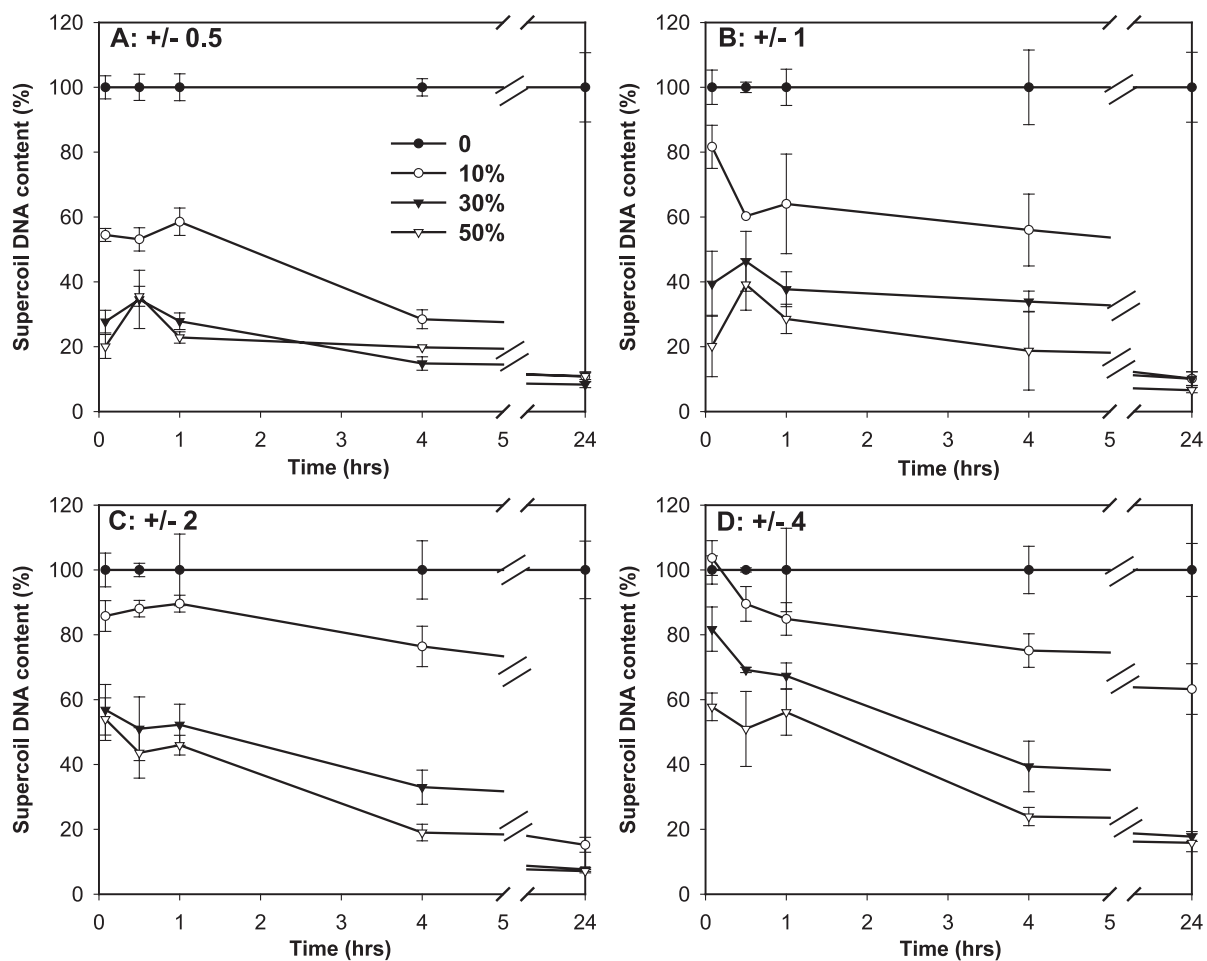


Fig. 3. The effect of serum concentration (0–50%) on the supercoiled DNA content of DOTAP:Chol (mol/mol 1:1)/DNA lipoplexes at different DOTAP<sup>+</sup>/DNA<sup>-</sup> charge ratios. Panel A, charge ratio 0.5; panel B, charge ratio 1; panel C, charge ratio 2; and panel D, charge ratio 4. Closed circle, no serum; open circle, 10% serum; closed triangle, 30% serum; open triangle, 50% serum. Symbols and error bars represent the mean  $\pm$  1 S.D. of triplicate samples.



Chol (mol/mol 1:1)/DNA prepared at different DOTAP<sup>+</sup>/DNA<sup>-</sup> ratios (0.5, 1, 2 and 4) and incubated at 37 °C in different concentrations of mouse serum (Fig. 1). For DOTAP-Chol/DNA lipoplexes in Tris buffer alone, at all DOTAP<sup>+</sup>/DNA<sup>-</sup> ratios examined, FRET efficiency varied in the first hour, then remained stable for 24 h, consistent with previous results [28]. However, the presence of serum caused a decrease of FRET efficiency, indicating that dissociation of lipid from DNA occurs under these conditions. Furthermore, the low FRET efficiency at our initial timepoint (2 min) indicates that much of the observed dissociation occurs immediately upon dilution of complexes into serum. The effect of serum components in weakening lipid–DNA interactions is more pronounced at higher serum concentrations, but is attenuated at higher charge ratios (Fig. 1). In the presence of serum, FRET efficiencies decreased over the time course of incubation at 37 °C. At charge ratio 0.5, the relative FRET efficiency was  $0.787 \pm 0.043$  at 2 min and  $0.447 \pm 0.043$  after 24 h in 10% mouse serum. In 50% serum, the relative FRET efficiency decreased to  $0.340 \pm 0.191$  at 2 min and  $0.043 \pm 0.085$  after 24 h (Fig. 1A). The very low FRET efficiency after 24 h suggests

that lipoplexes are almost completely dissociated under these conditions. At charge ratios 1, 2 and 4, the same trends with serum concentration and incubation time were observed. However, the dissociating effects of serum were clearly diminished at higher charge ratios, and this effect persisted throughout the 24-h incubation. For example, the relative FRET efficiencies of samples incubated in 50% serum were  $0.185 \pm 0.111$  at charge ratio 1,  $0.282 \pm 0.114$  at charge ratio 2, and  $0.545 \pm 0.1136$  at charge ratio 4 after a 24-h incubation (Fig. 1).

