

Effect of “helper lipid” on lipoplex electrostatics

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Received 10 May 2004; received in revised form 24 March 2005; accepted 15 April 2005

Available online 11 May 2005

Abstract

Lipoplexes, which are complexes between cationic liposomes (L^+) and nucleic acids, are commonly used as a nucleic acid delivery system in vitro and in vivo. This study aimed to better characterize cationic liposome and lipoplex electrostatics, which seems to play a major role in the formation and the performance of lipoplexes in vitro and in vivo. We characterized lipoplexes based on two commonly used monocationic lipids, DOTAP and DMRIE, and one polycationic lipid, DOSPA—each with and without helper lipid (cholesterol or DOPE). Electrical surface potential (Ψ_0) and surface pH were determined using several surface pH-sensitive fluorophores attached either to a one-chain lipid (4-heptadecyl hydroxycoumarin (C17HC)) or to the primary amino group of the two-chain lipids (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-carboxyfluorescein (CFPE) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-7-hydroxycoumarin) (HC-DOPE). Zeta potentials of the DOTAP-based cationic liposomes and lipoplexes were compared with Ψ_0 determined using C17HC. The location and relatively low sensitivity of fluorescein to pH changes explains why CFPE is the least efficient in quantifying the differences between the various cationic liposomes and lipoplexes used in this study. The fact that, for all cationic liposomes studied, those containing DOPE as helper lipid have the least positive Ψ_0 indicates neutralization of the cationic charge by the negatively-charged phosphodiester of the DOPE. Zeta potential is much less positively charged than Ψ_0 determined by C17HC. The electrostatics affects size changes that occurred to the cationic liposomes upon lipoplex formation. The largest size increase (based on static light scattering measurements) for all formulations occurred at DNA[−]/L⁺ charge ratios 0.5–1. Comparing the use of the one-chain C17HC and the two-chain HC-DOPE for monitoring lipoplex electrostatics reveals that both are suitable, as long as there is no serum (or other lipidic assemblies) present in the medium; in the latter case, only the two-chain HC-DOPE gives reliable results. Increasing NaCl concentrations decrease surface potential. Neutralization by DNA is reduced in a NaCl-concentration-dependent manner.

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Keywords: Cationic lipid; DNA; Surface potential; Hydroxycoumarin; Carboxyfluorescein; Ionic strength

Abbreviations: Chol, cholesterol; CFPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(carboxyfluorescein); C17HC, 4-heptadecyl-7-hydroxycoumarin; DC-Chol, 3β-[*N*-(*N*',*N*'-dimethyl-aminoethane)-carbonyl]-cholesterol; DMRIE, *N*-(1-(2,3-dimyristyloxypropyl)-*N*,*N*-dimethyl-(2-hydroxyethyl) ammonium bromide; DNA[−]/L⁺, mole charge ratio of DNA negatively-charged phosphate to positively-charged lipid; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOSPA, 2,3-dioleoyloxy-*N*-[2(sperminecarboxamido)-ethyl]-*N*,*N*-dimethyl-1-propanaminium trifluoro acetate; DOTAP, *N*-(1-(2,3-dioleoyloxy)propyl)-*N*,*N*,*N*-trimethylammonium chloride; HC-DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-hydroxycoumarin); HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*'-(2-ethanesulfonic acid); L⁺, positively charged lipid; LUV, large (≥100 nm) unilamellar vesicles; ODN, oligonucleotide; TMADPH, 1-(4-trimethyl-ammoniumphenyl)-6-phenyl-1,3,5-hexatriene; UHV, unsized heterogeneous vesicles

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1. Introduction

Delivery of nucleic acids into live cells in vitro and in vivo has gained increased interest as a novel modality to obtain transgene expression or to specifically inhibit expression of a gene by either antisense oligonucleotides (ODN) or by small inhibitory RNA (siRNA). Intensive efforts have been focused on developing a well-characterized “synthetic” delivery system for nucleic acids, which is efficient and harmless, in order to find alternatives that will overcome the drawbacks of viral delivery [1]. Cationic liposomes that complex with nucleic acids (DNA, ODN, RNA) to form lipoplexes have been shown to be promising candidates [1–3], although they are still much less efficient than viral vectors [4–6]. Improving our understanding of the structure–activity relationships of lipoplexes in gene delivery in cell culture and in vivo may aid in optimizing lipoplex performance.

In many (but not all) cases, the inclusion of “helper” lipids in the cationic liposomes improves lipoplex efficiency [7]. Helper lipids affect lipoplex electrostatics as well as the way the lipid self-assembles (micellar, lamellar, hexagonal, vesicular, etc.), the level of hydration, and DNA secondary and tertiary structure [8–10]. For in vitro transfection a favorable helper lipid is 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), which enables better matching of charge density of the lipid surface to DNA helices, facilitates counterion release from the lipid surface by DNA [11,12], and decreases lipid hydration [9,13]. On the other hand, for in vivo transfection, cholesterol is a much better helper lipid [14–17]. In general, it seems that in order to obtain efficient transfection in vitro [7,18] and in vivo [2,19,20], the lipoplex should remain cationic and should have membrane defects leading to size instability [10,18].

It is now obvious that electrostatics plays a major role in lipoplex formation and in various steps of the transfection process. Lipoplexes are formed spontaneously by electrostatic neutralization. The driving force for such complex formation is the removal of small counterions (cations from the DNA surface and anions from the cationic liposome surface) [14,15]. Counterion release depends on the liposome lipid composition, i.e., the type of cationic lipid and the type of neutral (helper) lipid present in the assembly [12,18], and on the medium composition (reviewed in [2]).

The commonly used measure of electrostatics is zeta potential, which is the electrical potential at the plane of shear of the liposomes or lipoplexes, i.e., the “border” between the fixed and diffuse ion layers [21]. This plane is further away from the plane of the quaternary amino group of the cationic lipid.

Fluorescent probes, which monitor electrical surface potential [22] and are evenly distributed over all liposome lipid bilayer(s), are a better tool to look at the intimate interaction between liposome and nucleic acid, as was demonstrated with the probe 4-heptadecyl-7-hydroxycoumarin (C17HC) previously [11,12,18]. However, C17HC

suffers from a major drawback related to being a single-chain amphiphile, being desorbed and equilibrated between all lipid-based assemblies present, including cell membranes and serum and lipoproteins [22]. To overcome this obstacle, we used two additional fluorescent phospholipids containing a fluorophore in their headgroups, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(carboxyfluorescein) (CFPE) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-hydroxycoumarin) (HC-DOPE). In addition to electrostatics, we also studied lipoplex instability. This was followed by change in degree of exposure of 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMADPH)-labeled cationic liposomes to water, monitoring membrane defects [13].

2. Materials and methods

2.1. Materials

2.1.1. Lipids and fluorescent probes

DOTAP, DOPE, DOPC, and CFPE were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol (purity 99%) was obtained from Sigma (St. Louis, MO). DC-Chol was a generous gift of Dr. L. Huang (Department of Pharmacology and Pharmaceutical Science, University of Pittsburgh, Pittsburgh, PA). *N*-(1-(2,3-dimyristoyloxypropyl)-*N,N*-dimethyl-(2-hydroxyethyl) ammonium bromide (DMRIE) and 2,3-dioleoyloxy-*N*-[2(spermincarboxamido)-ethyl]-*N,N*-dimethyl-1-propanaminium trifluoro acetate (DOSPA) were synthesized by Vical Inc. (San Diego, CA). Fluorescent probes C17HC and TMADPH were purchased from Molecular Probes (Eugene, OR). HC-DOPE was synthesized as described in International Patent Publication #WO 01/59156, 8/16/2001. All lipids and fluorescent probes were ≥98% purity based on TLC analysis [23].

All others chemicals were of analytical grade or better.

2.1.2. DNA preparation

An *Escherichia coli* containing the plasmid S16 hGH (size 4.8 kbp) was kindly supplied by Dr. O. Meyuhas of our department [24]. The plasmid S16 hGH was grown and isolated using a QIAGEN Mega Plasmid Kit (QIAGEN, Hilden, Germany). After the plasmid purification, DNA was dissolved in 20 mM HEPES (pH 7.4) with 1 mM EDTA, and its concentration was quantified by organic phosphate determination [25]. This concentration is equivalent to the DNA negative charge concentration [26]. Plasmid purity was determined using agarose gel (1%) electrophoresis [27] and ratio of absorbance at 260 nm to that at 280 nm. Agarose gel (1%) electrophoresis showed that the plasmid DNA was mostly in its supercoiled form (>70%), free of chromosomal DNA or RNA. In the different DNA batches, the A_{260}/A_{280} ratios were about 1.8–1.9, indicating lack of contamination by proteins [28].

For zeta potential measurements, sonicated DNA sodium salt Type I from calf thymus was purchased from Sigma (St. Louis, MO).

2.1.3. Liposome preparation

Fluorescent-labeled large (~ 100 nm) unilamellar vesicles (LUV) were prepared by mixing *tert*-butyl alcohol (TB) solutions of the different lipids with the fluorescent probes C17HC, CFPE, HC-DOPE, or TMADPH, using the mole ratio of total lipid to fluorophore of 400 (for C17HC, HC-DOPE, and CFPE) or 200 (for TMADPH). The mole ratio of cationic lipid to neutral lipid was 1. The mixture of lipids and fluorophore in TB was freeze-dried overnight. The lyophilized cake was hydrated with 20 mM HEPES buffer (pH 7.4) to reach a final concentration of total lipid of 31 mM. Then, the unsized heterogeneous liposomes formed, UHV, (>300 nm) were downsized to form LUV as described previously [13]. Recently, our cryo-transmission electron micrographs have revealed that the heterogeneous unsized cationic liposomes obtained after lipid hydration are actually ≥ 500 nm, uni-, bi-, or oligolamellar vesicles [2]. Particle size distribution of the LUV dispersions was determined by dynamic light scattering (DLS) using a Coulter N4SD (Coulter Electronics, Hialeah, FL) [25]. All the LUV preparations used in this study have a unimodal size distribution of similar mean size (100 ± 20 nm).

