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The use of fluorescence resonance energy transfer to monitor dynamic changes of lipid–DNA interactions during lipoplex formation

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Abstract

Fluorescence resonance energy transfer (FRET) was used to monitor interactions between Cy3-labeled plasmid DNA and NBD-labeled cationic liposomes. FRET data show that binding of cationic liposomes to DNA occurs immediately upon mixing (within 1 min), but FRET efficiencies do not stabilize for 1–5 h. The time allowed for complex formation has effects on in vitro luciferase transfection efficiencies of DOPE-based lipoplexes; i.e., lipoplexes prepared with a 1-h incubation have much higher transfection efficiencies than samples with 1-min or 5-h incubations. The molar charge ratio of DOTAP to negatively charged phosphates in the DNA ($DOTAP^+/DNA^-$) also affected the interaction between liposomes and plasmid DNA, and interactions stabilized more rapidly at higher charge ratios. Lipoplexes formulated with DOPE were more resistant to high ionic strength than complexes formulated with cholesterol. Taken together, our data demonstrate that lipid–DNA interactions and in vitro transfection efficiencies are strongly affected by the time allowed for complex formation. This effect is especially evident in DOPE-based lipoplexes, and suggests that the time allowed for lipoplex formation is a parameter that should be carefully controlled in future studies.

Keywords: Gene delivery; Nonviral vector; Stability; Fluorescence resonance energy transfer

1. Introduction

Problems associated with the use of viruses as gene delivery vehicles have renewed an interest in developing synthetic vectors with minimal immunogenicity [1–4]. Although transfection studies are careful to control certain parameters in the preparation of nonviral vectors (e.g., charge ratio, mixing conditions, DNA concentration), the time allowed for vectors to assemble is not typically optimized. This general statement is true for both in vitro and in vivo studies, and some clinical trials have employed pre-assembled vectors while others prepare complexes immediately prior to injection into the patient [5,6]. In an attempt to investigate the effects of complexation time on lipid–DNA complexes (lipoplexes), our study utilizes fluorescent resonance energy transfer (FRET) to examine the time-dependent interaction of cationic liposomes with plasmid DNA. In addition, we compare the changes we observe with FRET to alterations in NaCl sensitivity, transfection, and particle size.

Cationic liposomes used for gene delivery typically incorporate neutral helper lipids in order to increase the transfection efficiency of the resulting lipid–DNA complexes. It has been reported that dioleoylphosphatidylethanolamine (DOPE) increases the in vitro transfection efficiency, and cholesterol gives significant enhancement in vivo [7,8]. DOPE has a small head group and bulky acyl chains, which gives it a high propensity to form a hexagonal phase and promote membrane fusion [9–12]. Fusion of lipoplexes with the endosomal membrane is thought to result in cytosolic release of DNA and subsequent en-

Abbreviations: FRET, fluorescent 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, 7-nitrobenzo-2-oxa-1,3-diazol-4-yl phosphatidylethanolamine; $DOTAP^+/DNA^-$, molar charge ratio of DOTAP to negatively charged phosphates in the DNA; SDS, sodium dodecylsulfate; L_α^C, multilamellar structure; H_{II}^C, hexagonal lattice

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hanced gene expression [13–16]. Some studies of the effect of cholesterol on membrane structure have reported that cholesterol also promotes hexagonal phase formation [16,17]. However, other studies suggest that the lipids in cholesterol-based lipoplexes have a stable and rigid lamellar structure that does not readily fuse with lipid bilayers [18,19].

The electrostatic interactions between cationic liposomes and negatively charged nucleic acids result in spontaneous formation of lipoplexes [20,21]. The process of complexation is dynamic and has been shown to induce structural changes in both DNA and cationic liposomes [20–22]. However, the time needed for lipoplexes to stabilize is not well-studied, and various equilibration times (0–60 min) have been employed by different researchers [6,20,23–29]. A study by Gershon et al. [24] monitored ethidium bromide intercalation and showed that complexes at $DOTAP^+/DNA^-$ of 1.1 became stable after 40 min, while complexes at charge ratios of 0.5 and 1.5 stabilized within seconds. Their results suggest that the association between lipids and DNA in a lipoplex can fluctuate over time. Therefore, the time allowed for complexation is an experimental variable that can affect the physical properties of lipoplexes, and it seems prudent to investigate lipid–DNA interactions during complex formation more fully.

Previous studies have utilized FRET to evaluate the interaction between cationic lipids and DNA [30–34]. FRET can be observed when a fluorescently labeled lipid and DNA are in close proximity (approximately 2–9 nm), and is prevented upon dissociation [35]. Since FRET efficiency is inversely related to the sixth power of distance between the donor and acceptor probes, changes in FRET efficiency reflect alterations of the lipid and DNA interaction. FRET was determined by monitoring the decrease in fluorescence emission of 7-nitrobenzo-2-oxa-1,3-diazol-4-yl-phosphatidylethanolamine (NBD-PE) in the presence of the Cy3-DNA. In this work, we describe the use of NBD and Cy3 as fluorescent probes for monitoring lipid–DNA interactions by FRET. In addition, we report the results of experiments utilizing this technique, to investigate the effects of incubation time, $DOTAP^+/DNA^-$ charge ratio, and ionic strength on the stability of lipid–DNA interactions. Finally, we assess the effects of incubation time and $DOTAP^+/DNA^-$ charge ratio on particle size and in vitro cell transfection efficiency in an effort to elucidate the role of lipid–DNA interactions in biological activity.

2. Materials and methods

2.1. Plasmid DNA and lipids

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). Luciferase plasmid DNA is a kindly gift from Valentis 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 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 bought from Promega (Madison, WI).

2.2. Preparation of labeled liposomes and DNA

DOTAP, combined with DOPE or cholesterol (1:1 mole ratio), was mixed in chloroform with NBD-PE, to achieve a final concentration of 2.6 mol% labeled lipid. The lipid mixture was dried under a stream of nitrogen gas and placed under vacuum (10 mTorr) for 2 h to remove residual chloroform, and dried lipids were subsequently resuspended in autoclaved, distilled water. Cationic liposomes containing NBD-PE were prepared the day before the experiment, stored overnight at 4 °C, and sonicated immediately before use. DNA labeling with Cy3 was carried out following the protocol provided by the manufacturer. 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 the Cy3 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. At these concentrations of fluorescent probes, we do not observe self-quenching of the NBD-lipid or the Cy3-DNA, consistent with previous studies [36] and manufacturer's information (Mirus). Furthermore, the absence of any effects due to the labeling density and/or lipoplex concentration used in our experiments was demonstrated in separate experiments where virtually identical time- and charge ratio-dependent trends were observed despite either a 50% reduction in labeled lipid concentration or a 10-fold increase in DNA concentration (data not shown).

2.3. Preparation of lipoplexes

Lipoplexes with different $DOTAP^+/DNA^-$ charge ratios were prepared by adding different volumes of our stock lipid suspension (50 μg/ml DOTAP in addition to helper lipid in a 1:1 molar ratio) into 10 μl 16.7 μg/ml DNA solution. The suspension was subsequently diluted in 2.5 mM Tris–HCl buffer (pH 7.8) to reach a final volume of 500 μl. The final DNA concentration in the lipoplexes was held constant at 0.334 μg/ml (1 μM DNA phosphate) in all of our experiments.

2.4. FRET assay

FRET was determined by monitoring the decrease in fluorescence of NBD-PE (donor) in the presence of the Cy3-DNA (acceptor) on an Aviv automated titrating differential/ratio spectrofluorometer (model ATF105). Data are 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) in the presence of Cy3-DNA and unlabeled DNA, respectively.

To minimize potential problems associated with aggregation that could complicate interpretation of our data, experiments were performed at low DNA concentrations (0.334 μg/ml).