### 3.2. Effect of serum concentration and DOTAP<sup>+</sup>/DNA<sup>-</sup> charge ratio on turbidity

To investigate the effect of serum on lipoplex aggregation, turbidity was monitored using DOTAP:Chol (mol/mol 1:1)/DNA lipoplexes prepared at different DOTAP<sup>+</sup>/DNA<sup>-</sup> ratios (0.5, 1, 2 and 4) and incubated at 37 °C in different concentrations of mouse serum (Fig. 2). For DOTAP-Chol/DNA lipoplexes in Tris buffer alone, an increase in turbidity was observed during incubation at 37 °C, indicating a progressive aggregation with time. Turbidity increased sig-

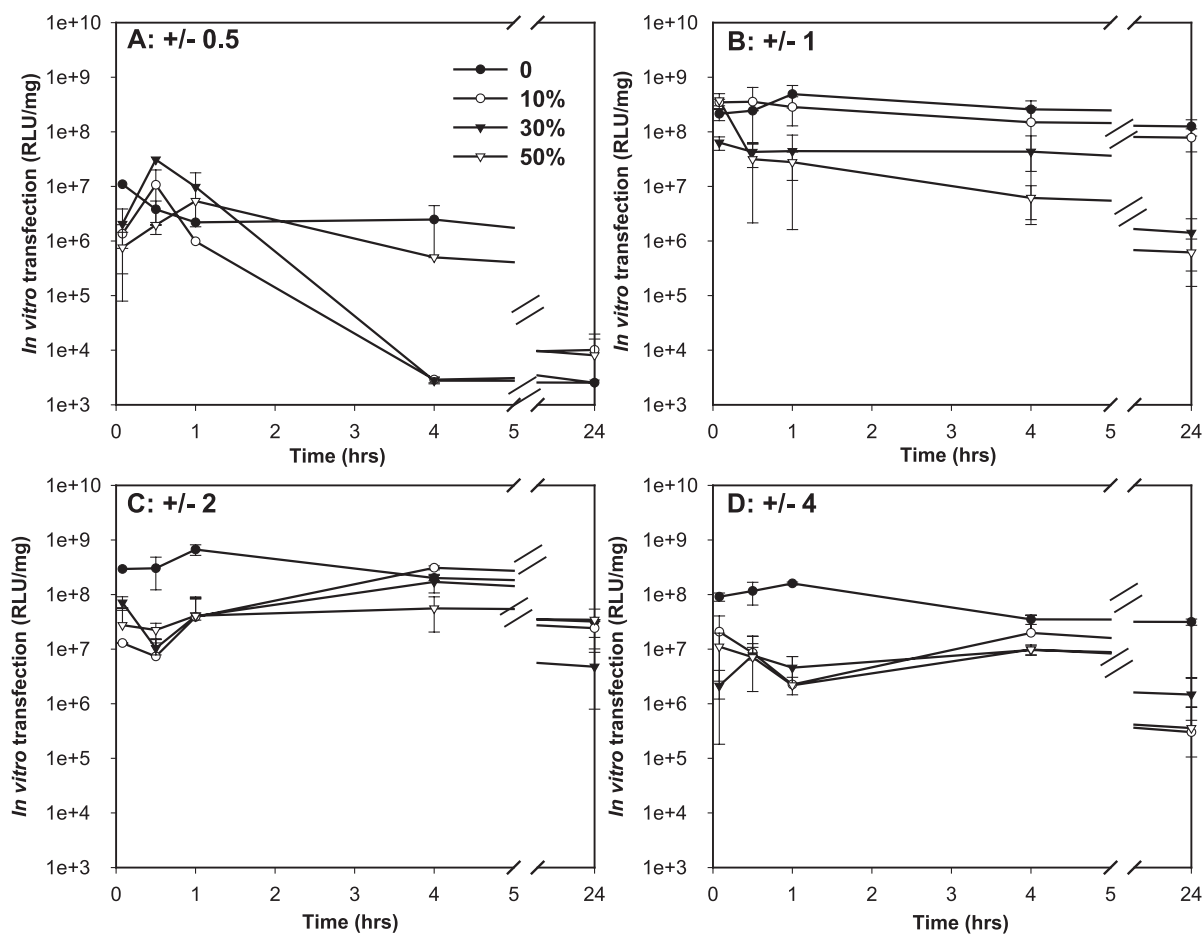


Fig. 4. The effect of serum concentration (0–50%) on the in vitro transfection efficiency of DOTAP:Chol (mol/mol 1:1)/DNA lipoplexes at different DOTAP<sup>+</sup>/DNA<sup>-</sup> charge ratios. Panel A, charge ratio 0.5; panel B, charge ratio 1; panel C, charge ratio 2; and panel D, charge ratio 4. Closed circle, no serum; open circle, 10% serum; closed triangle, 30% serum; open triangle, 50% serum. Symbols and error bars represent the mean  $\pm$  1 S.D. of triplicate samples.

nificantly in the presence of serum at all the charge ratios, suggesting that aggregation is enhanced in the presence of serum. Similar to the rapid dissociation observed in the FRET experiments (Fig. 1), much of the aggregation occurs within 2 min of exposure to serum (Fig. 2). Surprisingly, the presence of 30% serum caused more aggregation than other serum concentrations for lipoplexes at charge ratio 1 (Fig. 2B), and no consistent difference between aggregation in 30% and 50% serum was found at charge ratios 0.5 (Fig. 2A) and 4 (Fig. 2D). A similar effect at intermediate serum concentrations has been observed in other studies [31], but we did not observe this effect at charge ratio 2 (Fig. 2C). In general, turbidity tended to increase with serum concentration and time, reflecting trends similar to that seen with FRET. The clear exception to this general trend was observed at DOTAP<sup>+</sup>/DNA<sup>−</sup> of 1, where aggregation appeared to be completed within 2 min, and relatively constant turbidity was observed over time (Fig. 2B).

### 3.3. Effect of serum concentration and DOTAP<sup>+</sup>/DNA<sup>−</sup> charge ratio on DNA degradation

The presence of nuclease in the serum can degrade DNA, resulting in a loss of supercoil content and biological activity [32]. Not surprisingly, a progressive decrease in supercoil content was observed at higher serum concentrations regardless of charge ratio (Fig. 3). For DOTAP:Chol (mol/mol 1:1)/DNA lipoplexes at charge ratio 0.5, only 54 ± 2%, 28 ± 3%, and 20 ± 3% supercoiled DNA remained after 5 min in 10%, 30%, and 50% (v/v) serum, respectively (Fig. 3A). Furthermore, loss of supercoil was enhanced at longer incubation

times. Consistent with the FRET and turbidity data that indicated structural perturbations in the presence of serum, the loss of supercoil content suggests that these structural changes result in exposure of DNA to serum nucleases. As seen in the FRET results, the increase of DOTAP<sup>+</sup>/DNA<sup>−</sup> ratio resulted in enhanced DNA protection in all the serum concentrations, presumably due to the greater lipid–DNA interactions observed in Fig. 1. However, even at charge ratio 4, only 16 ± 3% supercoiled DNA remained after a 24-h incubation in 50% serum (Fig. 3D).