2.2. Methods

2.2.1. Fluorescence measurements

2.2.1.1. pH titration. Cationic liposomes were diluted in 3 ml of 20 mM HEPES buffer to a final cationic lipid concentration of 0.04 mM, and the pH adjusted to that desired. Then the samples were sonicated for about 5 s in a water bath in order to equilibrate the intra- and extraliposomal pH. The fluorescence measurements were performed on a Perkin-Elmer LS 50B luminescence spectrometer (Norwalk, CT) using a 1-cm light path cell under stirring conditions and at room temperature.

Fluorescence of C17HC was measured as described by Zuidam and Barenholz [11]. The excitation wavelength was scanned between 300 nm and 400 nm using an emission wavelength of 450 nm (bandwidths 5 nm) and with an emission filter at 430 nm. The dissociation degree of C17HC in the liposomes was calculated by taking the maximum value of 380/330 nm as 100% C17HC dissociation. The fluorescence intensity upon excitation at 380 nm represents the charged C17HC, while the fluorescence intensity upon excitation at 330 nm represents the pH-independent, isosbestic, point of C17HC and, therefore, the total C17HC in the lipid assembly [11]. Fluorescence of HC-DOPE was determined in a way similar to that for C17HC, except that

the ratio of excitation wavelengths of 408 nm for the charged fluorophore and 370 nm for the pH-independent isosbestic point (which represents the total HC-DOPE in the lipid assembly) were used as a result of a spectral shift. Emission wavelength was 454 nm.

The fluorescence intensity of CFPE-labeled liposomes was monitored at an excitation wavelength of 507 nm and an emission wavelength of 528 nm. The dissociation degree of CFPE in the liposomes was calculated by assuming that 100% dissociation corresponds to the maximum value at the emission wavelength of 528 nm.

The same procedures were performed when the effect of NaCl was studied, except that the HEPES buffer liposome medium was replaced at the dilution stage (see above) with HEPES buffer containing NaCl at the desired concentration.

2.2.1.2. Fluorescence and static light-scattering measurements in the presence of DNA. An aliquot of C17HC- or CFPE-fluorescent-labeled liposomes was diluted in 3 ml HEPES buffer (pH 7.4) or acetate buffer (pH 5.5), respectively, to reach a final cationic lipid concentration of 0.04 mM. Then, the fluorescence intensity of the liposomes and, 5 min after addition of DNA, the fluorescence intensity of the lipoplexes were measured. The amount of DNA is expressed as the amount of DNA phosphate negative charges. In the case of CFPE-labeled liposomes, the fluorescence intensity of the fluorescent-labeled liposomes without DNA is defined as F_0 , and the fluorescence intensity in the presence of DNA is referred to as F .

The fluorescence measurements in which TMADPH is used as a fluorophore were done as previously described [13].

90° static light-scattering (SLS) measurement of the same sample was obtained on the same spectrofluorometer described above using both excitation and emission wavelength at 600 nm, at which there is neither absorbance nor fluorescence emission [12].

2.2.1.3. Comparison of the stability of association of C17HC and HC-DOPE in DOTAP/Chol liposomes in various media. DOTAP/Chol (1/1 mol ratio) UHV (~ 500 nm) containing C17HC or HC-DOPE in a mole ratio of 1 to 50 of total UHV lipids were prepared in 5% dextrose with 20 mM HEPES (pH 7.4). DOTAP, 186 nmol, was mixed with DNA (ranging from 0 to 372 nmol) and the dispersions incubated for 30 min at room temperature. Then, an aliquot of 23 μ l lipoplexes or cationic liposomes was mixed with the appropriate amount of media: human serum, high-molecular-weight components of serum (HMWC) prepared as described elsewhere [29], 0.9% NaCl, or 5% dextrose so that the final DNA concentration was 50 μ g (150 nmol DNA⁻) DNA/ml medium. The medium containing lipoplexes was incubated for another 30 min, followed by dilution with 5% dextrose in 20 mM HEPES (pH 7.4) to a final total volume of 1 ml. The fluorescent intensities of HC-DOPE

and of C17HC were monitored as described in Section 2.2.1.1 above.

The high probe-to-lipid ratio of 1/50 was used in order to overcome serum-related artifacts in fluorescence determination such as inner filter effects. Lack of artifact due to this high ratio was demonstrated by showing lack of effect of the probe on the results in the absence of serum.

2.2.2. Determination of zeta potential

Zeta potential was measured at 25 °C using a Zetasizer Nano-Z, Malvern Instruments Ltd., Malvern, UK. An aliquot of 7 µl of LUV was diluted in 600 µl of 20 mM HEPES (pH 7.4) with or without NaCl. Sonicated DNA Type 1 from calf thymus was added to the diluted liposomes. The principle of measurement and equation for calculation are described elsewhere [30].

3. Results

3.1. Electrostatic parameters of cationic liposomes

4-heptadecyl-7-hydroxycoumarin (C17HC)- and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(carboxyfluorescein) (CFPE)-labeled cationic LUV of similar mean size ($\sim 100 \pm 20$ nm) were studied. Both fluorescent probes,

C17HC and CFPE, are pH-sensitive; C17HC, but not CFPE, has a pH-insensitive isosbestic point (at the excitation wavelength of 330 nm [11]). The dissociation degree of C17HC in the liposomes is monitored by the ratio of the excitation fluorescence intensities at 380 nm/330 nm and then translated as the percent of the maximum value (Fig. 1A). Titration curves of the C17HC- and CFPE-labeled cationic liposomes are presented in Fig. 1. The sigmoid curves in Figs. 1A and B were fitted to a modified Henderson–Hasselbach equation:

$$\text{pH}_{\text{bulk}} = \text{pK}_a + A \log \left(\frac{D - D_{\min}}{D_{\max} - D} \right) + \log \left(\frac{I_a}{I_b} \right)$$

where the constant A is ideally 1 since the protonation of C17HC is a one-to-one event (normally, we found values between 0.9 and 1.2, but when C17HC was incorporated into DMRIE/DOPE (1/1) liposomes we found a value for A of 1.7); pK_a is the apparent proton binding constant of C17HC; D is the dissociation degree; D_{\min} and D_{\max} are the minimum and maximum value of D , respectively. I_a/I_b is the ratio of the fluorescence intensity of the pH-independent isosbestic point of C17HC (excitation at 330 nm) and of the unprotonated charged C17HC (excitation at 380 nm). $\log(I_a/I_b)$ is 0 at an ideal isosbestic point; we found values between -0.1 and 0.1 .

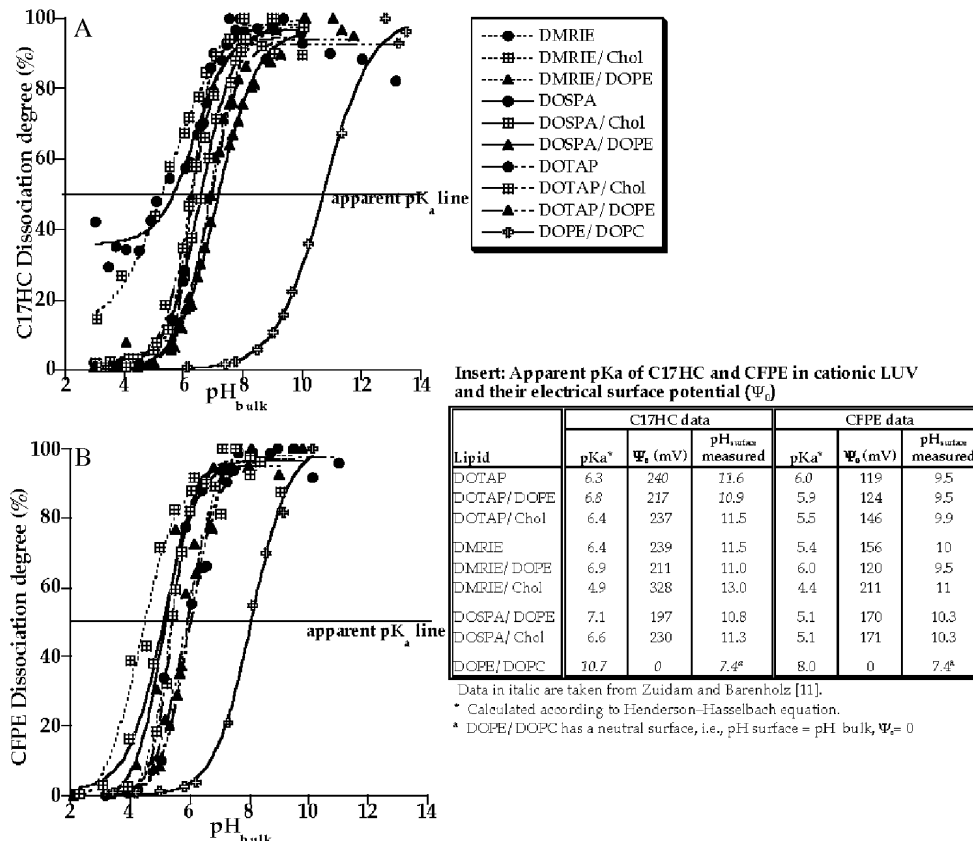


Fig. 1. Titration curves of C17HC (A) and CFPE (B) in cationic liposomes. See text. Insert: Apparent pK_a of C17HC and CFPE in cationic LUV and their electrical surface potential (Ψ_0).