2.5. In vitro transfection assay

African green monkey kidney cells (COS-7: ATCC No. CRL1651) were obtained from American Type Culture Collection (Rockville, MD). Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 50 U/ml penicillin G, and 50 g/ml streptomycin sulfate, and were propagated by reseeding at $1-3\times10^5$ cells/100-mm dish every 2–3 days. For in vitro transfection, cultures were freshly seeded at 2500 cells/well in 96-well plate 24 h before transfection. Lipoplexes (20 μl) containing 0.2 μg DNA were incubated at room temperature for 1 min, 1 and 5 h before applying to wells containing freshly washed (twice with phosphate-buffered saline, PBS) COS-7 cells in 80 μl serum-free, antibiotic-free DMEM. The cells were incubated with lipoplexes for 4 h before the medium was replaced with 100 μl DMEM containing serum and antibiotics as previously described [37]. Forty hours after transfection, the culture medium was discarded, and the cells were washed twice with 100 μl PBS and then lysed with 20 μl of lysis buffer (Promega). A single freeze–thaw was performed to ensure complete lysis. Twenty microliters of cell lysis solution were used to assay for luciferase expression using the luciferase assay kit (Promega), according to the manufacturer's protocol. The signal was quantified using a TD-20e Luminometer (Turner Designs, Inc., Mountain View, CA).

2.6. Dynamic light scattering analysis

Samples (20 μl) containing 0.5 μg plasmid and corresponding liposomes were prepared with equal volumes of DNA and cationic lipids and incubated at room temperature for 1 min, 1 and 5 h before diluting to 500 μl with 2.5 mM Tris buffer. Diluted samples were transferred to a cuvette for dynamic light scattering analysis on a Nicomp 370 Submicron Particle Sizer (Particle Sizing Systems, Santa Barbara, CA). Channel width was set automatically based on the rate of fluctuation of scattered light intensity. The data were volume-weighted, and the analysis assumed that lipoplexes are solid particles [38].

3. Results

3.1. FRET experimental conditions

Preliminary studies attempted to utilize NBD-labeled liposomes and carboxy-rhodamine-labeled DNA to monitor lipid–DNA interactions by FRET. In our hands, an increase in carboxy-rhodamine emission was readily observed by exciting NBD-labeled lipid, indicating that FRET had occurred between the two probes. However, no corresponding decrease in the NBD emission was observed, as would be expected if energy is transferred from the NBD-labeled liposome to the rhodamine-labeled DNA. A search for other fluorescent probes that exhibited FRET as indicated by both a decrease in donor probe emission and a corresponding increase in acceptor probe emission, revealed that NBD-labeled liposomes combined with Cy3-labeled DNA satisfied this criterion. To our knowledge, the use of this pair of fluorescent probes to monitor FRET has not been previously reported.

The excitation and emission spectra of NBD-PE and Cy3-DNA are illustrated in Fig. 1. The donor probe, NBD, has an excitation maximum at 463 nm and an emission peak at 528 nm. The acceptor probe, Cy3, emits at 565 nm with an excitation maximum near 545 nm. As shown in Fig. 1, there is more than 90% overlap between the excitation spectrum of Cy3 and the emission spectrum of NBD. This overlap allows FRET to occur when the two probes are separated by a distance comparable to the Förster distance (approximately 2–9 nm).

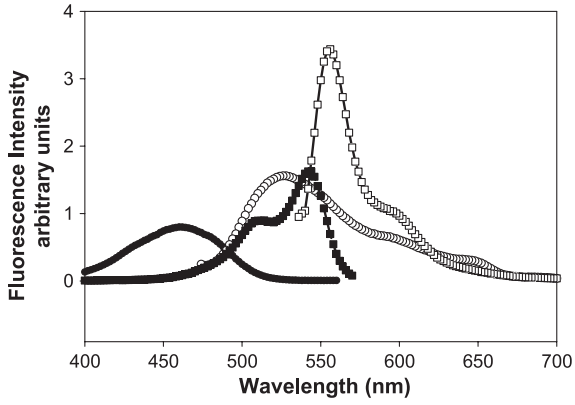

Fig. 1. The fluorescence spectra of NBD and Cy3 in 2.5 mM Tris–HCl buffer (pH 7.8): excitation spectrum of NBD-PE (closed circle); emission spectrum of NBD-PE (open circle); excitation spectrum of Cy3-DNA (closed square); emission spectrum of Cy3-DNA (open square).

Fig. 2 shows the decrease in fluorescence emission of NBD-labeled liposomes and the corresponding increase in fluorescence emission of Cy3-labeled DNA, indicating that resonance energy is successfully transferred from donor to acceptor. In monitoring the Cy3-DNA emission maximum from lipoplexes containing both Cy3-DNA and NBD-liposomes, a slight red shift of approximately 6 nm is observed (Fig. 3A). In contrast, the emission maximum of NBD-PE-lipid exhibits only a decrease in the fluorescence intensity, with no shift of the peak (Fig. 3B). In experiments utilizing FRET to assess lipid–DNA interactions, the FRET efficiency was monitored by following the decrease in NBD-PE emission maximum as demonstrated in Fig. 3B. In the calculation of FRET efficiency, previous researchers [30,32] appear to have used a value for F_D that is the measured fluorescence intensity of the donor-labeled molecule (in our case, NBD-PE) in the absence of any acceptor-labeled molecule (in our case, Cy3-DNA). Considering that the microenvironment of the probes can significantly affect their fluorescent properties, this approach assumes that the microenvironment of the donor probe is unaltered by complex formation. While this assumption may be valid for certain conditions, our results with the lipid–DNA system indicate that the fluorescence of NBD-lipids is altered by complexation (Fig. 2). For example, the fluorescence emission of NBD-PE in DOTAP-cholesterol liposomes at 528 nm shows no change when complexed with Cy3-DNA, suggesting no energy transfer between NBD-PE:DOTAP-cholesterol and Cy3-DNA. However, an increase in Cy3-DNA emission is observed in the same condition, which indicates that energy transfer between NBD-lipid and Cy3-DNA has occurred (Fig. 2B). This discrepancy can be explained by a change in the fluorescent probe's environment that causes higher fluorescence emission of NBD-PE:DOTAP-cholesterol after complexation with DNA. Thus, FRET efficiency calculations are most accurate when NBD-PE:DOTAP-cholesterol complexed with unlabeled DNA is used as the value for F_D. Similarly, complexation with unlabeled DNA increases the intensity of NBD-PE in DOTAP-DOPE liposomes, but the increase is not as great as that observed with