### 3.4. Effect of serum concentration and DOTAP<sup>+</sup>/DNA<sup>−</sup> ratio on *in vitro* transfection efficiency

At DOTAP<sup>+</sup>/DNA<sup>−</sup> ratio 0.5, the *in vitro* transfection efficiencies of DOTAP:Chol (mol/mol 1:1)/DNA lipoplexes were relatively low regardless of the presence of serum (Fig. 4A). Furthermore, transfection was virtually eliminated in these formulations after 24 h (Fig. 4A). Enhanced *in vitro* transfection efficiencies were found at higher charge ratios, and serum concentration and incubation time had much smaller effects on transfection efficiency in formulations of DOTAP<sup>+</sup>/DNA<sup>−</sup> of 1, 2, and 4 (Fig. 4B–D). However, there was a clear trend of reduced transfection observed at higher serum concentrations and longer incubation times. Taken together, the data in Figs. 1–4 indicate enhanced stability at higher charge ratios. Although transfection remained relatively high at a DOTAP<sup>+</sup>/DNA<sup>−</sup> of 1, the size of particles is relatively large even before exposure to serum (Table 1a), so formulations at charge ratios 2 and 4 were used in further experiments to investigate the role of charge density. In

Table 1

(a) Particle diameter (nm) of lipoplexes with different helper lipid compositions at different DOTAP<sup>+</sup>/DNA<sup>−</sup> charge ratios (0.5, 1, 2 and 4) in 2.5 mM Tris buffer pH 7.5. The mean and the standard deviation of triplicate samples are shown.

	+/- 0.5	+/- 1	+/- 2	+/- 4
DOTAP:Chol (2:1)	251.0 ± 94.7	761.2 ± 116.6	216.3 ± 11.8	153.8 ± 14.0
DOTAP:Chol (1:1)	192.4 ± 11.6	1480.0 ± 197.9	212.5 ± 8.5	197.8 ± 41.0
DOTAP:Chol (1:2)	127.3 ± 18.5	642.9 ± 102.2	146.9 ± 20.9	140.4 ± 3.7
DOTAP:Chol (1:4)	113.2 ± 16.8	469.6 ± 98.6	260.0 ± 17.8	120.7 ± 15.0
DOTAP:Chol (1:8)	657.8 ± 193.7	322.4 ± 11.2	285.8 ± 79.8	520.6 ± 18.4
DOTAP:Chol (1:16)	598.5 ± 56.0	405.4 ± 35.3	428.3 ± 10.6	833.2 ± 34.3
DOTAP:Chol:POPC (1:1:0.75)	182.0 ± 9.4	2088.3 ± 461.9	447.9 ± 139.5	405.6 ± 223.8
DOTAP:Chol:DOPE (1:1:0.75)	92.0 ± 9.8	285.2 ± 7.2	160.9 ± 37.9	136.5 ± 24.9

(b) Zeta potential (mV) of lipoplexes with different helper lipid compositions at different DOTAP<sup>+</sup>/DNA<sup>−</sup> charge ratios (0.5, 1, 2 and 4) in 2.5 mM Tris buffer pH 7.5. The mean and the standard deviation of triplicate samples are shown.

	+/- 0.5	+/- 1	+/- 2	+/- 4
DOTAP:Chol (2:1)	−24.1 ± 0.4	−21.9 ± 1.9	26.7 ± 5.1	27.8 ± 4.1
DOTAP:Chol (1:1)	−21.7 ± 3.4	−27.8 ± 5.7	35.3 ± 1.4	32.9 ± 1.5
DOTAP:Chol (1:2)	−33.4 ± 2.7	−36.3 ± 0.3	33.6 ± 4.1	43.2 ± 5.7
DOTAP:Chol (1:4)	−37.8 ± 2.1	−41.8 ± 1.2	45.7 ± 0.3	51.7 ± 1.6
DOTAP:Chol (1:8)	−33.1 ± 0.5	−34.1 ± 2.0	28.9 ± 1.1	47.7 ± 0.2
DOTAP:Chol (1:16)	−34.9 ± 2.8	−34.6 ± 1.8	29.7 ± 2.1	49.5 ± 3.6
DOTAP:Chol:POPC (1:1:0.75)	−26.5 ± 3.2	−29.3 ± 1.3	19.5 ± 1.5	41.0 ± 3.9
DOTAP:Chol:DOPE (1:1:0.75)	−27.0 ± 6.4	−28.8 ± 3.8	35.1 ± 1.4	37.9 ± 4.5

addition, given that 50% serum is the most relevant condition for *in vivo* applications, we utilized these conditions to compare the stability of different lipoplex formulations in subsequent experiments.

### 3.5. Effect of lipid charge density on lipoplex serum stability at DOTAP<sup>+</sup>/DNA<sup>-</sup> charge ratio 2 in 50% serum

In the experiments depicted in Figs. 1–4, the DOTAP:Chol ratio was held constant at 1. Although the DOTAP<sup>+</sup>/DNA<sup>-</sup> charge ratio was altered in those experiments, the charge density of the lipid component remained constant. The data shown in Fig. 5 represent experiments where the DOTAP:Chol ratio was varied while maintaining DOTAP<sup>+</sup>/DNA<sup>-</sup> at 2, thereby altering the charge density of the lipid component. As shown in Fig. 5A, all the formulations exhibited dramatic reductions in FRET within the first hour, indicating a rapid perturbation of lipoplex structure in 50% (v/v) serum. This rapid initial reduction in FRET was

followed by a more gradual dissociation, and the relative FRET efficiencies ranged from 0.15 to 0.3 after a 24-h incubation (Fig. 5A). No major differences in relative FRET efficiencies were detected in DOTAP:Chol/DNA lipoplexes containing different mole concentrations of cholesterol. However, formulations showed dramatic differences in their tendency to aggregate in 50% serum, as indicated by changes in turbidity (Fig. 5B). A decrease in DOTAP:Chol mole ratio from 1:1 to 1:2 to 1:4 resulted in a progressive decrease in lipoplex aggregation. This apparent trend was not followed by the DOTAP:Chol 2:1 formulation which demonstrated turbidity changes that were intermediate to the 1:1 and 1:2 formulations. Surprisingly, no aggregation was detected in the DOTAP:Chol 1:4 formulation even after 24 h in 50% serum (Fig. 5B).