The dissociation degrees of C17HC in DMRIE or DOSPA or DOTAP liposomes combined with cholesterol as helper lipid were always lower than the dissociation degrees of C17HC in the same cationic lipid alone or with DOPE as helper lipid (Fig. 1A).

The pK_a 's of C17HC in zwitterionic, uncharged DOPE/DOPC or DOPC liposomes are 10.7 and 10.5, respectively [11]. Since DOPC liposomes are neutral throughout all the pH range used in this study [31], ΔpK_{el} (which is the shift in pK_a due to change in surface potential) of C17HC in charged liposome membranes can be calculated. For this estimation, the pK_a of C17HC in DOPC liposomes is taken as a neutral reference. Then, the values of ΔpK_{el} were used to calculate ψ_0^{C17HC} , the electrical surface potential at the location of the fluorophore in the charged liposomes—by rearrangement of the Boltzmann equation [31–33]:

$$\psi_0^{C17HC} = -\frac{\Delta pK_{el} k T \ln 10}{e} = -\frac{pK_a^{\text{charged}} - pK_a^{\text{neutral}} k T \ln 10}{e}$$

where k is the Boltzmann constant ($1.38 \times 10^{-23} \text{ J K}^{-1}$), T is the absolute temperature (here 295 K), e is the electron charge ($1.6 \times 10^{-19} \text{ C}$) and pK_a^{charged} and pK_a^{neutral} are the pK_a 's in charged bilayers and in neutral DOPC bilayers, respectively. pH_{surface} was also determined by using the Boltzmann equation for a given potential [33]:

$$pH_{\text{surface}} = 7.4 + \frac{\psi_0 e}{k T} \ln 10$$

High surface pHs were found for all the cationic liposomes (see insert Fig. 1) DOPE lowered the surface potential of the three cationic liposomes DOTAP, DMRIE, and DOSPA.

The dissociation degrees of CFPE in the various cationic liposomes are presented in Fig. 1B. One can observe that the apparent pK_a 's of CFPE in cationic liposomes with cholesterol as helper lipid are shifted to lower pH values compared to the pK_a 's of CFPE in cationic liposomes without any helper lipid. The insert in Fig. 1 shows the pK_a 's of CFPE in cationic liposomes and their electrical surface potentials. As expected, the pK_a of CFPE in the neutral DOPE/DOPC liposomes was higher than of the cationic liposomes. The values of pK_a 's and surface potentials of CFPE in cationic and neutral liposomes are lower compared to the values of pK_a 's and ψ_0^{C17HC} obtained with the fluorophore C17HC.

3.2. Effect of DNA on the electrical properties of labeled cationic liposomes

Increasing DNA^-/L^+ ratio results in decreasing the electrical surface potential in cationic liposomes (Fig. 2). At $\text{DNA}^-/\text{L}^+ = 0$, DMRIE/Chol liposomes have the highest, and DC-Chol/DOPE liposomes, the lowest electrical surface potential.

Formation of lipoplexes by addition of DNA led to reduction in the electrical surface potential of C17HC in a DNA^-/L^+ -dependent manner (Fig. 2). However, the effect was sigmoidal in nature, with a small effect at low DNA^-/L^+ charge ratio (especially for DOTAP and DMRIE/Chol lipoplexes), with the major diminution above mole ratio $\text{DNA}^-/\text{L}^+ 0.5$, followed by a plateau starting at DNA^-/L^+ charge ratio 1.0. The smallest effect upon DNA addition was observed with DC-Chol/DOPE

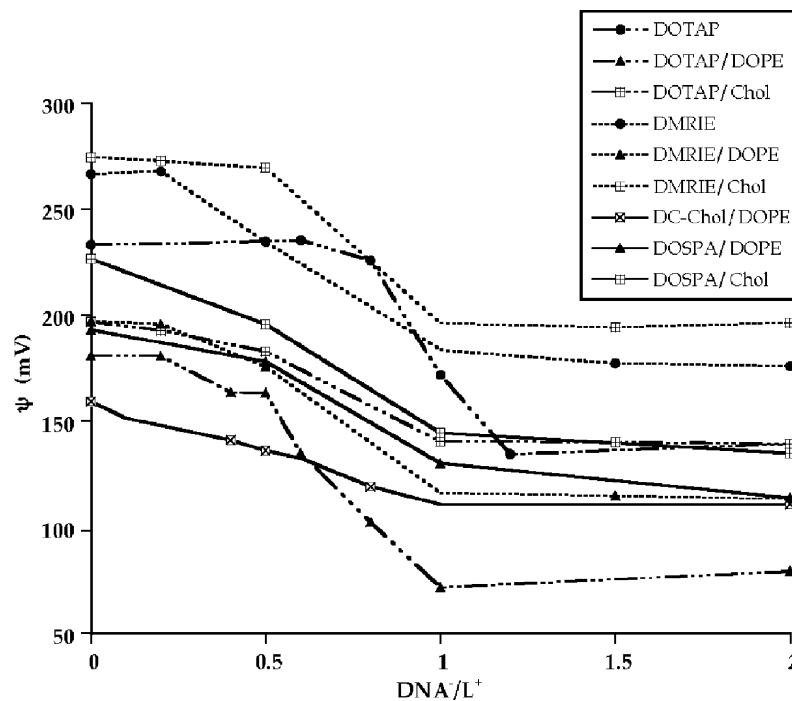


Fig. 2. Changes in surface potential of C17HC in lipoplexes after addition of plasmid DNA to cationic liposomes as a function of mole ratio of DNA^-/L^+ followed by means of C17HC-labeled cationic liposomes.

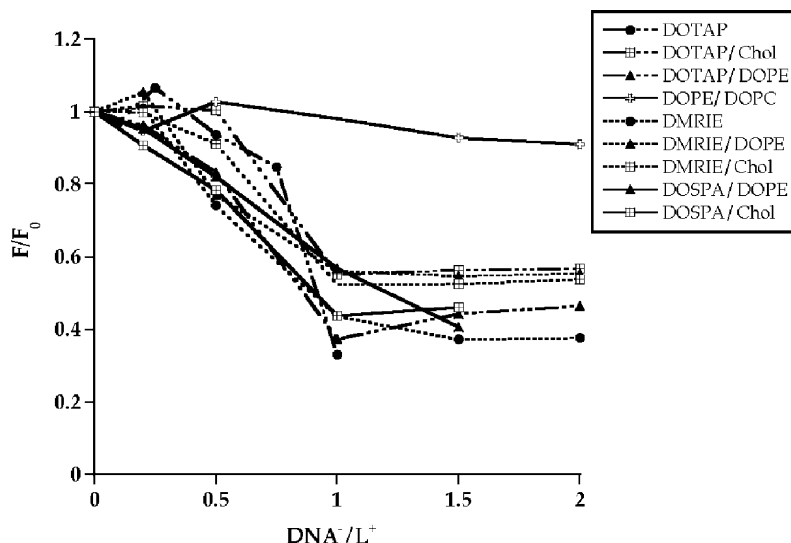


Fig. 3. Change in fluorescence intensity of CFPE in lipoplexes as a function of mole ratio of DNA^-/L^+ . F/F_0 : F_0 is fluorescence intensity of CFPE-labeled cationic liposomes; F is fluorescence intensity of CFPE-labeled lipoplexes.

liposomes. Fig. 2 shows that none of the cationic formulations were completely neutralized by DNA even when DNA^-/L^+ mole ratio was 2.

The reduction in fluorescence intensities of CFPE-labeled cationic liposomes as a function of the mole ratio of DNA^-/L^+ is shown in Fig. 3. In order to better

monitor the interaction of DNA with cationic liposomes, the pH of the medium was reduced to 5.5 (acetate buffer), which is the pH-sensitive part of the pH titration curve [34].