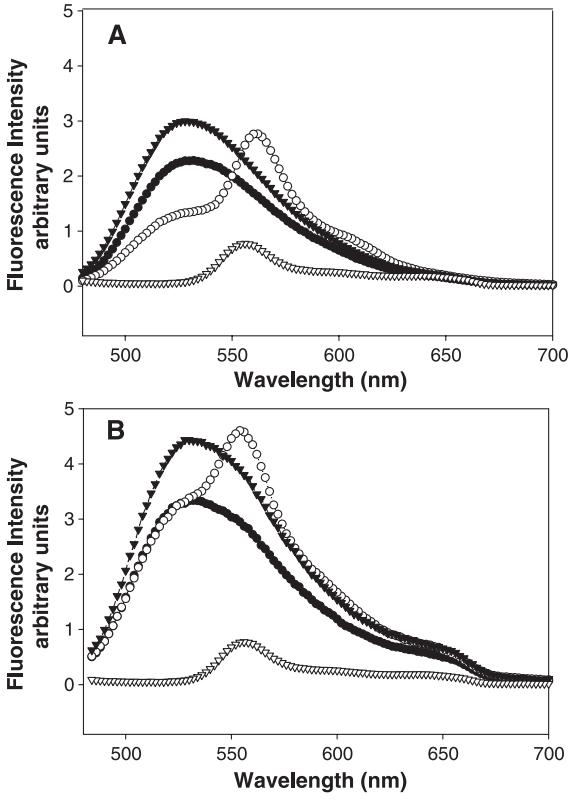

Fig. 2. (A) Fluorescence emission spectra of NBD-PE:DOTAP-DOPE, Cy3-DNA and the lipoplexes (NBD-PE:DOTAP-DOPE in the presence of Cy3-DNA and unlabeled DNA, respectively) in 2.5 mM Tris–HCl buffer (pH 7.8): Lipoplex 1 (NBD-PE:DOTAP-DOPE and Cy3-DNA mixture, open circle); NBD-PE:DOTAP-DOPE alone (closed circle); Lipoplex 2 (NBD-PE:DOTAP-DOPE and unlabeled DNA mixture, closed triangle); Cy3-DNA alone (open triangle); (B) Fluorescence emission spectra of NBD-PE:DOTAP-cholesterol, Cy3-DNA and the lipoplexes (NBD-PE:DOTAP-cholesterol in the presence of Cy3-DNA and unlabeled DNA, respectively) in 2.5 mM Tris–HCl buffer (pH 7.8): Lipoplex 3 (NBD-PE:DOTAP-cholesterol and Cy3-DNA mixture, open circle); NBD-PE:DOTAP-cholesterol alone (closed circle); Lipoplex 4 (NBD-PE:DOTAP-cholesterol and unlabeled DNA mixture, closed triangle); Cy3-DNA alone (open triangle). All lipoplexes were prepared at a $DOTAP^+/DNA^-$ charge ratio of 1.

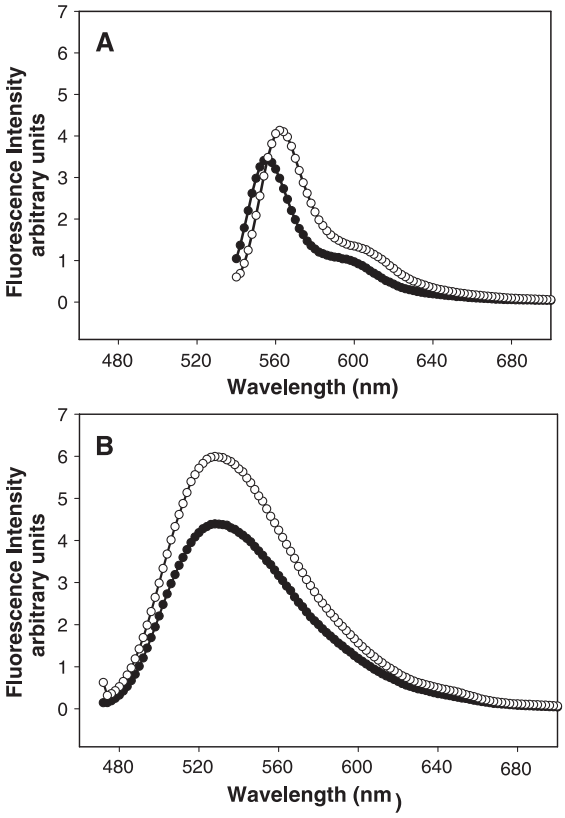

Fig. 3. (A) Emission spectra of Cy3-DNA (closed circle), and lipoplex of NBD-PE:DOTAP-DOPE and Cy3-DNA mixture (open circle) excited at 528 nm; (B) Emission spectra of NBD-PE:DOTAP-DOPE (closed circle), and lipoplex of NBD-PE:DOTAP-DOPE and unlabeled DNA mixture (open circle) excited at 463 nm. Lipoplexes were prepared at a $DOTAP^+/DNA^-$ charge ratio of 1.

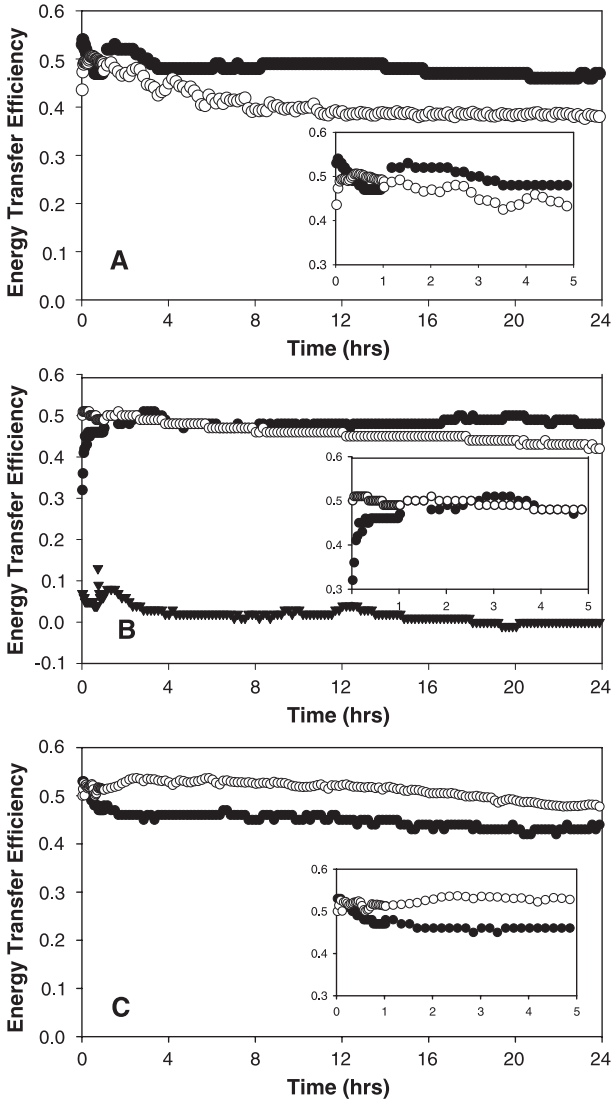

Fig. 4. The dynamic change of FRET efficiency between NBD-lipids and Cy3-DNA at DOTAP^{+}/DNA^{-} charge ratio 0.5 (panel A), 1 (panel B) and 2 (panel C). DOTAP-DOPE (closed circle); DOTAP-cholesterol (open circle) and POPC (triangle). Insets: expanded *X*-axis of the first 5 h.

cholesterol-based liposomes (Fig. 2A). These different effects are consistent with previous studies demonstrating that the choice of helper lipid contributes to lipoplex structure [16]. To account for structural changes that might alter fluorescence, NBD-labeled liposomes complexed with unlabeled DNA were utilized to determine the value for F_D used to calculate FRET.

In the scenario depicted above, any changes in fluorescence intensity resulting from complexation should be fully reversible upon dissociation of the lipoplexes. In order to test for reversibility, 0.05 M sodium dodecylsulfate (SDS), which has been shown to dissociate cationic lipids from DNA [37], was added to NBD-PE:DOTAP-DOPE/Cy3-DNA lipoplexes, and NBD-PE:DOTAP-DOPE liposomes. After boiling for 5 min, the fluorescence emission intensities of samples were measured and compared to each other. The results showed that the fluorescence intensity at 528 nm was not significantly different in these samples, indicating that alterations in fluorescence upon complexation are fully reversible upon dissociation of the lipoplexes (data not shown). The dissociation of lipoplexes by SDS was verified by agarose gel electrophoresis (data not shown).