The supercoiled DNA content of all formulations decreased rapidly within the first hour, although the 1:1 and 1:2 formulations clearly showed greater abilities to protect DNA at early timepoints (Fig. 5C). After a 24-h incubation,

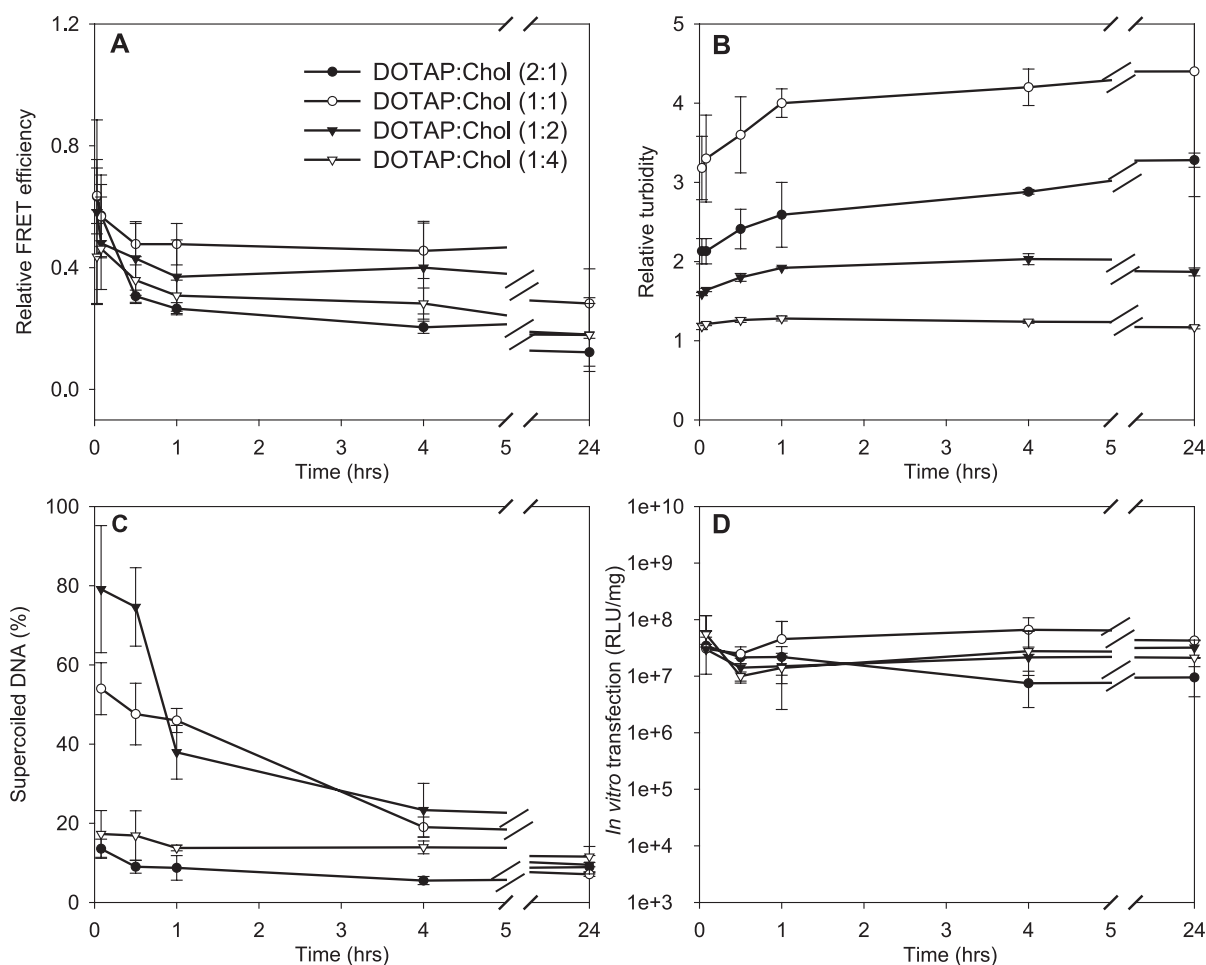


Fig. 5. The effect of lipid charge density on the stability of DOTAP:Chol/DNA lipoplexes in 50% serum at DOTAP<sup>+</sup>/DNA<sup>-</sup> charge ratio 2. Panel A, relative FRET efficiency; panel B, relative turbidity; panel C, supercoiled DNA content; and panel D, *in vitro* transfection efficiency. Closed circle, DOTAP:Chol (mol/mol 2:1); open circle, DOTAP:Chol (mol/mol 1:1); closed triangle, DOTAP:Chol (mol/mol 1:2); open triangle, DOTAP:Chol (mol/mol 1:4). Symbols and error bars represent the mean  $\pm$  1 S.D. of triplicate samples.



supercoil contents of all formulations were reduced to 7–11% of initial levels. In contrast to supercoil content, *in vitro* transfection efficiency was largely independent of DOTAP:Chol ratio and remained relatively constant over 24 h (Fig. 5D).

### 3.6. Effect of lipid charge density on lipoplex serum stability at DOTAP<sup>+</sup>/DNA<sup>-</sup> ratio 4 in 50% serum

Similar to that seen at charge ratio 2, FRET efficiencies decreased in the first hour, then remained relatively stable up to 24 h (Fig. 6A). The relative FRET efficiencies were higher in all DOTAP:Chol/DNA lipoplexes at charge ratio 4 as compared to those at charge ratio 2 (compare Figs. 5A and 6A), suggesting that the higher charge ratio enhanced the association between lipid and DNA. After 24 h, the relative FRET efficiencies were retained at values of approximately 0.5 for DOTAP:Chol 1:1 and 1:4, suggesting that these vectors did not undergo the extensive dissociation that was generally observed at lower charge ratios (Fig. 6A). Despite

the enhanced associations indicated by FRET, the turbidity and DNA degradation results were similar to those observed at charge ratio 2 (compare Figs. 5B–C and 6B–C). More specifically, DOTAP:Chol 1:4 was able to resist aggregation in 50% serum (Fig. 6B), but all formulations experienced a dramatic reduction in supercoiled DNA content that was further reduced below 30% after 24 h (Fig. 6C). In contrast to that observed at DOTAP<sup>+</sup>/DNA<sup>-</sup> 2, the formulations displayed sharp differences in transfection efficiency, and were comparatively low and variable with incubation time (Fig. 6D). However, the DOTAP:Chol 1:4 formulation exhibited high and stable transfection efficiency over the time course of this experiment (Fig. 6D). Furthermore, the transfection efficiency of this formulation in 50% serum ( $9.1 \times 10^7$  RLU/mg protein) was greater than that observed in the absence of serum ( $1.3 \times 10^7$  RLU/mg protein) after 24 h. In general, we conclude that there is no consistent correlation of lipid–DNA interactions, aggregation, nuclease protection or *in vitro* transfection efficiency with lipid charge density at DOTAP<sup>+</sup>/DNA<sup>-</sup> 2 or 4.