As observed with C17HC-labeled cationic liposomes, a plateau was reached above a mole ratio DNA^-/L^+ of 1.0 for

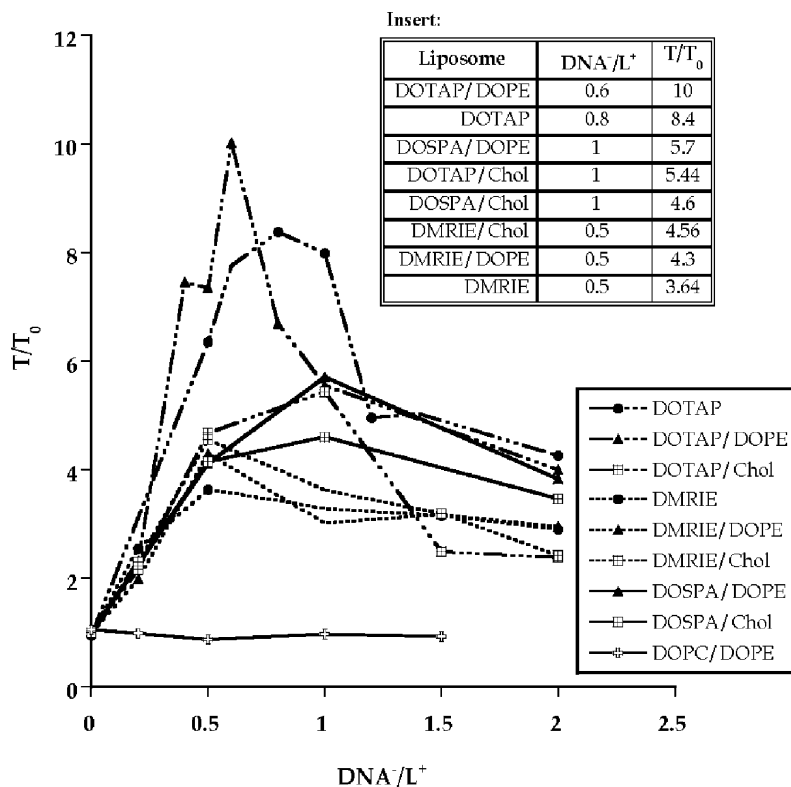


Fig. 4. Changes in 90° static light scattering (SLS) of cationic liposomes upon DNA addition. SLS data are presented as T/T_0 , where T is the 90° SLS after the addition of DNA to the cationic liposomes and T_0 is the 90° SLS of the same cationic liposomes prior to DNA addition. Insert: Values of SLS (T/T_0) in decreasing order.

most of the cationic formulations. As mentioned above for C17HC, CFPE also demonstrates that even at DNA⁻/L⁺ ratio of 2 neutralization and charge reversal of the supermolecular assemblies were not complete. However, unlike C17HC, which demonstrates large differences between different cationic liposomes, no major differences were noted between the various cationic liposome formulations above the DNA⁻/L⁺ ratio of 1.0.

3.3. Effect of DNA on TMADPH fluorescence intensity

Effects of DNA on TMADPH fluorescence intensity of TMADPH-labeled cationic liposomes were found in all formulations (data not shown). The ratio F/F_0 decreased because DNA induced TMADPH quenching. For most cationic liposome formulations, the maximum quenching was obtained at DNA⁻/L⁺ mole ratio <0.8. Above this value, a plateau was reached, followed in some formulations

(DMRIE/DOPE; DOTAP/DOPE) by an increase in the ratio F/F_0 .

3.4. Static light scattering

The change in 90° static light scattering (SLS) was followed in parallel to the fluorescence measurements as a measure of particle size change. The SLS data were presented as T/T_0 , where T is the 90° SLS after the addition of DNA to the cationic liposomes and T_0 is the 90° SLS of the same cationic liposomes before DNA addition. T/T_0 is presented as a function of DNA⁻/L⁺ mole ratio (Fig. 4). Using the kinetic mode of the Perkin-Elmer LS 50B spectrofluorimeter, we followed time-dependent changes in T . Lipoplex formation can be monitored through the change in T or T/T_0 . The curves describing T/T_0 as a function of DNA⁻/L⁺ mole ratio have a bell shape, reaching their highest value at DNA⁻/L⁺

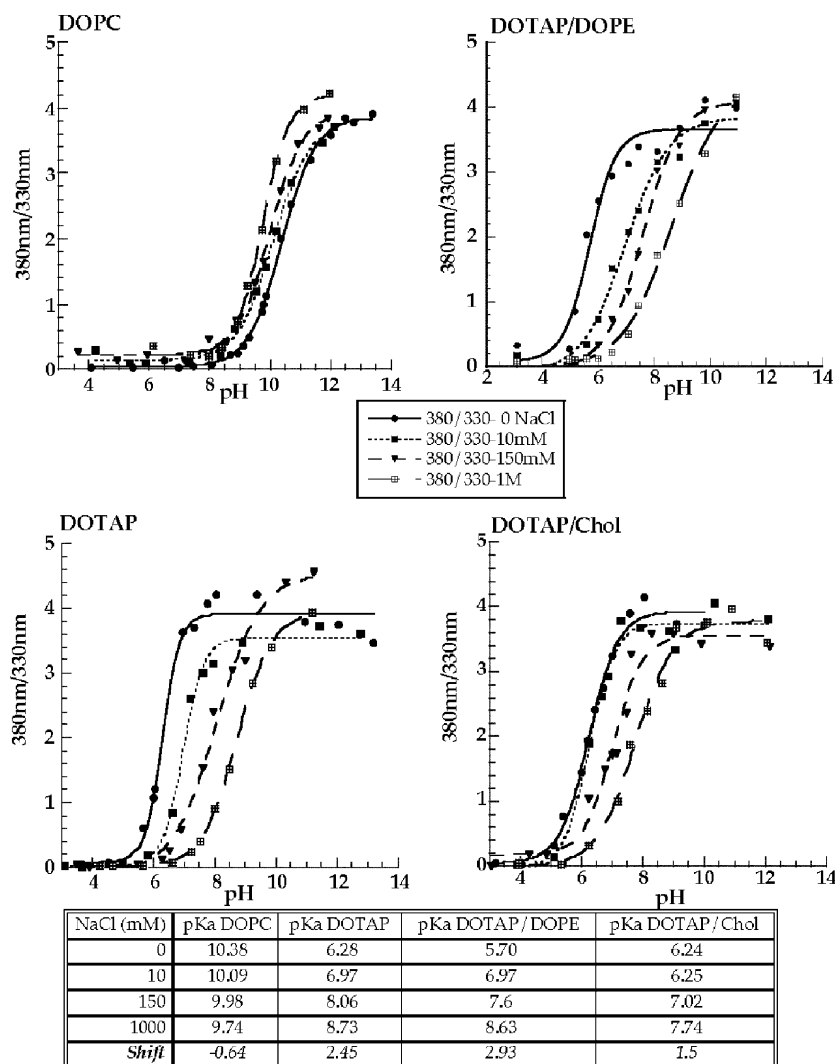


Fig. 5. Effect of NaCl on titration curves of C17HC in liposomes of DOPC, DOTAP, DOTAP/DOPE, and DOTAP/Chol. Insert: pK_a 's for C17HC in DOPC liposomes and those containing DOTAP at each NaCl concentration.

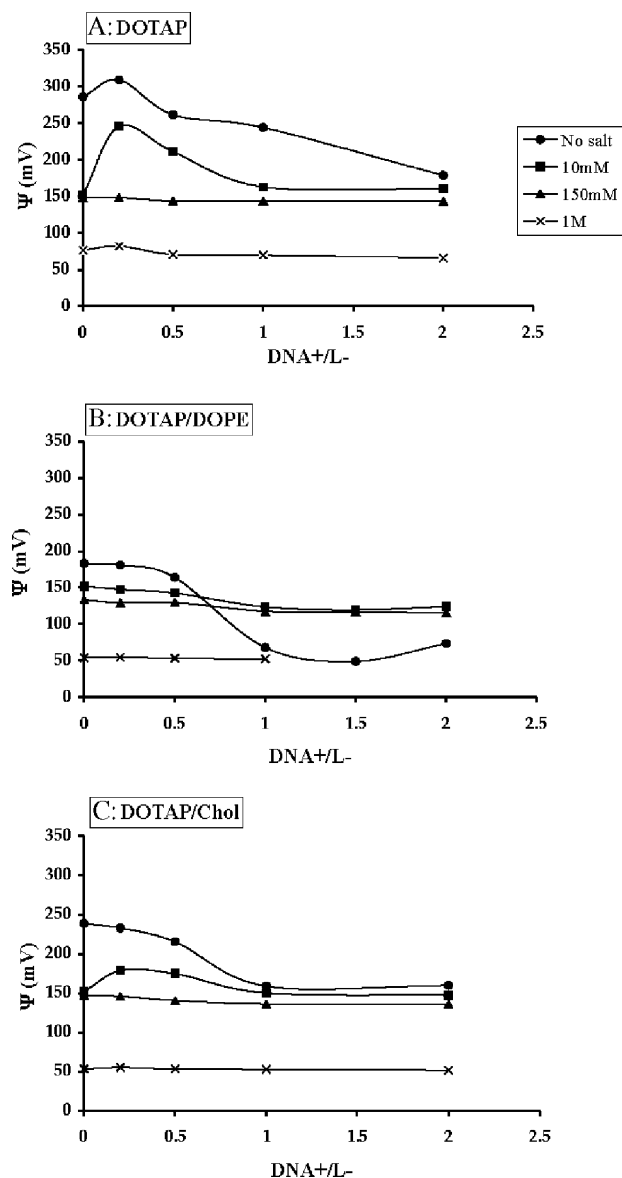


Fig. 6. Effect of NaCl on surface potential of C17HC-labeled liposomes DOTAP (A), DOTAP/DOPE (B), and DOTAP/Chol (C), after addition of DNA.

mole ratio ≥ 0.5 –1.0. The values were dependent on the liposome lipid composition. The order of maximum T/T_0 (from high to low) is shown in Fig. 4, insert.

3.5. Effect of NaCl on the electrostatics of cationic liposomes and on lipoplex formation

In general, salt should reduce the electrical surface potential of the liposomes, and therefore apparent pK_a should shift to higher bulk pH [35], as indeed occurred (Fig. 5). However, the various liposome compositions used differed in their response. As NaCl concentration increased, the largest significant shift in apparent pK_a to higher values (2.93 pH units) is observed for DOTAP/DOPE liposomes (see Fig. 5, insert). DOTAP/Cholesterol

liposomes were the least sensitive to increases in NaCl concentrations among all the cationic liposome formulations used.

The effect of NaCl concentrations on the electrical surface potential of C17HC-labeled lipoplexes is described in Fig. 6.