3.2. Time course of complexation

DOTAP-DOPE/DNA and DOTAP-cholesterol/DNA lipoplexes were prepared at DOTAP^{+}/DNA^{-} ratios of 0.5, 1, and 2, and FRET efficiency was followed for 24 h to investigate the kinetics of the complexation process. As shown in Fig. 4, FRET measurements indicate that DNA and cationic liposomes interact immediately after mixing (within 1 min). However, the fluorescent signal does not stabilize for 1–5 h. More specifically, FRET efficiency did not stabilize for approximately 5, 4, and 1 h(s) in lipoplexes prepared at charge ratios of 0.5, 1, and 2, respectively (Fig. 4). These findings suggest that higher concentrations of cationic liposomes (relative to DNA) stabilize interactions within the lipoplexes, and shorten the time necessary to achieve a stable structure. Furthermore, in all three charge ratios tested, the most significant changes happened within

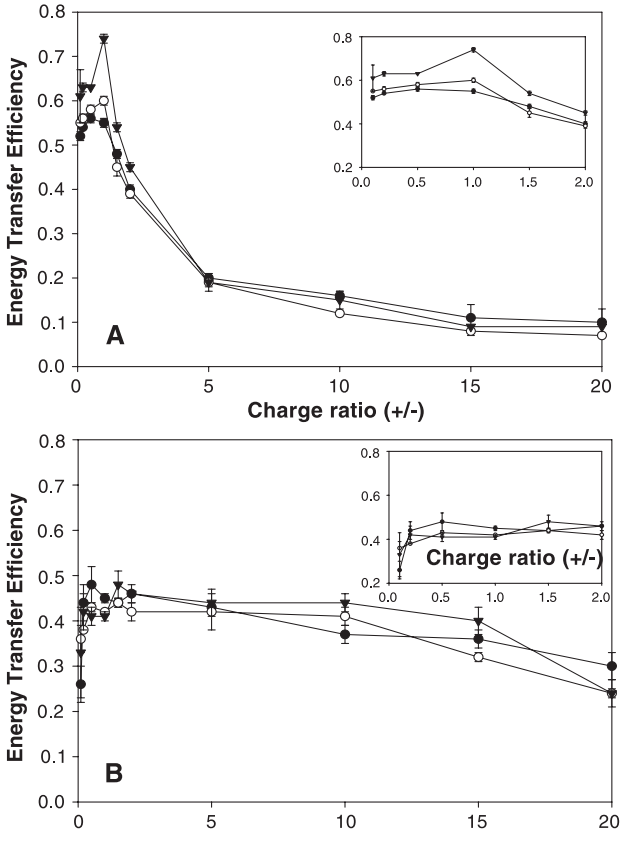

Fig. 5. Effect of DOTAP^{+}/DNA^{-} ratio on the FRET efficiency between NBD-lipids and Cy3-DNA after a 1 min (closed circle), 1 h (open circle) and 5 h (triangle) incubation. (A) DOTAP-DOPE; (B) DOTAP-cholesterol. Insets: expanded *X*-axis from charge ratio 0.1 to 2.

the first hour (Fig. 4, inserts). In contrast to cationic liposomes, there was essentially no FRET between DNA and neutral liposomes composed of POPC (Fig. 4B), consistent with previous studies demonstrating that electrostatic interactions are responsible for lipoplex formation [20,21].

3.3. The effect of charge ratio on FRET

Another parameter that is commonly varied in transfection studies is the $DOTAP^+/DNA^-$ charge ratio. To investigate the effects of this parameter on lipid–DNA interactions, FRET efficiency was monitored at charge ratios from 0.1 to 20 in lipoplexes. Considering that interactions within the lipoplex vary over the first 5 h (Fig. 4), FRET efficiency of lipoplexes was monitored at three time points: 1 min, 1 and 5 h after mixing. In DOTAP-DOPE/DNA lipoplexes, FRET efficiency is maximized at an $DOTAP^+/DNA^-$ ratio of approximately 1 (Fig. 5A). Although incubation time of DOPE-based complexes had little effect on FRET efficiencies at different charge ratios, samples given a 5-h incubation exhibit the highest FRET efficiency (0.72 at $DOTAP^+/DNA^-=1$). FRET efficiency declines progressively in each of these lipoplexes above neutrality, consistent with a binding saturation at the DNA–lipid interface followed by a decreasing fraction of the lipids having access to DNA (Fig. 5A). In contrast, maximum FRET efficiency (≈ 0.4) is achieved at a charge ratio of 0.2 in DOTAP-cholesterol/DNA lipoplexes and maintained at this level up to a charge

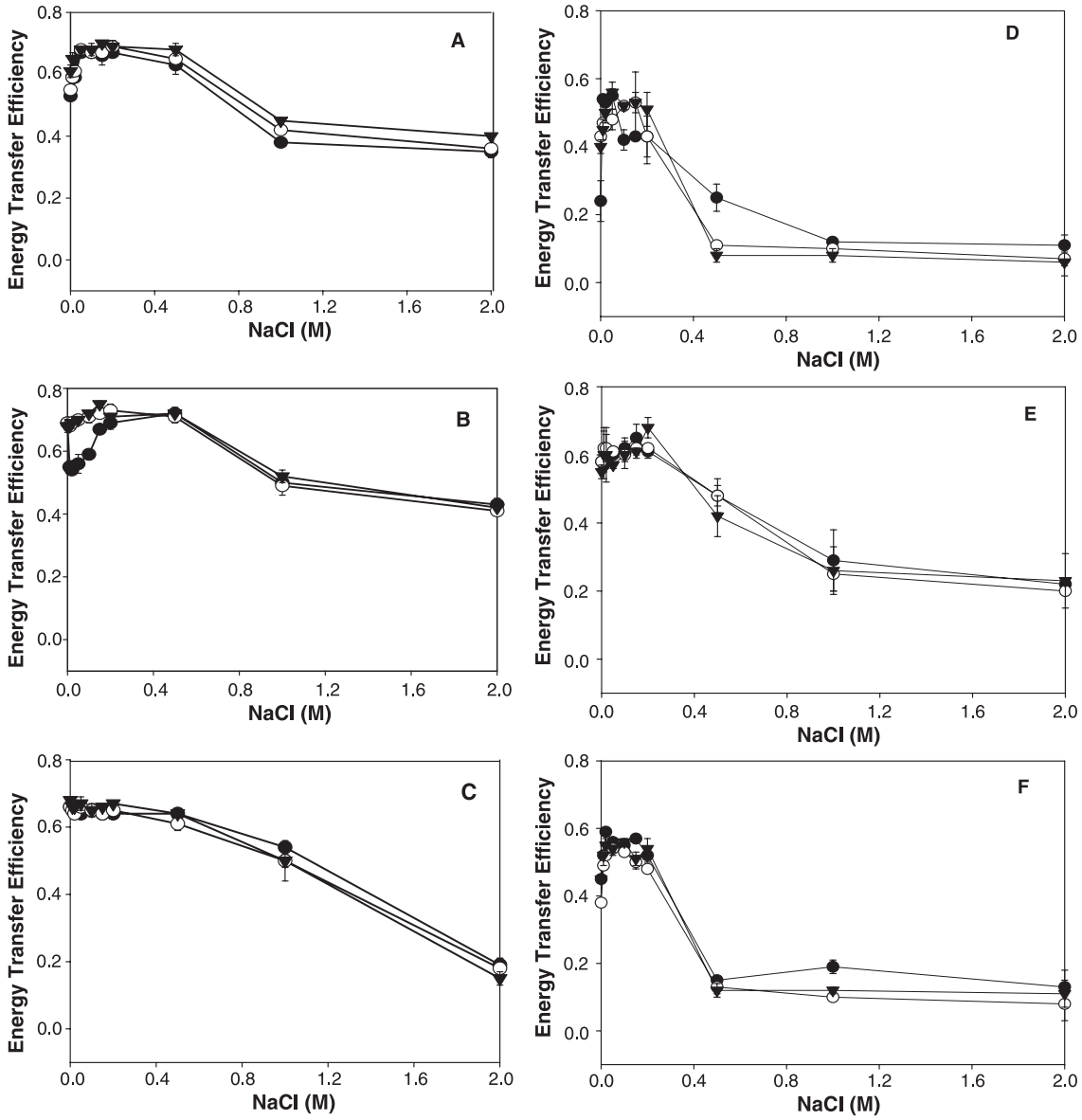

Fig. 6. Effect of NaCl concentration on the FRET efficiency of lipoplexes at $DOTAP^+/DNA^-$ ratios of 0.5 (A, D), 1 (B, E) and 2 (C, F) after a 1 min (closed circle), 1 h (open circle) and 5 h (triangle) incubation. A–C, DOTAP-DOPE; D–F, DOTAP-cholesterol.

ratio of 15 (Fig. 5B). These results demonstrate that different behaviors are observed for lipoplexes containing cholesterol as compared to DOPE.