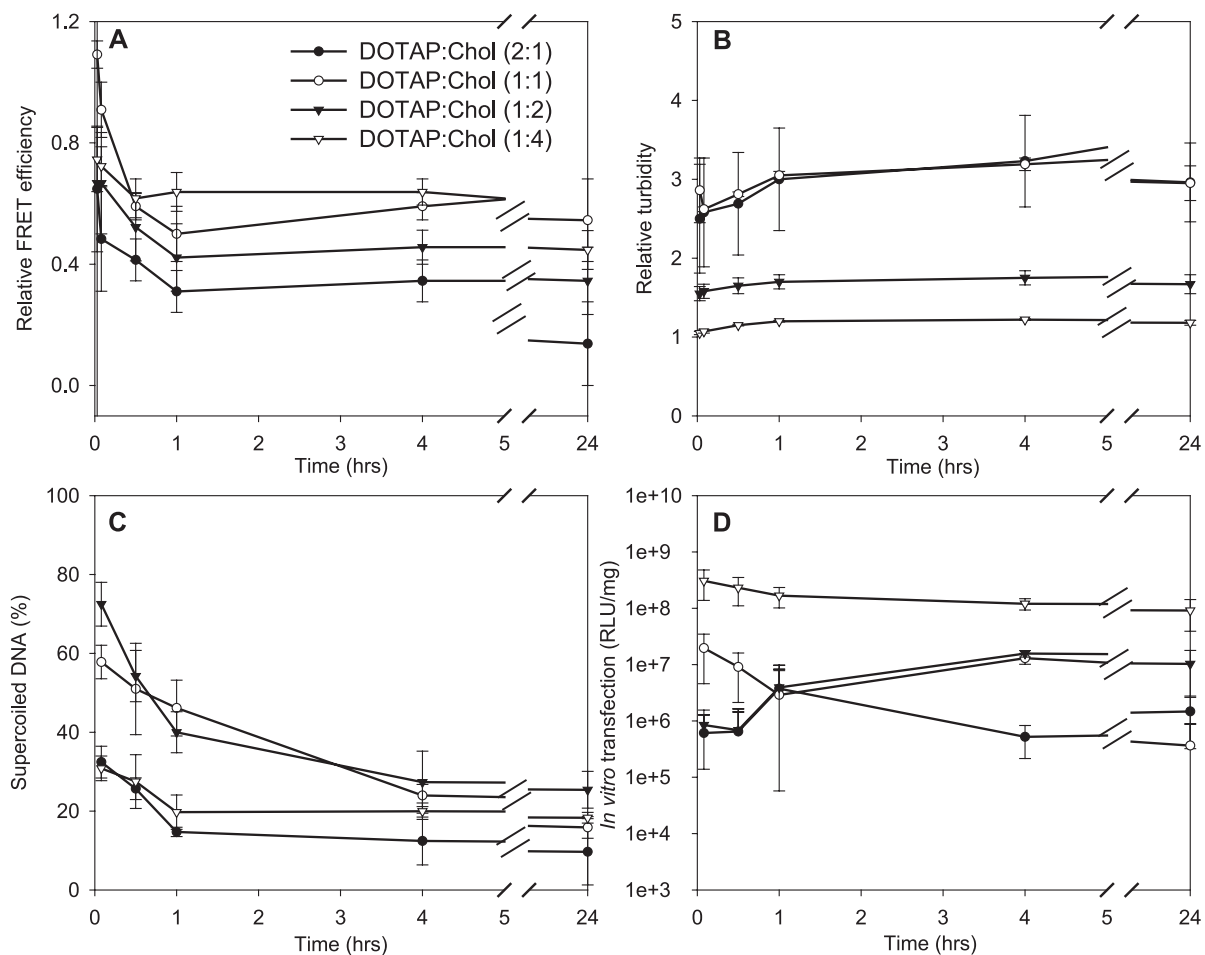


Fig. 6. The effect of lipid charge density on the stability of DOTAP:Chol/DNA lipoplexes in 50% serum at DOTAP<sup>+</sup>/DNA<sup>-</sup> charge ratio 4. Panel A, relative FRET efficiency; panel B, relative turbidity; panel C, supercoiled DNA content; and panel D, *in vitro* transfection efficiency. Closed circle, DOTAP:Chol (mol/mol 2:1); open circle, DOTAP:Chol (mol/mol 1:1); closed triangle, DOTAP:Chol (mol/mol 1:2); open triangle, DOTAP:Chol (mol/mol 1:4). Symbols and error bars represent the mean  $\pm$  1 S.D. of triplicate samples.

### 3.7. Effect of lipid composition on lipoplex serum stability

The results described above do not show a strong correlation between enhanced serum stability and reduced lipid charge density. It follows that some of the improved characteristics observed with high cholesterol formulations might be specifically due to the increased cholesterol content rather than a reduction in charge density per se. To investigate this hypothesis, the serum stability of lipoplexes with the same charge density but different helper lipid composition was assessed. Previous studies have shown that mixing DNA with cationic liposomes can lead to lipoplexes with either a lamellar structure or a columnar inverted hexagonal lattice [33]. Thus, a lipid that promotes lamellar structure (POPC) and a lipid that facilitates hexagonal phase formation (DOPE) were selected for use as helper lipids in combination with cholesterol. Data from X-ray diffraction studies have reported that the molecular area of PC is  $62.7 \text{ \AA}^2$ , cholesterol is  $35 \text{ \AA}^2$ , and condensed PC in the presence of cholesterol is  $46.7 \text{ \AA}^2$  [34,35]. Thus,

one cholesterol molecule occupies approximately the same area as 0.75 PC molecules when these lipids are combined. To obtain the same charge density as DOTAP:Chol (mol/mol 1:2), DOTAP:Chol:POPC (mol/mol/mol 1:1:0.75) was used as a model lipid formulation. These formulations were compared with DOTAP:Chol:DOPE (mol/mol/mol 1:1:0.75), under the assumption that the volume occupied by DOPE in the bilayer is similar to that of POPC. At DOTAP<sup>+</sup>/DNA<sup>-</sup> ratio 2, no difference in FRET efficiency was observed between DOTAP:Chol (1:2) and POPC containing lipoplexes (Fig. 7A). Surprisingly, the inclusion of DOPE resulted in no FRET, suggesting a rapid disintegration of these lipoplexes in 50% serum (Fig. 7A), which was confirmed by turbidity data (Fig. 7B). The relative FRET efficiency for DOPE-containing lipoplexes below zero can be understood in the context of the FRET efficiency calculation:  $E = 1 - F_{DA}/F_D$ . The values of  $F_{DA}$  ( $\sim 135$  at 24 h) and  $F_D$  ( $\sim 130$  at 24 h) in 50% serum were very close due to the dissociation of DNA and DOTAP:Chol:DOPE. This small difference ( $<4\%$ ) result-