NaCl reduces the ability of DNA to neutralize the cationic lipid (as monitored by the level of Ψ_0^{C17HC} reduction) in a DNA[−]/L⁺ mole ratio-dependent manner (Figs. 6 A–C). In Figs. 6A and C, one can see that at 10 mM and 150 mM NaCl, above the mole ratio DNA[−]/L⁺ of 1.0, Ψ_0 is not much affected by NaCl. At 1 M NaCl, addition of DNA had no effect. To increase sensitivity at this NaCl concentration the experiment was done at higher pH's, at which changes in Ψ_0 are more sensitive; pHs 9.4, 8.9, and 8.2 for DOTAP, DOTAP/DOPE, and DOTAP/Chol liposomes, respectively, were used (Fig. 5).

Changes in 90° SLS (T/T_0) of DOTAP/DOPE liposomes upon DNA addition as a function of different NaCl concentrations are presented in Fig. 7. As NaCl concentration increases, T/T_0 decreases. In 1 M NaCl, most of the effect is contributed by increase in T , that is, there is less DNA-induced aggregation. Competition between Cl[−] and DNA[−] can explain these results.

3.6. Zeta potential of cationic liposomes and lipoplexes

All DOTAP liposomes showed positive zeta potentials, as expected (Table 1). As NaCl concentrations increased, the zeta potential decreased for all DOTAP liposomes (Table 1). In the absence of NaCl, DOTAP/DOPE liposomes had the lowest zeta potential among the DOTAP liposomes.

At DNA[−]/L⁺ mole ratio of 0.5, the zeta potentials were not affected by the presence of NaCl. On the other hand, at DNA[−]/L⁺ mole ratio of 1.0, a reversal from positive values to negatives values of zeta potential occurred.

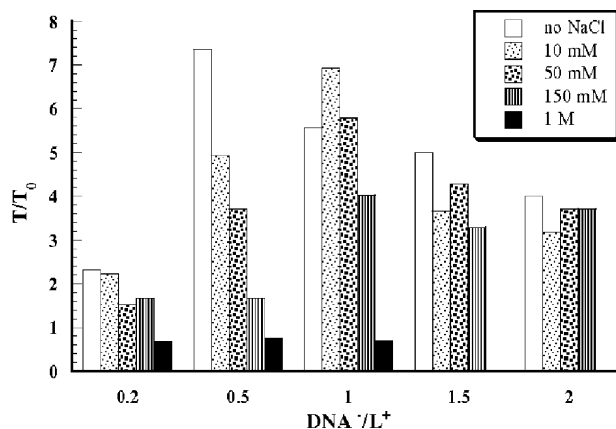


Fig. 7. Effect of increasing NaCl concentration and DNA[−]/L⁺ on 90° SLS in DOTAP/DOPE liposomes. SLS data are presented as T/T_0 where T is the 90° SLS of the lipoplexes and T_0 is the 90° SLS of the same cationic liposomes prior to DNA addition.

Table 1

Zeta potential (ζ) and electrical surface potential (Ψ_0) of liposomes and lipoplexes in the presence of different NaCl concentrations

Liposome	Buffer	DNA ⁻ /L ⁺	ζ (mV)	Ψ_0 (C17H) (mV)
DOTAP	20 mM HEPES, pH 7.4	—	62.19	286.15
	HEPES + 10 mM NaCl	—	75.88	151.77
	HEPES + 50 mM NaCl	—	56.56	NA
	HEPES + 150 mM NaCl	—	47.6	148.22
	HEPES + 1 M NaCl	—	13.39	76.39
DOTAP/DOPE	20 mM HEPES, pH 7.4	—	54.9	183.07
	HEPES + 10 mM NaCl	—	57.07	151.77
	HEPES + 50 mM NaCl	—	45.4	NA
	HEPES + 150 mM NaCl	—	35.41	133.22
	HEPES + 1 M NaCl	—	16.8	54.21
DOTAP/Chol	20 mM HEPES, pH 7.4	—	80.51	238.4
	HEPES + 10 mM NaCl	—	89.92	151.77
	HEPES + 50 mM NaCl	—	56.6	NA
	HEPES + 150 mM NaCl	—	37.31	147.12
	HEPES + 1 M NaCl	—	14.74	53.76
DOPE/DOPC	20 mM HEPES, pH 7.4	—	-26.22	0
	HEPES + 10 mM NaCl	—	-30.92	NA
	HEPES + 50 mM NaCl	—	NA	NA
	HEPES + 150 mM NaCl	—	-11.725	NA
	HEPES + 1 M NaCl	—	1.01	NA
DOTAP/DOPE	20 mM HEPES, pH 7.4	—	47.31	183.07
	20 mM HEPES, pH 7.4	0.5	52.55	163.9
	20 mM HEPES, pH 7.4	1	-74.73	67.83
DOTAP/DOPE	HEPES + 10 mM NaCl	—	58.12	151.77
	HEPES + 10 mM NaCl	0.5	57.68	142.34
	HEPES + 10 mM NaCl	1	-78.83	123.4
DOTAP/DOPE	HEPES + 150 mM NaCl	—	33.19	133.22
	HEPES + 150 mM NaCl	0.5	26.21	129.6
	HEPES + 150 mM NaCl	1	-49.73	117.33
DOTAP/DOPE	HEPES + 1 M NaCl	—	8.09	54.21
	HEPES + 1 M NaCl	0.5	-7.39	53.33
	HEPES + 1 M NaCl	1	-12.82	52.31
DNA alone, no lipids	20 mM HEPES, pH 7.4	Amount of 0.5	-49.76	
DNA alone, no lipids	HEPES + 10 mM NaCl	Amount of 0.5	-50.48	
DNA alone, no lipids	HEPES + 150 mM NaCl	Amount of 0.5	-34.67	
DNA alone, no lipids	HEPES + 1 M NaCl	Amount of 0.5	-16.42	
DNA alone, no lipids	HEPES + 10 mM NaCl	Amount of 1.0	-51.95	
DNA alone, no lipids	HEPES + 1 M NaCl	Amount of 1.0	-22.74	

3.7. Comparing the performance of the probes HC-DOPE and C17HC in DOTAP/Chol liposomes in various media

Fig. 8 demonstrates the difference in the behavior of the two probes, the one-chain C17HC and the two-chain phospholipid HC-DOPE in DOTAP/Chol liposomes or in their lipoplexes, in the presence of various media. C17HC-labeled DOTAP/Chol liposomes when mixed with serum or HMWC showed a large (>75%) decrease in the 380/330 nm fluorescence intensity ratio, while in 0.9% NaCl there was a much smaller (<30%) decrease in this ratio, and in 5% dextrose, there was no effect on this ratio (Fig. 8 A1). However, HC-DOPE-labeled DOTAP/Chol liposomes exhibited no appreciable changes in the 408/370 nm intensity ratio in all four media (Fig. 8 B1).

In lipoplexes, the results (Figs. 8 A2, B2) reflect those of the cationic liposomes: as the mole ratio DNA⁻/L⁺ increases, the 380/330 nm intensity ratio of C17HC-labeled lipoplexes in all four media decreases (Fig. 8 A2). A much

larger decrease, which is independent of DNA⁻/DOTAP⁺ mole ratio, is observed in serum and HMWC (Fig. 8 A2). When HC-DOPE-labeled lipoplexes were used (Fig. 8 B2), a decrease in the intensity ratio 408/370 nm is also observed in all four media, and it is significantly lower than in C17HC-labeled lipoplexes and similar for all four media.

4. Discussion

4.1. General aspects

A better understanding of the physicochemical properties of cationic lipid–DNA complexes and the forces leading to “productive” lipoplexes is essential in order to understand the contribution of these properties to transfection efficiency. Such understanding may enable one to optimize lipoplex-mediated transfection in vitro and in vivo. In this study, we focused on a more detailed characterization of

A: C17HC

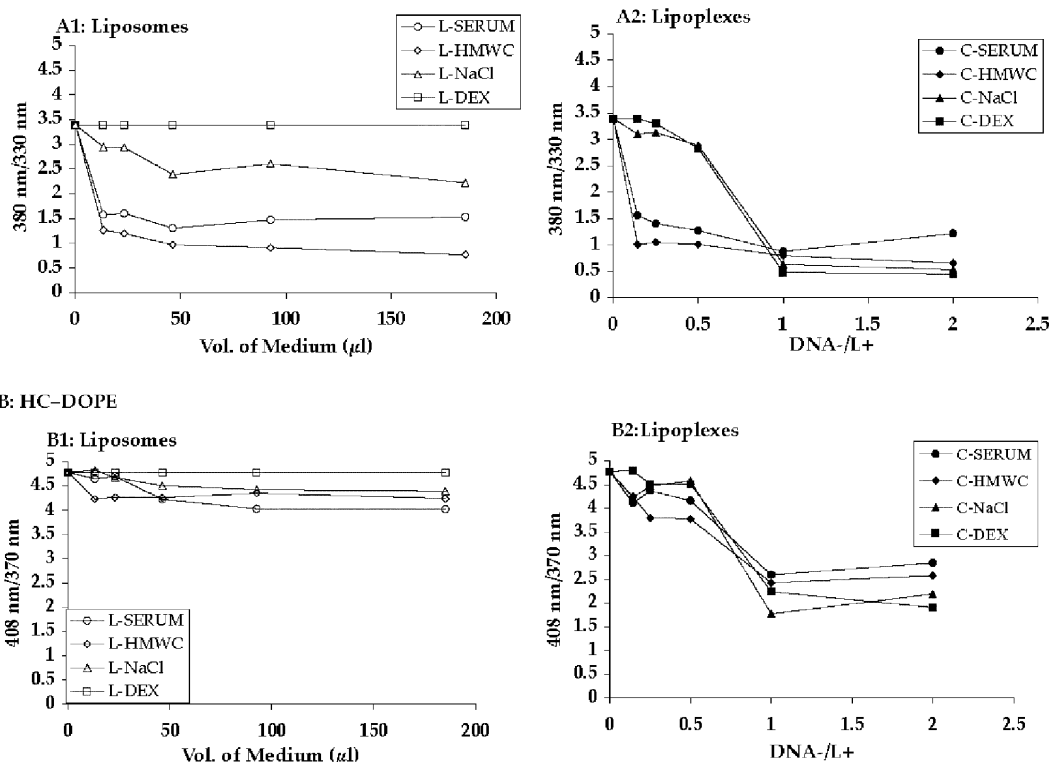


Fig. 8. Effect of various media (human serum, serum high-molecular-weight components (HMWC), 0.9% NaCl, or 5% dextrose) on the ratio of fluorescence intensities of C17HC (A) or HC-DOPE (B) in DOTAP/Chol liposomes (A1 and B1) and after addition of DNA (A2 and B2). L denotes liposomes and C denotes complex (lipoplex).

electrical surface potential of cationic liposomes and lipoplexes under a broad range of conditions. Electrical surface potential was determined using pH-sensitive fluorescent probes.