3.4. Ionic strength effects on lipid–DNA interactions

Salt has been implicated as a contributing factor in facilitating lipoplex dissociation, and there are conflicting reports in the literature regarding the effect of salt on lipoplex stability [22,39,40]. In fact, high salt (2 M) has been used to dissociate nonviral vectors prior to analysis of DNA tertiary structure [41,42]. To investigate the effect of increasing NaCl concentrations on lipid–DNA interactions, FRET efficiency was monitored using lipoplexes prepared at three different $DOTAP^+/DNA^-$ ratios (0.5, 1, 2) and incubated in NaCl solutions ranging in concentration from 0 to 2 M (Fig. 6). As in the previous experiments, three different incubation times (1 min, 1 and 5 h) were used to study both DOPE- and cholesterol-based lipoplexes. It is important to note that lipoplexes are originally prepared as suspensions lacking NaCl, incubated for 1 min, 1 or 5 h, and then incubated in appropriate salt solutions for an additional h prior to FRET analysis. Thus, these experiments are designed to assess the ability of salt to dissociate lipoplexes that are formed in the absence of NaCl. For DOTAP-DOPE/DNA lipoplexes at $DOTAP^+/DNA^-$ ratios of 0.5 and 1, low concentrations of NaCl (0.01–0.5 M) increase the FRET efficiency, while higher concentrations decrease FRET efficiency, suggesting that low NaCl concentrations enhance lipid–DNA interactions, and high salt concentrations weaken the association between lipid and DNA (Fig. 6A,B). However, even at 2 M NaCl, FRET efficiency remains at approximately 0.4, indicating that although some dissociation occurs, complexes are still largely intact. In contrast, low salt has little effect at high charge ratios, although high NaCl concentrations have a greater ability to dissociate lipoplexes under these conditions (Fig. 6C). With the exception of neutral lipoplexes at low salt, incubation time had little effect on the ability of salt to dissociate DOPE-based complexes. Curiously, at charge ratio of 0.5, a slightly greater resistance to salt was observed with longer incubation times, but this trend is not evident at higher charge ratios (Fig. 6A–C).

For DOTAP-cholesterol/DNA lipoplexes (Fig. 6D–F), low NaCl concentrations (0.01–0.2 M) increase FRET efficiency, and higher concentrations (≥0.5 M) decrease FRET efficiency; similar to that observed with DOPE-based complexes. However, incubation time has clear effects on FRET efficiency for lipoplexes prepared at $DOTAP^+/DNA^-$ ratio of 0.5 incubated at low NaCl concentration (<0.5 M). FRET efficiency at high salt is lower in cholesterol-based lipoplexes than that in DOPE-based lipoplexes, suggesting that the choice of helper lipid can have effects on disassembly in addition to lipoplex formation.

3.5. Incubation time and charge ratio on in vitro transfection efficiency

Our FRET data have shown that incubation time and charge ratio have effects on the interaction between DNA and cationic lipids. We also conducted experiments to determine whether incubation time affected in vitro transfection in COS-7 cells. Our results indicate that incubation time plays an important role in transfection by DOPE-based lipoplexes (Fig. 7). The high variability in luciferase expression observed in samples prepared with a 1-min incubation is consistent with the instability of lipoplexes during the initial period of complexation. The in vitro luciferase expression increases several fold after a 1-h incubation as compared to a 1-min incubation at all $DOTAP^+/DNA^-$ ratios (Fig. 7). Five-hour incubations produced lipoplexes with more consistent, but lower transfection efficiencies (Fig. 4). For cholesterol-based lipoplexes, no distinct differences in transfection efficiency were found among lipoplexes prepared with three incubation periods, but the very low luciferase expression for lipoplexes at charge ratios of 0.5 and 1 makes it difficult to observe changes in transfection efficiency under these con-

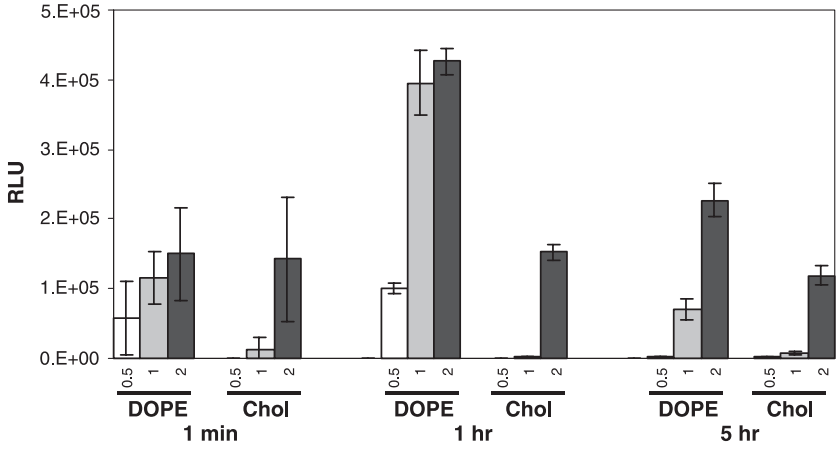

Fig. 7. Effect of incubation time and charge ratio on in vitro transfection efficiency of DOTAP-DOPE and DOTAP-cholesterol lipoplexes. $DOTAP^+/DNA^-$ ratios of 0.5 (white bars), 1 (gray bars), and 2 (black bars).

ditions. In contrast, cholesterol-based lipoplexes prepared at charge ratio 2 exhibited relatively constant transfection efficiencies, consistent with the minor changes in FRET observed at different incubation times (Fig. 4C). Taken together, the FRET efficiency and in vitro transfection efficiency data suggest that incubation time plays an important role in lipid–DNA interactions that is reflected by changes in transfection efficiency.

3.6. Incubation time and charge ratio on particle size

It is well known that lipoplex size is a major determinant of in vitro transfection efficiency [43,44]. To test if the variation in transfection was a result of complex aggregation, particle size was measured for lipoplexes prepared at different $DOTAP^+/DNA^-$ ratios (0.5, 1 and 2) and incubation times (1 min, 1 and 5 h). The results showed that lipoplexes prepared with a 1-min incubation had larger particle sizes as compared to those with 1- or 5-h incubations, with the exception of cholesterol-based lipoplexes at charge ratio 2. After a 1-h incubation, lipoplex size appeared to stabilize, and only minor differences were seen after a 5-h incubation. Taken together, these measurements indicate that the higher transfection efficiency observed with lipoplexes incubated for 1 h is not due to changes in particle size. Instead, we suggest that subtle rearrangements in the lipid–DNA interactions, as reflected in the FRET experiments, may be responsible for the observed differences in transfection.