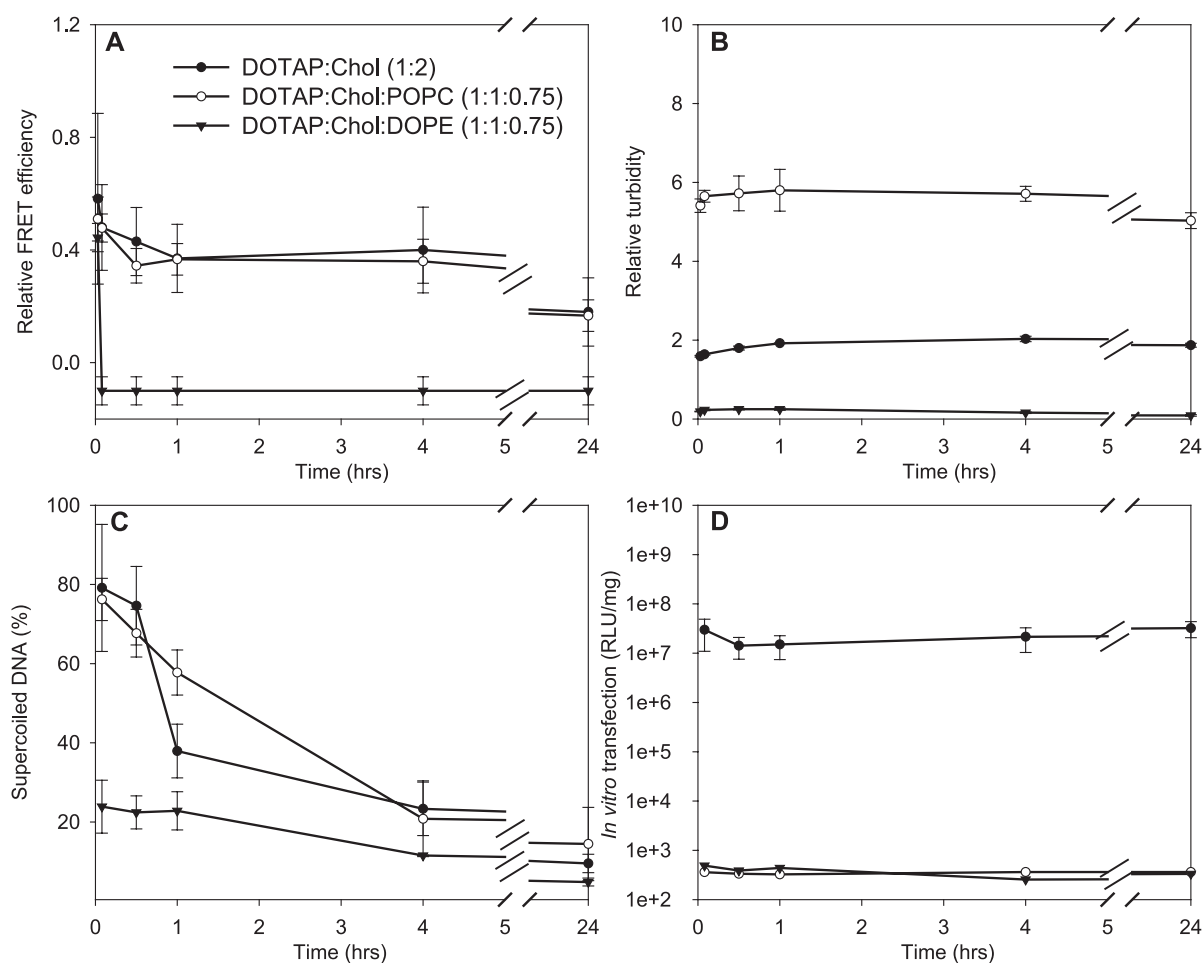


Fig. 7. The effect of lipid composition on the stability of lipoplexes in 50% serum at DOTAP<sup>+</sup>/DNA<sup>-</sup> ratio 2. Panel A, relative FRET efficiency; panel B, relative turbidity; panel C, supercoiled DNA content; and panel D, in vitro transfection efficiency. Closed circle, DOTAP:Chol (mol/mol 1:2); open circle, DOTAP:Chol:POPC (mol/mol/mol 1:1:0.75); closed triangle, DOTAP:Chol:DOPE (mol/mol/mol 1:1:0.75). Symbols and error bars represent the mean  $\pm$  1 S.D. of triplicate samples.

ing from slight assay variability led to negative FRET efficiency after calculation. For reference, the value of  $F_{DA}$  was 90.4 and  $F_D$  was 102 at time zero in Tris buffer alone for DOPE containing lipoplexes. Similarly, the turbidity of DOPE-containing lipoplexes in Tris buffer alone at time zero was 0.445 and it remained constant for 24 h (data not shown). As mentioned above, the relative turbidity of DOPE-containing lipoplexes less than 1 (Fig. 7B) can only be explained by the dissociation of lipoplexes in the presence of 50% serum. POPC-containing lipoplexes rapidly aggregated and generated high turbidity in 50% serum (Fig. 7B), consistent with the loss of biological activity (Fig. 7D). The increase of DOTAP<sup>+</sup>/DNA<sup>-</sup> ratio from 2 to 4 resulted in stronger associations between lipid and DNA, which was reflected by the enhanced FRET efficiencies in all formulations (compare Figs. 7A and 8A). However, even at this higher charge ratio, DOPE containing lipoplexes dissociated and lost transfection activity after 24 h in 50% serum (Figs. 8A and 8D).

POPC containing lipoplexes exhibited high turbidity and low transfection similar to that observed at DOTAP<sup>+</sup>/DNA<sup>-</sup> 2. In all formulations at both charge ratios, the protection of DNA from serum nucleases was minimal after the first few hours, although slightly higher supercoil contents were seen at DOTAP<sup>+</sup>/DNA<sup>-</sup> 4 as compared to 2 (Figs. 7C and 8C).

#### 4. Discussion

Detrimental interaction with serum components is one of the major barriers which intravenous nonviral gene delivery has to overcome. A previous study reported that the immediate effect of serum on cationic lipoplexes was aggregation, and that further interactions with serum led to lipoplex dissociation, DNA release and degradation [10]. The inclusion of cholesterol in lipoplex formulations has been shown to result in a slower dissociation in serum,