All fluorescent lipids used were able to provide information on cationic liposomes and lipoplex electrostatics. The difference between the results as monitored via C17HC or CFPE demonstrated a different location of the fluorophore part of the molecule with respect to the lipid headgroup; the 7-hydroxycoumarin moiety of C17HC is closer to the quaternary amino group of DOTAP. Moreover, the HC-DOPE probe proved to be the most stable in cationic liposomes and lipoplexes in the presence of salt or serum.

4.2. Electrostatic monitoring through the different fluorescent probes

DOPE when present in the cationic liposomes or lipoplexes decreases the positive electrical surface potential for all 3 cationic lipids (DOTAP, DMRIE, and DOSPA) studied (insert, Fig. 1). This is due to a salt bridge between, and partial neutralization of, the positively charged quaternary amino group of these 3 lipids and the negatively charged phosphate group of DOPE [17], while the DOPE amino group may interact with the negatively-charged phosphates of the DNA [20]. Several advantages were

attributed to DOPE in the lipoplex formation and intracellular processing that are related to the above salt formation: it induces an easier counterion release from the lipid surface by DNA [12], lower hydration of the lipid surface [9], and enables a better matching of charge density between lipid and DNA surfaces. In addition, DOPE confers instability to the liposomes because of its “curvature-loving” properties, leading to coexistence of hexagonal and lamellar phases [10]. All these contribute to the superiority of DOPE as a helper lipid under the more controlled situation of *in vitro* transfection.

Unlike DOPE, the helper lipid cholesterol lacks an ionogenic group. Its β^- OH group at C3 introduced only minimal polarity, just enough to make it compatible with the lipid bilayer. Its presence in the cationic liposome bilayer slightly increases (or does not affect) the electrical surface potential of the cationic liposomes compared to the cationic liposomes composed of the cationic lipid alone. The presence of cholesterol is known to cause a significant dehydration of the cationic lipid bilayer [9], thereby making the cationic charge more accessible, and therefore the positive electrical surface potential observed with C17HC is slightly higher than for DOTAP alone. Recent data showed that as cholesterol mole % increased in DOTAP liposomes, the interlamellar distance in lipid/ODN systems expands [36]. For transfection *in vivo*, but not *in vitro*,

lipoplexes based on DOTAP/Chol are much superior to those based on DOTAP/DOPE [2]. DOTAP/Chol lipoplexes, for example, showed successful delivery to the murine lungs [37] and to tumor lesions [38] *in vivo*.

Fig. 2 shows that DNA, even at a 2-fold charge excess, did not cause complete neutralization and charge reversal of the supermolecular assemblies. The incomplete neutralization can be explained in two ways: (1) not all the positive charges of the cationic LUV are accessible to the DNA phosphates and/or (2) the C17HC fluorophore is located closer to the cationic lipid than to DNA phosphates and therefore the measurement is biased in favor of the cationic lipids.

CFPE showed a similar pattern of electrostatics as C17HC, although results were less detailed, i.e., the differences between the various lipid compositions were smaller than with C17HC. This can be explained by the different location of the fluorophores of the two probes in the lipid bilayer. The C17HC is located at the lipid bilayer with its HC fluorophore in the plane of the DOTAP quaternary amino group (and DOPE phosphate group), while its long-chain tail of 17 carbons is parallel to the lipid acyl chains [22] (Fig. 9). C17HC cannot monitor all the amino groups of DOSPA which has a larger polycationic headgroup (Fig. 9). The interaction between DNA and lipid assemblies also occurred at this plane.

The fluorescein moiety of the CFPE molecule, being larger and bulkier than the C17HC hydroxycoumarin moiety and attached through a spacer of phosphoethanolamine, is located further out from the above plane of interaction between DNA and the cationic lipids, and therefore it is less sensitive to the variation of lipid packing in the bilayers (Fig. 9). The ^1H NMR study (data not shown) confirms that the C17HC moiety affects the packing of methyl groups of the quaternary amine of DOTAP, indicating that this probe is

in the proximity of this headgroup. The results of this study (compare Fig. 2 with Fig. 3) clearly demonstrate that C17HC is the superior probe to determine the fine details of cationic liposome and lipoplex electrostatics. However, C17HC suffers from a major drawback, demonstrated in Fig. 8, which shows the differences between C17HC and HC-DOPE in liposomes and lipoplexes. The C17HC, being a one-acyl chain amphiphile, desorbs from the lipid bilayer to the lipid-containing components of the serum (i.e., lipoproteins), while the HC-DOPE, being a two-acyl chain phospholipid, remains located in the liposome or lipoplex. The DOPE moiety, having two acyl chains instead of the single heptadecyl chain as part of the lipid assembly bilayers, confers to the HC-DOPE probe a higher stability. Hence, C17HC application is limited and cannot be used when other lipidic assemblies (i.e., lipoproteins or cells) are present in the system, while HC-DOPE can be used, even in *in vivo* systems. Therefore, HC-DOPE is very useful for understanding lipoplex interactions with various components, including those of serum [2].

4.3. Size instability

The 90° SLS is a good measure of relative changes in particle size [39,40]. It gives important information on size instability, which is one of the major factors in determining transfection efficiency in cell culture [7,8,10,37]. Parallel measurements of change in 90° SLS and in cationic lipid neutralization of L^+ due to addition of DNA demonstrated that increase in SLS requires the presence of a cationic lipid. When the mole ratio DNA^-/L^+ is lower than 1.5, there are large differences between the various formulations (Fig. 4). This is in agreement with previous data showing a higher proportion of lipoplexes with larger diameters at mole ratio DNA^-/L^+ of 0.5 and 0.7 [41]. Likewise, Pires et al. showed

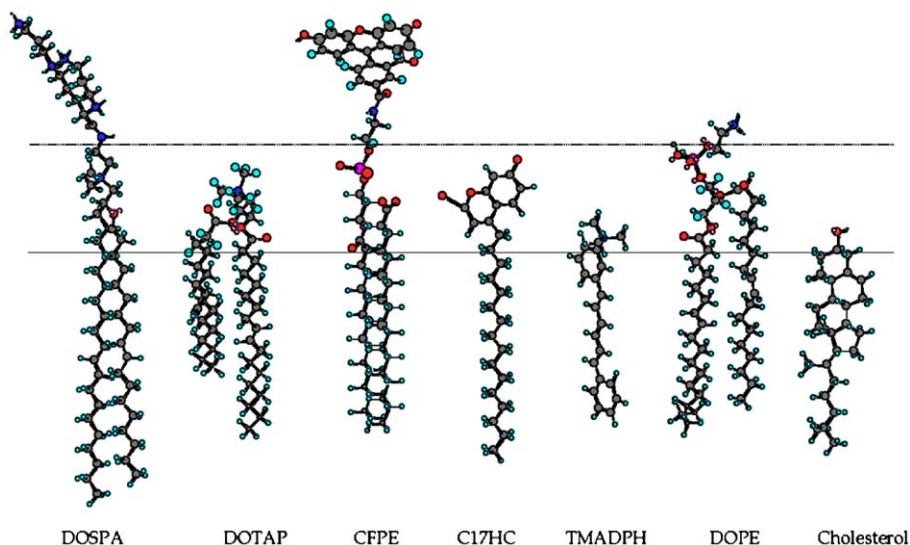


Fig. 9. Molecular models of the fluorophores (CFPE, C17HC, and TMADPH) together with the cationic lipids (DOTAP and DOSPA) and the helper lipids (DOPE and cholesterol). The software used was CSC Chem3DPlus™ (version 7.0, Cambridge Scientific Computing, Cambridge, MA).

a significant reduction in zeta potential as well as an increase in particle size at charge ratio DNA^-/L^+ under 1.0 [42].

The DOTAP-based lipoplexes seemed to reach the largest particle sizes, and DOTAP/DOPE > DOTAP/Cholesterol. This is in agreement with recent data of atomic force microscopy showing that lipoplexes based on polycationic liposomes have a globular shape and more compact structures than lipoplexes based on monocationic liposomes [43].