4. Discussion

The lipids used in this study were modified with the NBD probe covalently attached to the PE head group. As a result, the cationic liposomes employed in this study have the fluorescent probe located at the lipid–water interface. It has been shown by Barenholz et al. [22,45] that the interaction of DNA with cationic liposomes primarily affects the region close to the lipid–water interface. Therefore, the location of the fluorophore on the head of the lipid should accurately reflect the extent of interaction between the DNA and lipids. Previous studies have shown that electrostatic interactions between cationic liposomes and DNA cause each component to dehydrate during lipoplex formation [20,22,46,47]. Dehydration likely affects the fluorescence properties of the probes, and must be considered when determining FRET. In addition, fluorescence is sensitive to the polarity of the microenvironment; the fluorescence quantum yield of NBD is low in aqueous solutions and high in hydrophobic environments [48]. Thus, alteration of the microenvironment by dehydration and/or partial burial in the acyl chain region of the membrane, might explain the enhanced fluorescence observed upon binding to unlabeled DNA (Figs. 2 and 3). Regardless of the exact mechanism responsible for the altered fluorescence properties, it is important that such studies utilize lipoplexes with unlabeled DNA, rather than liposomes alone, to calculate FRET efficiency more accurately. Under these conditions, the fluorescence intensity of NBD-PE:DOTAP-cholesterol emission in the presence of unlabeled DNA is about 1.5 times that observed in the absence of unlabeled DNA. Calculation of FRET efficiencies using the traditional labeled liposome reference would result in a value of zero for DOTAP-cholesterol/DNA complexes despite a distinct increase in the fluorescence intensity of Cy3-DNA emission which can only be explained by FRET (Fig. 2B). In comparison, NBD-PE:DOTAP-DOPE complexed with unlabeled DNA increases the fluorescence intensity of NBD emission by 32% (Fig. 2A). In this case, the traditional calculation would yield FRET efficiencies that are 14% lower than those computed when effects of complexation on the NBD microenvironment are considered. The observation that NBD fluorescence is affected more by DNA binding to DOTAP-cholesterol than DOTAP-DOPE liposomes is consistent with a greater degree of dehydration in the former case [22].

Perhaps the finding that is most relevant to gene delivery is that the association of lipid with DNA in a lipoplex is not stable immediately upon complex formation. Instead, results from FRET experiments suggest that associations within the lipoplex may require up to 5 h to stabilize. The time required for stabilization is dependent on both $DOTAP^+/DNA^-$ charge ratio and the nature of the helper lipid (Fig. 4). FRET efficiencies are clearly more stable at higher charge ratios, suggesting that the kinetics of lipid–DNA binding contribute to the observed instability. The fact that FRET efficiency fluctuates with time instead of progressively increasing indicates that structural rearrangements that alter the association between lipid and DNA continue to occur after binding is achieved (Fig. 4). It is interesting that in vitro transfection efficiency was higher with lipoplexes given a 1-h incubation as compared to samples for either 1-min or 5-h incubations (Fig. 7). Furthermore, these differences do not appear to be related to changes in particle size of the lipoplexes (Fig. 8).

Our findings are consistent with reports by Yang and Huang [27,49], who showed that increases in the $DOTAP^+/DNA^-$ charge ratio and extension of the time (up to 1 h) allowed for complexation can be used to overcome instability in media containing serum. These authors described this "maturation" process in terms of the ability of complexes to maintain transfection rates, and we suggest that our FRET measurements reflect a similar process during which structural alterations occur within the lipoplex that contribute to biological activity. Since the time allowed for complexation is not a parameter that is typically monitored closely, such time-dependent structural changes might contribute to the high variability in transfection rates that is often observed. Therefore, we suggest that variability in transfection rates might be reduced if future studies employ protocols that strictly regulate complexation time. This is particularly important in light of our results showing that transfection efficiency of lipoplexes incubated for prolonged periods (5

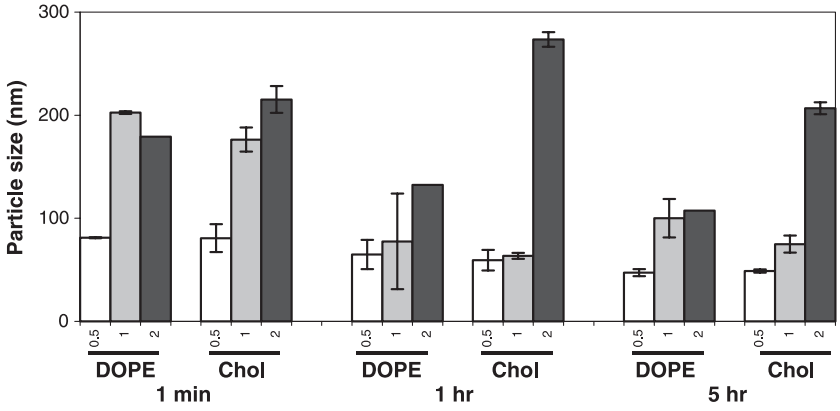

Fig. 8. Effect of incubation time and charge ratio on particle size of DOTAP-DOPE and DOTAP-cholesterol containing lipoplexes. $DOTAP^+/DNA^-$ ratios of 0.5 (white bars), 1 (gray bars), and 2 (black bars).

h) can be lower than that observed after shorter incubation times (Fig. 7).

Previous studies have also reported time-dependent changes after cationic liposomes interact with DNA; however, these reports have primarily described changes occurring within the first minute after mixing. Using time-resolved multiangle laser light scattering, Eva and van Zanten [50] demonstrated that lipoplex formation is kinetically controlled and governed by charge ratio. These authors concluded that lipoplexes eventually reach a stable state within a few minutes. Similarly, Barreleiro et al. [51] utilized a stopped-flow technique and showed that interactions happened on a millisecond time scale, and that relatively stable multilamellar structures were formed on a time scale of minutes. Although these studies employed very different experimental techniques than the FRET approach used in our study, the findings consistently demonstrate that lipoplex structure continues to evolve after the initial binding event. Thus, it seems prudent to consider complexation time as a variable that should be controlled and optimized for individual experimental conditions. Furthermore, the significant structural alterations observed in the early minutes of lipoplex formation suggest that clinical results might be altered substantially if samples were not injected immediately after complex formation [5,6].

Our data also clearly show that peak FRET efficiencies are not observed at the lowest $DOTAP^+/DNA^-$ ratios tested, as would be expected if lipid binding to DNA was strongest under conditions where DNA was in excess (Fig. 5). Instead, the results suggest that the accumulation of a given amount of lipid on the plasmid induces a structural transition that allows more intimate lipid–DNA interactions. This conclusion is bolstered by our experiments with circular dichroism (data not shown) and consistent with previous studies demonstrating alterations in DNA structure upon complex formation [47,48,52–54].

Lipoplexes are usually prepared in low ionic strength solution in order to minimize precipitation. It has been reported that precipitation caused by high ionic strength is more pronounced during lipoplex formation than in the preformed lipoplexes [55]. In this study, lipoplexes were prepared in the absence of NaCl, and then the disassembly at different ionic strengths was assessed. At low ionic strength (0.01–0.5 M), there is an increase in FRET efficiencies at $DOTAP^+/DNA^-$ ratios of 0.5 and 1 in DOTAP-DOPE lipoplexes, suggesting enhanced association of cationic liposomes with DNA under these conditions (Fig. 6A,B). These observations can be explained by the charge-shielding of DNA phosphates that is thought to enhance DNA binding to cationic liposomes at low ionic strength [56,57]. At a charge ratio of 2 in DOTAP-DOPE lipoplexes, there is no significant change in FRET efficiencies from 0.01 to 0.5 M NaCl concentration with different incubation time periods, suggesting maximal association occurs simultaneously with lipoplex formation (Fig. 6C). This effect at high $DOTAP^+/DNA^-$ might be related to more rapid "maturation" of lipoplexes under these conditions, as described by Yang and Huang [27]. These findings are also consistent with reports that reduced $DOTAP^+/DNA^-$ ratios are required for lipoplex formation at low ionic strength as compared to high ionic strength [40].