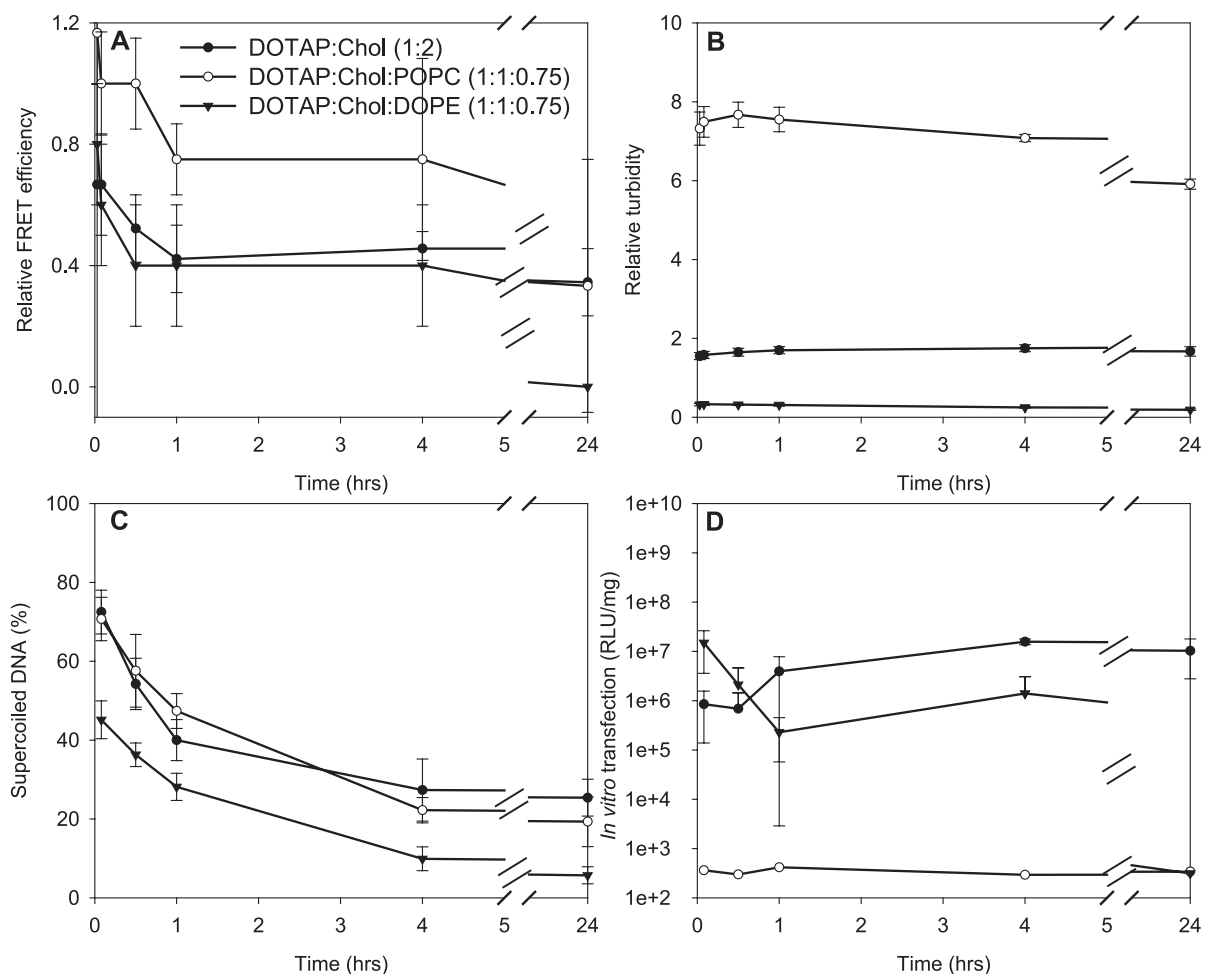


Fig. 8. The effect of lipid composition on the stability of lipoplexes in 50% serum at DOTAP<sup>+</sup>/DNA<sup>-</sup> ratio 4. Panel A, relative FRET efficiency; panel B, relative turbidity; panel C, supercoiled DNA content; and panel D, in vitro transfection efficiency. Closed circle, DOTAP:Chol (mol/mol 1:2); open circle, DOTAP:Chol:POPC (mol/mol/mol 1:1:0.75); closed triangle, DOTAP:Chol:DOPE (mol/mol/mol 1:1:0.75). Symbols and error bars represent the mean  $\pm$  1 S.D. of triplicate samples.

which likely contributes to the greater *in vivo* transfection observed with cholesterol-containing lipoplexes [10,18,21,22,25–27]. Similarly, Crook et al. [22] reported that cholesterol-containing lipoplexes maintained their ability to bind to cells in the presence of serum, and suggested that this effect was due to the decreased lipid charge density in lipoplexes incorporating cholesterol. More recently, Lin et al. [36] have suggested that the lipid charge density is the key universal parameter that determines transfection, although these authors were careful not to extend their conclusions to experiments conducted in the presence of serum. The goal of our study was to investigate the role of charge density in determining serum stability. In trying to define “stability” in this context, we felt that the degree of lipoplex dissociation, aggregation, and the ability to protect plasmids from serum nucleases were the critical parameters that would likely be important for maintaining transfection rates. Accordingly, we directly monitored lipid–DNA interactions with FRET, assessed the extent of aggregation in serum with turbidity, determined changes in supercoil content after exposure to serum, and evaluated transfection in mouse endothelial cells. FRET had been carried out under physiological conditions due to its lower sensitivity to coexisting components [10,20,37]. However, in the previous FRET studies, either FRET between lipid molecules [10,20] or FRET between DNA molecules [37] was used to reflect the interactions between cationic vectors and DNA indirectly.

Although it is widely recognized that the presence of serum can dramatically alter the performance of nonviral vectors, previous studies on serum stability have tended to conduct experiments in fetal bovine serum and/or at low serum concentrations that mimic cell culture conditions [9,12,13,15,19,20]. However, the goal of many studies is to develop delivery systems that can be utilized *in vivo*, and therefore it behooves researchers to utilize serum conditions that more accurately mimic physiological conditions. In this context, it is important to recognize that previous studies have documented distinct differences between various sera, and shown that mouse serum more accurately mimics human serum conditions [10,38]. Furthermore, it should not be surprising that vector stability is dependent on serum concentration, and it was recently suggested that serum stability studies should be performed at physiological serum concentrations ( $\approx 50\%$  v/v) in order to approximate *in vivo* conditions [31]. Our results are consistent with this suggestion, and demonstrate that higher serum concentrations have progressively greater negative impacts on lipoplex performance (Figs. 1–4). Also consistent with previous reports, our data show that the stability of lipoplexes in serum can be dramatically improved by utilizing higher  $+/ -$  charge ratios [9,26]. Thus, in investigating the role of lipid charge density in serum stability, we employed lipoplexes at high DOTAP<sup>+</sup>/DNA<sup>−</sup> charge ratios (2 and 4) in experiments conducted in physiological concentrations of mouse serum.