Another way to study size instability is to study membrane defects through the change in degree of exposure of TMADPH to water, which can be followed as changes in probe fluorescence intensity [13]. The increase in the TMADPH fluorescence intensity (above the ratio F/F_0 of 1.0) in the presence of DOPE in lipoplexes based on DOTAP and DMRIE implies a specific effect of this helper lipid at $\text{DNA}^-/\text{L}^+ > 1$ (data not shown). In this mole charge ratio range, there is a homogeneous lipid condensation, suggesting a reduction in the TMADPH exposure to water molecules [13]. The largest dehydration upon lipoplex formation was also observed with DOTAP/DOPE using laurdan fluorescence and differential scanning calorimetry [9]. These results suggest a tight compaction between DOTAP/DOPE and DNA. This is supported by the results of Even-Chen and Barenholz (unpublished data) showing that the association of DNA to DOTAP/DOPE is stronger than to DOTAP/Chol, even though DOTAP/Chol is more positively charged.

4.4. Effect of ionic strength

NaCl is an important component of most biological fluids, including serum. Therefore, a better understanding of the lipoplex fate in a biological milieu requires understanding the effect of NaCl and separating it from the effect of the high molecular weight components (mainly proteins) present in the relevant biological fluid. As expected from the Gouy–Chapman equation [35], NaCl, being an electrolyte, should decrease the electrical surface potential of cationic liposomes in a concentration-dependant manner (as determined by the upper shift in the apparent pK_a of C17HC). Zeta potential data confirmed electrical surface potential results (see Table 1). This also explains why the NaCl has a large effect on reducing neutralization of the cationic DOTAP by DNA (Figs. 5 and 6).

This reduction in neutralization of cationic lipid by DNA is explained by the fact that increasing ionic strength reduces the fraction of small counterions removed from the DNA and the cationic liposome upon mixing the two polyelectrolytes, that is, there is less intimate DNA–cationic lipid interaction, and therefore lower degree of neutralization.

As demonstrated in Fig. 6 the neutralization of the three types of DOTAP-based liposomes by DNA is reduced by the addition of 10 mM NaCl. There is no neutralization at 1 M NaCl. Constructing a DNA–cationic liposome phase diagram, with the aid of ultracentrifugation, reveals that

95% of the DNA is in the lipoplexes at 150 mM NaCl at DNA^-/L^+ mole ratios of 0.5 and 1.0, while at 1.5 M NaCl at the DNA^-/L^+ ratio of 1.0, the level of free DNA is dependent on lipid composition: ~30% DNA in DOTAP/DOPE lipoplexes and 9% DNA in DOTAP/Chol lipoplexes (Even-Chen and Barenholz, unpublished data). It was shown before [44,45] that at high ionic strength, there is reduced electrostatic interaction between DNA and the cationic lipid [44], and therefore neutralization occurred at lower DNA^-/L^+ .

4.5. Zeta potential versus electrical surface potential

At DNA^-/L^+ ratios of 0.5, the zeta potential results in the presence of NaCl, confirmed the electrical surface potential data, i.e., as NaCl concentration increased, there is no major effect of DNA. On the other hand, at DNA^-/L^+ ratios of 1.0, zeta potential values became negative while the electrical potential remained positive. Zeta potential measurements reflect the electrical surface potential of the external leaflet

Table 2

Comparisons of (1) physicochemical characteristics and (2) transfection in vitro and in vivo of DOTAP/DOPE and DOTAP/Cholesterol liposomes and their lipoplexes

Characteristics	DOTAP/cholesterol	DOTAP/DOPE
Electrical surface potential of liposome before lipoplex formation	More positive (274 mV)	Less positive (181 mV)
Electrical surface potential of liposome after lipoplex formation	Less neutralization by DNA	More neutralization by DNA
Interaction with DNA	Weaker	Stronger
Increase in T/T_0	Smaller	Larger
NaCl effect on liposome electrical surface potential and surface pH	Smaller (especially at low salt concentration)	Larger
NaCl (high concentration, 1.5 M) effect on lipoplex stability ^a	Less stable	More stable
Aggregation ^b in: NaCl (0.9%)	Yes	Yes
Dextrose	No	No
Serum effect on electrical surface potential of HC-DOPE-labeled lipoplexes ^b	No effect	Large effect
Serum effect (cryo-TEM) ^b	Liposomes remain intact and are impermeable to serum proteins	Liposomes do not remain intact
Transfection in vitro ^c	Low	High
Transfection in vivo ^b (in the murine lungs)	High	Low

^a Stability measured as free DNA (by agarose gel electrophoresis) (data from Even-Chen and Barenholz, unpublished data).

^b Data from Simberg et al. [2].

^c Data from Kerner et al. [7].

of the assembly at its plane of shear further away from the lipid headgroup plane [21]. The location of the plane of shear [21] is affected by the medium ionic strength. Hence, the electrical surface potential, measured by the various fluorescent probes used in this study, give different values, which reflect their fluorophore location with respect to the charged headgroup (Fig. 9). However, while surface potential measurements by the fluorophores used in this study monitor the assembly lipid/water interface only, zeta potential at DNA⁻/L⁺ ratios of 1.0 reflect also the excess of DNA, which is not complexed with the lipids. Indeed, 10%–30% of the DNA is free at this DNA⁻/L⁺ mole ratio of 1.0 [46].

In parallel to its effect on electrostatics, NaCl also increases the 90° SLS of the DOTAP/DOPE liposomes (T_0), from 16.8 ± 2.85 in 20 mM HEPES buffer, pH 7.4 (without NaCl) to 633.95 ± 71.2 in 20 mM HEPES buffer, pH 7.4 in 1 M NaCl. Serum also increased size of cationic liposomes [42]. In fact, the small counterions interact with the headgroups of the lipids, inducing condensation of the headgroups due to charge masking, thereby causing defects on the liposome surface; these defects are responsible for exposure of hydrophobic parts of the lipid bilayer to the aqueous medium, leading to aggregation and fusion of the liposomes [46,47], and therefore an increase in particle size is observed.

The ratio T/T_0 , i.e., the change in 90° SLS due to DNA addition to DOTAP/DOPE liposomes, decreases as NaCl concentration is increased. At 1 M NaCl, DNA did not cause any change in 90° SLS of DOTAP/DOPE liposomes.

4.6. DOPE versus cholesterol as helper lipid

Table 2 summarizes the differences between DOTAP/Chol and DOTAP/DOPE lipoplexes determined in this study (3.1–3.5 and 4.1–4.4 above) and elsewhere (reviewed in [37]): DOTAP/Chol, being more positively charged, is neutralized by DNA to a lesser extent and is less affected by NaCl and serum. The integrity of the DOTAP/Chol lipoplexes in serum is better [2].

DMRIE alone and DMRIE/DOPE liposomes and their lipoplexes behave similarly to the equivalent DOTAP-based systems in both physicochemical properties (this work) and transfection efficiency [10]. DMRIE/Chol cationic liposomes and their lipoplexes have the highest electrical surface potential of all systems studied, especially when monitored by C17HC (Fig. 2), and to a lesser degree by CFPE (Fig. 3). However, more studies are needed in order to prove if these differences in the electrostatics explain the transfection superiority of DMRIE/Chol over DOTAP/Chol (most DNA⁻/L⁺ ratios) [10]. DOSPA-based systems are more complex, as DOSPA alone forms micelles and not liposomes. DOSPA mixed with the helper lipids form micellar/lamellar phases that coexist, which may be the dominant factor in explaining the transfection superiority of these systems over those based on monocationic lipids [10].

Correlation of these physicochemical data with transfection efficiency in vitro [7,10,14,18] and in vivo [2] suggests that lipoplex instability is a “major player” in transfection efficiency. However, while in the absence of serum (or in low serum levels), the instability imposed by DOPE [10,18] is an advantage; upon contact with serum proteins, disintegration of lipoplexes is followed by massive aggregation and loss of efficient transfection [2]. It seems that this behavior is related to the DOPE properties of being a curvature-loving lipid, having a large packing parameter [37].

These fluorophores used in this study proved to be powerful tools to study interactions of cationic liposomes with nucleic acids, allowing one to follow lipoplex formation and the interaction of lipoplexes with salts and biological fluids such as serum, as well as to compare cationic liposomes and lipoplexes of different lipid compositions.

Acknowledgments

Mr. S. Geller is acknowledged for help in editing the manuscript and Mrs. B. Levene for helping in the typing.

References

- [1] D. Lasic, *Liposomes in Gene Delivery*, CRC Press, Boca Raton, FL, 1997.
- [2] D. Simberg, S. Weisman, Y. Talmon, A. Faerman, T. Shoshani, Y. Barenholz, The role of organ vascularization and lipoplex–serum initial contact in intravenous murine lipofection, *J. Biol. Chem.* 278 (2003) 39858–39865.
- [3] C. Dass, Biochemical and biophysical characteristics of lipoplexes pertinent to solid tumour gene therapy, *Int. J. Pharm.* 241 (2002) 1–25.
- [4] M. Kay, J. Glorioso, L. Naldini, Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics, *Nat. Med.* 7 (2001) 33–40.
- [5] T. Takehara, N. Hayashi, Y. Miyamoto, M. Yamamoto, E. Mita, H. Fusamoto, T. Kamada, Expression of the hepatitis C virus genome in rat liver after cationic liposome-mediated in vivo gene transfer, *Hepatology* 21 (1995) 746–751.
- [6] M.J. Wright, L.M. Wightman, C. Lilley, M. de Alwis, S.L. Hart, A. Miller, R.S. Coffin, A. Thrasher, D.S. Latchman, M.S. Marber, In vivo myocardial gene transfer: optimization, evaluation and direct comparison of gene transfer vectors, *Basic Res. Cardiol.* 96 (2001) 227–236.
- [7] M. Kerner, O. Meyuhas, D. Hirsch-Lerner, L. Rosen, Z. Min, Y. Barenholz, Interplay in lipoplexes between type of pDNA promoter and lipid composition determines transfection efficiency of human growth hormone in NIH3T3 cells in culture, *Biochim. Biophys. Acta* 1532 (2001) 128–136.
- [8] N. Zuidam, Y. Barenholz, A. Minsky, Chiral DNA packaging in DNA–cationic liposome assemblies, *FEBS Lett.* 457 (1999) 419–422.
- [9] 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.
- [10] D. Simberg, D. Danino, Y. Talmon, A. Minsky, M. Ferrari, C. Wheeler, Y. Barenholz, Phase behavior, DNA ordering, and size instability of cationic lipoplexes. Relevance to optimal transfection activity, *J. Biol. Chem.* 276 (2001) 47453–47459.