Our results with cholesterol are much different from those observed with DOPE-based complexes (Figs. 4–8). The different effects observed with lipoplexes prepared with DOPE versus cholesterol might result from the distinctly different structures that are formed when these helper lipids are employed [15,18,19,58]. For example, studies with small angle X-ray scattering studies by Koltover et al. [15] have demonstrated that mixing DNA with cationic liposomes can lead to lipoplexes with either a multilamellar structure (L_α^C) or a columnar inverted hexagonal lattice (H_{II}^C). DOPE induces the $L_\alpha^C \rightarrow H_{II}^C$ structural transition by controlling the spontaneous curvature of the lipid monolayer. In contrast to L_α^C lipoplexes, H_{II}^C lipoplexes more easily fuse with anionic vesicles and release DNA, resulting in increased transfection efficiency [16,58]. Cholesterol-based lipid formulations in lipoplexes form a rigid L_α^C structure and do not fuse as readily with lipid bilayers [18,19]. These previous reports con-

cerning the greater flexibility of DOPE- versus cholesterol-based lipid formulations might explain the higher FRET efficiencies we observed with DOPE that are consistent with a more intimate interaction between the lipid and DNA.

In summary, the time necessary for lipid–DNA interactions to stabilize is strongly dependent on the $DOTAP^{+}/DNA^{-}$ ratio. Charge ratio has less effect on interactions between DNA and cationic lipids in cholesterol-based lipoplexes than those in DOPE-based lipoplexes. The fact that transfection efficiencies were also greatly affected by incubation time (especially in DOPE-based lipoplexes) suggests that incubation time should be carefully controlled just like other experimental parameters (e.g., charge ratio) to achieve more consistent transfection. Considering that the observed changes in transfection cannot be attributed to alterations in particle size, we conclude that the fluctuations observed with FRET reflect time-dependent structural rearrangements within the lipoplex that ultimately affect transfection rates. Future studies monitoring structural rearrangements with FRET should be useful in determining formulation parameters that result in enhanced lipoplex stability in physiological fluids (e.g., serum).

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References

[1] J.H. Felgner, R. Kumar, C.N. Sirdhar, C.J. Wheeler, Y.J. Tsai, R. Border, P. Ramsey, M. Martin, P.L. Felgner, Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations, J. Biol. Chem. 269 (1994) 2550–2561.

[2] N.J. Caplen, E.W. Alton, P.G. Middleton, J.R. Dorin, B.J. Stevenson, X. Gao, S.R. Durham, P.K. Jeffery, M.E. Hodson, C. Coutelle, Liposome-mediated CFTR gene transfer to the nasal epithelium of patients with cystic fibrosis, Nat. Med. 1 (1995) 39–46.

[3] E. Fortunati, A. Bout, M.A. Zanta, D. Valerio, M. Scarpa, In vitro and in vivo gene transfer to pulmonary cells mediated by cationic liposomes, Biochim. Biophys. Acta 1278 (1996) 41–50.

[4] N. Somia, I.M. Verma, Gene therapy: trials and tribulations, Nat. Rev., Genet. 1 (2000) 91–99.

[5] G.J. Nabel, A. Chang, E.G. Nabel, G. Plautz, B.A. Fox, L. Huang, S. Shu, Immunotherapy of malignancy by in vivo gene transfer into tumors, Hum. Gene Ther. 3 (1992) 399–410.

[6] G.J. Nabel, E.G. Nabel, Z.Y. Yang, B.A. Fox, G.E. Plautz, X. Gao, L. Huang, S. Shu, D. Gordon, A.E. Chang, Direct gene transfer with DNA–liposome complexes in melanoma: expression, biologic activity, and lack of toxicity in humans, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 11307–11311.

[7] Y. Liu, L.C. Mounkes, H.D. Liggitt, C.S. Brown, I. Solodin, T.D. Heath, R.J. Debs, Factors influencing the efficiency of cationic liposome-mediated intravenous gene delivery, Nat. Biotechnol. 15 (1997) 167–173.

[8] A. Noguchi, T. Furuno, C. Kawaura, M. Nakanishi, Membrane fusion plays an important role in gene transfection mediated by cationic liposomes, FEBS Lett. 433 (1998) 169–173.

[9] P.R. Cullis, B. de Kruijff, Lipid polymorphism and the functional roles of lipids in biological membranes, Biochim. Biophys. Acta 559 (1979) 399–420.

[10] P.R. Cullis, B. de Kruijff, M.J. Hope, A.J. Verkleij, R. Nayar, S.B. Farren, C. Tilcock, T.D. Madden, M.B. Bally, Structural properties of lipids and their functional roles in biological membranes, in: R.C. Aloia (Ed.), Membrane Fluidity in Biology, vol. 1, Academic Press, New York, 1983, pp. 39–81.

[11] K.L. Hong, W.W. Zheng, A. Baker, D. Papahadjopoulos, Stabilization of cationic liposome–plasmid DNA complexes by polyamine and poly(ethylene glycol)-phospholipid conjugates for efficient in vivo gene delivery, FEBS Lett. 400 (1997) 233–237.

[12] F. Liu, H. Qi, L. Huang, D. Liu, Factors controlling the efficiency of cationic lipid-mediated transfection in vivo via intravenous administration, Gene Ther. 4 (1997) 517–523.

[13] H. Farhood, N. Serbina, L. Huang, The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer, Biochim. Biophys. Acta 1235 (1995) 289–295.

[14] I. Wrobel, D. Collins, Fusion of cationic liposomes with mammalian cells occurs after endocytosis, Biochim. Biophys. Acta 1235 (1995) 296–304.

[15] I. Koltover, T. Salditt, J.O. Rädler, C.R. Safinya, An inverted hexagonal phase of cationic liposome–DNA complexes related to DNA release and delivery, Science 281 (1998) 78–81.

[16] I.M. Hafez, N. Maurer, P.R. Cullis, On the mechanism whereby cationic lipids promote intracellular delivery of polynucleic acids, Gene Ther. 8 (2001) 1188–1196.

[17] C.P.S. Tilcock, Lipid polymorphism, Chem. Phys. Lipids 40 (1986) 109–125.

[18] N.S. Templeton, D.D. Lasic, P.M. Frederik, H.H. Strey, D.D. Roberts, G.N. Pavlakis, Improved DNA:liposome complexes for increased systemic delivery and gene expression, Nat. Biotechnol. 15 (1997) 647–652.

[19] F. Sakurai, T. Nishioka, H. Saito, T. Baba, A. Okuda, O. Matsumoto, T. Taga, F. Yamashita, Y. Takakura, M. Hashida, Interaction between DNA-cationic liposome complexes and erythrocytes is an important factor in systemic gene transfer via the intravenous route in mice: the role of the neutral helper lipid, Gene Ther. 8 (2001) 677–686.

[20] N.J. Zuidam, Y. Barenholz, Electrostatic and structural properties of complexes involving plasmid DNA and cationic lipids commonly used for gene delivery, Biochim. Biophys. Acta 1368 (1998) 115–128.

[21] V.M. Meidan, J.S. Cohen, N. Amariglio, D. Hirsch-Lerner, Y. Barenholz, Interaction of oligonucleotides with cationic lipids: the relationship between electrostatics, hydration and state of aggregation, Biochim. Biophys. Acta 1464 (2000) 251–261.

[22] D. Hirsch-Lerner, Y. Barenholz, Hydration of lipoplexes commonly used in gene delivery: follow-up by laurdan fluorescence changes and quantification by differential scanning calorimetry, Biochim. Biophys. Acta 1461 (1999) 47–57.

[23] N.J. Zuidam, Y. Barenholz, A. Minsky, Chiral DNA packaging in DNA-cationic liposome assemblies, FEBS Lett. 457 (1999) 419–422.