To test the role of lipid charge density in serum stability, increasing amounts of cholesterol were formulated with DOTAP at a constant DOTAP<sup>+</sup>/DNA<sup>−</sup> charge ratio. Our results do not show any consistent correlations of changes in lipid–DNA interactions, aggregation, nuclease resistance, or transfection with altered lipid charge density of the formulations. However, we did observe a progressive decrease in serum-induced aggregation as cholesterol content was increased from 50% (DOTAP:Chol 1:1) to 80% (DOTAP:Chol 1:4) at both charge ratios 2 and 4 (Figs. 5B and 6B), but this trend was not followed by the high charge density formulation with the lowest cholesterol content (DOTAP:Chol 2:1). Although lower lipid charge densities did not provide enhanced stability in our experiments, the lowest charge density formulation with high cholesterol content did exhibit remarkable stability in terms of maintaining particle size and transfection efficiency in 50% serum. This effect was especially striking at charge ratio 4 where even the FRET data indicate that approximately half of the lipid–DNA associations were maintained after 24 h (Fig. 6A). To test if higher cholesterol contents would lead to further improvements in serum stability, DOTAP:Chol (mol/mol 1:8) and DOTAP:Chol (mol/mol 1:16) were prepared. Unfortunately, large particle sizes were measured for liposomes alone ( $580.2 \pm 49.1$  and  $919.7 \pm 39.7$  nm for DOTAP:Chol 1:8 and 1:16 liposomes, respectively), and lipoplexes ( $>500$  nm) at charge ratio 4 (Table 1), indicating that these formulations would not be suitable for *in vivo* administration. Also, the *in vitro* transfection efficiency of these formulations in 50% serum proved to be approximately 1/10 (DOTAP:Chol mol/mol 1:8) and 1/15 (DOTAP:Chol mol/mol 1:16) of that in DOTAP:Chol (mol/mol 1:4) formulations (data not shown). Thus, additional serum stability studies were not conducted with these formulations.

Considering the trend noted above of less aggregation at lower lipid charge densities, it is important to recognize that the lipid charge densities were reduced in our formulations by increasing the relative amount of cholesterol. Thus, the enhanced resistance to aggregation could be due to either decreased lipid charge density or the increased cholesterol content utilized in these formulations. Accordingly, additional experiments were conducted using formulations incorporating different neutral lipids at a constant lipid charge density. Our results demonstrate that lipoplexes possessing equivalent lipid charge densities exhibit very different serum stabilities as determined by all of our criteria (Figs. 7 and 8). While differences in formulations containing lipids that stabilize a lamellar structure (e.g., POPC) versus lipids that promote hexagonal phase formation (e.g., DOPE) might be expected [39], neither of these formulations displayed characteristics similar to that seen with the DOTAP:Chol lipoplexes when incubated in 50% serum. We conclude from these experiments that serum stability is not governed simply by lipid charge density, and that cholesterol must possess properties that allow lipoplexes to resist aggregation and maintain transfection in serum. In

similar studies with liposomes, the presence of at least 25 mol% cholesterol was found to be required to preserve serum stability [22,40].

With regards to the mechanism by which cholesterol endows lipoplexes with enhanced serum stability, we can only speculate that increasing the amount of cholesterol causes a resistance to perturbations induced by the binding of serum proteins. A similar mechanism was proposed by Templeton et al. [25] who concluded that cholesterol enhances bilayer stability that results in greater resistance to mechanical breakage upon serum adsorption. It is important to point out that the decreased FRET observed in all formulations exposed to serum clearly demonstrates that perturbations of the lipoplex structure have occurred even in formulations where particle size and transfection are maintained. Furthermore, the highly positive zeta potentials for each of the formulations at DOTAP<sup>+</sup>/DNA<sup>-</sup> 4 were reduced to slightly negative values (–2 to –8 mV) upon exposure to serum (data not shown). These results indicate that the enhanced serum stability observed for the DOTAP:Chol (mol/mol 1:4)/DNA lipoplexes was not due to diminished protein binding [22], but to the ability of this formulation to withstand the detrimental effects of such binding. It is interesting to note that studies have shown that cholesterol contents greater than approximately 30% (mol/mol) result in a phase separated system in the neutral vesicle bilayer [34]. Although the complexes formed between DNA and cationic lipids are very different from conventional liposomes, cholesterol interacted with cationic lipids in much the same way as it did with other phospholipids [41]. Thus, our DOTAP:Chol formulations (1:1, 1:2, and 1:4) possess an increasing amount of phase separated cholesterol that correlates with the enhanced resistance to serum by protecting lipoplexes against mechanical breakage or aggregation upon adsorption of serum components [25,42]. In addition, the fact that the DOTAP:Chol 2:1 formulation did not follow the general trend of reduced aggregation with increased cholesterol content may be because this lipoplex does not possess a substantial amount of phase-separated cholesterol. Furthermore, it is unlikely that the formulations incorporating POPC or DOPE in addition to cholesterol (Figs. 7 and 8) are phase-separated, and this may contribute to the poor stability observed with these lipoplexes. Alternatively, the increased amounts of cholesterol may further promote hexagonal phase formation upon binding to the endosomal membrane [43,44], facilitate DNA release into cytoplasm and lead to improved transfection efficiency.

We consistently observe no correlation between the loss of supercoil content and transfection, as suggested by other studies [45,46]. In fact, even our most stable formulation lost approximately 70% of its original supercoil content within 2 min of serum exposure despite maintaining transfection efficiency (Figs. 6C and 6D). However, it should be noted that this formulation retained approximately 20% of its initial supercoil content even after 24 h in serum. Thus, it could be argued that it is the intact DNA that is responsible

for the observed transfection. According to this argument, only a fraction of the lipoplexes in the heterogeneous particle population are responsible for transfection, and these “active” vectors are assembled in a structure that protects DNA from serum nucleases [20,26]. Alternatively, since the open circle form of DNA is also capable of transfection [47,48], it may be that the loss of supercoil content per se does not accurately reflect the capacity of lipoplexes to transfect cells.

We wish to point out that only one cationic lipid was investigated here, but studies have shown that the membrane order promoted by cholesterol is independent of lipid headgroup structure, charge, or the nature of chain-backbone links [42]. Therefore, we assume that the enhancement of serum stability of DOTAP:Chol resulting from the inclusion of cholesterol is also applicable to other cationic liposomes.

In summary, our results are consistent with previous studies demonstrating that the concentration of serum can have a tremendous effect on the observed “stability” as assessed by several parameters. We also observe an increased serum stability at high DOTAP<sup>+</sup>/DNA<sup>-</sup> ratios, and this effect was further enhanced at high cholesterol contents. However, the greater serum stability did not directly correlate with decreases in lipid charge density, and appeared to be related to the use of increasing amounts of cholesterol as a helper lipid. We speculate that the high cholesterol contents result in a phase-separated cholesterol domain that contributes to the observed increase in serum stability. Finally, our results with DOTAP:Chol (mol/mol 1:4)/DNA lipoplexes at DOTAP<sup>+</sup>/DNA<sup>-</sup> 4 indicate that this formulation is surprisingly stable in serum, and future work will characterize its circulating half-life and ability to transfect cells in vivo.

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