- [11] N. Zuidam, Y. Barenholz, Electrostatic parameters of cationic liposomes commonly used for gene delivery as determined by 4-heptadecyl-7-hydroxycoumarin, *Biochim. Biophys. Acta* 1329 (1997) 211–222.
- [12] N. Zuidam, Y. Barenholz, Electrostatic and structural properties of plasmid DNA–lipid complexes commonly used for gene delivery, *Biochim. Biophys. Acta* 1368 (1998) 115–128.
- [13] D. Hirsch-Lerner, Y. Barenholz, Probing DNA–cationic lipid interactions with the fluorophore trimethylammonium diphenyl-hexatriene (TMADPH), *Biochim. Biophys. Acta* 1370 (1998) 17–30.
- [14] B. Sternberg, K. Hong, W. Zheng, D. Papahadjopoulos, Ultrastructural characterization of cationic liposome–DNA complexes showing enhanced stability in serum and high transfection activity in vivo, *Biochim. Biophys. Acta* 1375 (1998) 23–35.
- [15] N. Templeton, D. Lasic, P. Frederik, H. Strey, D. Roberts, G. Pavlakis, Improved DNA: liposome complexes for increased systemic delivery and gene expression, *Nat. Biotechnol.* 15 (1997) 647–652.
- [16] S. Li, W. Tseng, D. Stolz, S. Wu, S. 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.
- [17] I. Zuhorn, W. Visser, U. Bakowsky, J. Engberts, D. Hoekstra, Interference of serum with lipoplex–cell interaction: modulation of intracellular processing, *Biochim. Biophys. Acta* 1560 (2002) 25–36.
- [18] N. Zuidam, D. Hirsch-Lerner, S. Margulies, Y. Barenholz, Lamellarity of cationic liposomes and mode of preparation of lipoplexes affect transfection efficiency, *Biochim. Biophys. Acta* 1419 (1999) 207–220.
- [19] R. Mahato, K. Anwer, F. Tagliaferri, C. Meaney, P. Leonard, M. Wadhwa, M. Logan, M. French, A. Rolland, Biodistribution and gene expression of lipid/plasmid complexes after systemic administration, *Hum. Gene Ther.* 9 (1998) 2083–2099.
- [20] N. Templeton, D. Lasic, P. Frederik, H. Strey, D. Roberts, G. Pavlakis, Improved DNA: liposome complexes for increased systemic delivery and gene expression, *Nat. Biotechnol.* 15 (1997) 647–652.
- [21] E.M. Egorova, The validity of the Smoluchowski equation in electrophoretic studies of lipid membranes, *Electrophoresis* 15 (1994) 1125–1131.
- [22] R. Pal, W.A. Petri, V. Ben Yashar, R.R. Wagner, Y. Barenholz, Characterization of the fluorophore 4-heptadecyl-7-hydroxycoumarin: a probe for the headgroup region of lipid bilayers and biological membranes, *Biochemistry* 24 (1985) 573–581.
- [23] D. Simberg, D. Hirsch-Lerner, R. Nissim, Y. Barenholz, Comparison of different commercially available cationic lipid-based transfection kits, *J. Liposome Res.* 10 (2000) 1–13.
- [24] S. Levy, D. Avni, N. Aariharan, R. Perry, O. Meyuhas, Oligopyrimidine tract at the 5' end of mammalian ribosomal protein mRNAs is required for their translational control, *Proc. Natl. Acad. Sci. U. S. A.* 88 (1991) 3319–3323.
- [25] Y. Barenholz, S. Amselem, Quality control assays in the development and clinical use of liposome-based formulations, in: G. Gregoriadis (Ed.), *Liposome Technology*, 2nd ed. *Liposome Preparation and Related Techniques*, vol. I, CRC Press, Boca Raton, FL, 1993, pp. 527–616.
- [26] P. Felgner, Y. Barenholz, J. Behr, S. Cheng, P. Cullis, L. Huang, J. Jese, L. Seymour, F. Szoka, A. Thierry, E. Wagner, G. Wu, Nomenclature for synthetic gene delivery systems, *Hum. Gene Ther.* 8 (1997) 511–512.
- [27] S. Even Chen, Y. Barenholz, DOTAP cationic liposomes prefer relaxed over supercoiled plasmids, *Biochim. Biophys. Acta* 1509 (2000) 176–188.
- [28] T. Maniatis, E. Fritsch, J. Sambrook, *Molecular cloning*, A Laboratory Manual, Cold Spring Harbor Laboratory, 1982.
- [29] H. Eliyahu, N. Serval, A.J. Domb, Y. Barenholz, Lipoplex-induced hemagglutination: potential involvement in intravenous gene delivery, *Gene Ther.* 9 (2002) 850–858.
- [30] O. Garbuzenko, S. Zalipsky, M. Qazen, Y. Barenholz, Electrostatics of PEGylated micelles and liposomes containing charged and neutral lipopolymers, *Langmuir* 21 (2005) 2560–2568.
- [31] H.S. Harned, B.B. Owen, *The Physical Chemistry of Electrolytic Solutions*, Reinhold, New York, 1958.
- [32] S.A. Tatlalian, *Phospholipids Handbook*, Marcel Dekker, New York, 1993.
- [33] G. Cevc, Membrane electrostatics, *Biochim. Biophys. Acta* 1031 (1990) 311–382.
- [34] V. Meidan, J. 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.
- [35] G. Cevc, Electrostatic characterization of liposomes, *Chem. Phys. Lipids* 64 (1993) 163–186.
- [36] S. Weisman, D. Hirsch Lerner, Y. Barenholz, Y. Talmon, Nanostructure of cationic lipid–oligonucleotide complexes, *Biophys. J.* 87 (2004) 609–614.
- [37] D. Simberg, S. Weisman, Y. Talmon, Y. Barenholz, DOTAP (and other cationic lipids): chemistry, biophysics, and transfection, *Crit. Rev. Ther. Drug Carrier Syst.* 21 (2004) 257–317.
- [38] K. Anwer, G. Kao, B. Proctor, A. Rolland, S. Sullivan, Optimization of cationic lipid/DNA complexes for systemic gene transfer to tumor lesions, *J. Drug Target.* 8 (2000) 125–135.
- [39] A. Kenworthy, S. Simon, J. McIntosh, Structure and phase behavior of lipid suspensions containing phospholipids with covalently attached poly(ethylene glycol), *Biophys. J.* 68 (1995) 1903–1920.
- [40] S. Belsito, R. Bartucci, L. Sportelli, Lipid chain length effect on the phase behaviour of PCs/PEG-2000-PEs mixtures. A spin label electron spin resonance and spectrophotometric study, *Biophys. Chem.* 93 (2001) 11–22.
- [41] H. Lee, S.K. Ratanathanawongs Williams, S.D. Allison, T.J. Anchor-douy, Analysis of self-assembled cationic-lipid–DNA gene carrier complexes using flow field-flow fractionation and light scattering, *Anal. Chem.* 73 (2001) 837–843.
- [42] P. Pires, S. Simões, S. Nir, R. Gaspar, N. Düzgünes, M.C. Pedrosa de Lima, Interaction of cationic liposomes and their DNA complexes with monocytic leukemia cells, *Biochim. Biophys. Acta* 1418 (1999) 71–84.
- [43] L.A. Wangerek, H.M. Dahl, T.J. Senden, J.B. Carlin, D.A. Jans, D.E. Dunstan, P.A. Ioannou, R. Williamson, S.M. Forrest, Atomic force microscopy imaging of DNA–cationic liposome complexes optimised for gene transfection into neuronal cells, *J. Gene Med.* 3 (2001) 72–81.
- [44] M.T. Kennedy, E.V. Pozharski, V.A. Rakhmanova, R.C. MacDonald, Factors governing the assembly of cationic phospholipids–DNA complexes, *Biophys. J.* 78 (2000) 1620–1633.
- [45] S.J. Eastman, C. Siegel, J. Tounsiant, A.E. Smith, S.H. Cheng, R.K. Scheule, Biophysical characterization of cationic lipid: DNA complexes, *Biochim. Biophys. Acta* 1325 (1997) 41–62.
- [46] M. Mora, F. Mir, M. de Madariaga, M. Sagrista, Aggregation and fusion of vesicles composed of N-palmitoyl derivatives of membrane phospholipids, *Lipids* 35 (2000) 513–524.
- [47] M. Nagata, T. Yotsuyanagi, K. Ikeda, NaCl-induced aggregation of egg phosphatidylcholine liposomes, *Chem. Pharm. Bull.* 34 (1986) 1391–1394.