[24] H. Gershon, R. Ghirlando, S.B. Guttman, A. Minsky, Mode of formation and structural features of DNA-cationic liposome complexes used for transfection, Biochemistry 32 (1993) 7143–7151.

[25] T. Stegmann, J.Y. Legendre, Gene transfer mediated by cationic lipids: lack of correlation between lipid mixing and transfection, Biochim. Biophys. Acta 1325 (1997) 71–79.

[26] A.R. Thierry, P. Rabinovich, B. Peng, L.C. Mahan, J.L. Bryant, R.C. Gallo, Characterization of liposome-mediated gene delivery: expression, stability and pharmacokinetics of plasmid DNA, Gene Ther. 4 (1997) 226–237.

[27] J.P. Yang, L. Huang, Time-dependent maturation of cationic liposome–DNA complex for serum resistance, Gene Ther. 5 (1998) 380–387.

[28] S. Li, W.-C. Tseng, D.B. Stolz, S.-P. Wu, S.C. Watkins, L. Huang,

Dynamic changes in the characteristics of cationic lipidic vectors after exposure to mouse serum: implications for intravenous lipofection, Gene Ther. 6 (1999) 585–594.
[29] S.D. Allison, M.D.C. Molina, T.J. Anchordoquy, Stabilization of lipid/DNA complexes during the freezing step of lyophilization process: the particle isolation hypothesis, Biochim. Biophys. Acta 1468 (2000) 127–138.
[30] A. Kõiv, P.K.J. Kinnunen, Binding of DNA to liposomes containing different derivatives of sphingosine, Chem. Phys. Lipids 72 (1994) 77–86.
[31] A. Kõiv, J. Palvimo, P.K.J. Kinnunen, Evidence for ternary complex formation by histone H1, DNA, and liposomes, Biochemistry 34 (1995) 8018–8027.
[32] O. Zelphati, F.C. Szoka, Mechanism of oligonucleotide release from cationic liposomes, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 11493–11498.
[33] M. Subramanian, J.M. Holopainen, T. Paukku, O. Eriksson, I. Huhtaniemi, P.K.J. Kinnunen, Characterisation of three novel cationic lipids as liposomal complexes with DNA, Biochim. Biophys. Acta 1466 (2000) 289–305.
[34] C.M. Wiethoff, M.L. Gill, G.S. Koe, J.G. Koe, C.R. Middaugh, The structural organization of cationic lipid–DNA complexes, J. Biol. Chem. 277 (2002) 44980–44987.
[35] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, 2nd ed., Kluwer Academic/Plenum Publishers, New York, 1999.
[36] D. Hoekstra, Fluorescence method for measuring the kinetics of Ca2+-induced phase separations in phosphatidylserine-containing lipid vesicles, Biochemistry 21 (1982) 1055–1061.
[37] T.J. Anchordoquy, J.F. Carpenter, D.J. Kroll, Maintenance of transfection rates and physical characterization of lipid/DNA complexes after freeze-drying and rehydration, Biochim. Biophys. Acta 348 (1997) 199–206.
[38] M.C. Molina, S.D. Allison, T.J. Anchordoquy, Maintenance of nonviral vector particle size during the freezing step of the lyophilization process is insufficient for preservation of activity: insight from other structural indicators, J. Pharm. Sci. 90 (2000) 1445–1455.
[39] S.J. Eastman, C. Siegel, J. Tousignant, A.E. Smith, S.H. Cheng, R.K. Scheule, Biophysical characterization of cationic lipid:DNA complexes, Biochim. Biophys. Acta 1325 (1997) 41–62.
[40] M.T. Kennedy, E.V. Pozharski, V.A. Rakhmanova, R.C. MacDonald, Factors governing the assembly of cationic phospholipid–DNA complexes, Biophys. J. 78 (2000) 1620–1633.
[41] C. Plank, M.X. Tang, A.R. Wolfe, F.C. Szoka Jr., Branched cationic peptides for gene delivery: role of type and number of cationic residues in formation and in vitro activity of DNA polyplexes, Hum. Gene Ther. 10 (1999) 319–332.
[42] D.V. Schaffer, N.A. Fidelman, N. Dan, D.A. Lauffenburger, Vector unpacking as a potential barrier for receptor-mediated polyplex gene delivery, Biotechnol. Bioeng. 67 (2000) 598–606.
[43] P.C. Ross, S.W. Hui, Lipoplex size is a major determinant of in vitro lipofection efficiency, Gene Ther. 6 (1999) 651–659.
[44] D.R. Staggs, D.W. Burton, L.J. Deftos, Importance of liposome complexing volume in transfection optimization, BioTechniques 21 (1996) 792–795.
[45] D. Hirsch-Lerner, Y. Barenholz, Probing DNA–cationic lipid interactions with the fluorophore trimethylammonium diphenyl-hexatriene (TMADPH), Biochim. Biophys. Acta 1370 (1998) 17–30.
[46] S. Choosakoonkriang, C.M. Wiethoff, T.J. Anchordoquy, G.S. Koe, J.G. Smith, C.R. Middaugh, Infrared spectroscopic characterization of interaction of cationic lipids with plasmid DNA, J. Biol. Chem. 276 (2001) 8037–8043.
[47] S. Choosakoonkriang, C.M. Wiethoff, L.A. Kueltzo, C.R. Middaugh, Characterization of synthetic gene delivery vectors by infrared spectroscopy, in: M.A. Findeis (Ed.), Nonviral Vectors for Gene Therapy, Humana Press, Totowa, NJ, 2001, pp. 285–318.
[48] K. Rajarathnam, J. Hochman, M. Schindler, M.S. Ferguson, Synthesis, location, and lateral mobility of fluorescently labeled ubiquinone 10 in mitochondrial and artificial membranes, Biochemistry 28 (1989) 3168–3176.
[49] J.P. Yang, L. Huang, Overcoming the inhibitory effect of serum on lipofection by increasing the charge ratio of cationic liposome to DNA, Gene Ther. 4 (1997) 950–960.
[50] E. Lai, J.H. van Zanten, Real time monitoring of lipoplex molar mass, size and density, J. Control. Release 82 (2002) 149–158.
[51] P.C. Barreleiro, R.P. May, B. Lindman, Mechanism of formation of DNA–cationic vesicle complexes, Faraday Discuss. 122 (2002) 191–201.
[52] T. Akao, T. Fukumoto, H. Ihara, A. Ito, Conformational change in DNA induced by cationic bilayer membranes, FEBS Lett. 391 (1996) 215–218.
[53] N.J. Zuidam, Y. Barenholz, Characterization of DNA–lipid complexes commonly used for gene delivery, Int. J. Pharm. 183 (1999) 43–46.
[54] C.S. Braun, L.A. Kueltzo, C.R. Middaugh, Ultraviolet absorption and circular dichroism spectroscopy of nonviral gene delivery systems, in: M.A. Findeis (Ed.), Nonviral Vectors for Gene Therapy, Humana Press, Totowa, NJ, 2001, pp. 253–284.
[55] M.C. Pedroso de Lima, S. Simões, P. Pires, H. Faneca, N. Düzgünes, Cationic lipid–DNA complexes in gene delivery: from biophysics to biological applications, Adv. Drug Deliv. 47 (2001) 277–294.
[56] N. Dan, Formation of ordered domains in membrane-bound DNA, Biophys. J. 71 (1996) 1267–1272.
[57] N. Dan, Multilamellar structures of DNA complexes with cationic liposomes, Biophys. J. 73 (1997) 1842–1846.
[58] A.L. Bailey, P.R. Cullis, Membrane fusion with cationic liposomes: effects of target membrane lipid composition, Biochemistry 36 (1997) 1628–1634.