Structural and Fusogenic Properties of Cationic Liposomes in the Presence of Plasmid DNA

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ABSTRACT The structural and fusogenic properties of large unilamellar vesicles (LUVs) composed of the cationic lipid *N*-[2,3-(dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) and 1,2-dioleoyl-3-phosphatidylethanolamine (DOPE) have been examined in the presence of pCMV5 plasmid and correlated with transfection potency. It is shown, employing lipid mixing fusion assays, that pCMV5 plasmid strongly promotes fusion between DOTMA/DOPE (1:1) LUVs and DOTMA/1,2-dioleoyl-3-phosphatidylcholine (DOTMA/DOPC) (1:1) LUVs such that at a cationic lipid-to-DNA charge ratio of 3.0, ~80% fusion is observed. The anions citrate and chloride can also trigger fusion, but at much higher concentrations. Freeze-fracture electron microscopy studies demonstrate the tendency of cationic vesicles to form clusters at low pCMV5 content, whereas macroscopic fused aggregates can be observed at higher plasmid levels. ^{31}P NMR studies of the fused DNA-DOTMA/DOPE (1:1) complexes obtained at high plasmid levels (charge ratio 1.0) reveal narrow "isotropic" ^{31}P NMR resonances, whereas the corresponding DOPC containing systems exhibit much broader "bilayer" ^{31}P NMR spectra. In agreement with previous studies, the transfection potency of the DOPE-containing systems is dramatically higher than for the DOPC-containing complexes, indicating a correlation between transfection potential and the motional properties of endogenous lipids. Interestingly, it was found that the complexes could be separated by centrifugation into a pellet fraction, which exhibits superior transfection potencies, and a supernatant fraction. Again, the pellet fraction in the DOPE-containing system exhibits a significantly narrower ^{31}P NMR resonance than the corresponding DOPC-containing system. It is suggested that the ^{31}P NMR characteristics of complexes exhibiting higher transfection potencies are consistent with the presence of nonbilayer lipid structures, which may play a direct role in the fusion or membrane destabilization events vital to transfection.

INTRODUCTION

Liposomes composed of equimolar mixtures of *N*-[2,3-(dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) and 1,2-dioleoyl-3-phosphatidylethanolamine (DOPE) can act as agents that mediate intracellular delivery of plasmid DNA into cells. This has been shown to result in efficient transgene expression in vitro (Felgner et al., 1987, 1989; Lu et al., 1989; Pinnaduwage et al., 1989; Jarnagin et al., 1992) and limited transgene expression in vivo (Nabel et al., 1992; Zhu et al., 1993; Egilmez et al., 1996). In these systems, plasmid DNA is mixed with preformed small unilamellar vesicles (SUVs) or large unilamellar vesicles (LUVs) to form DNA-lipid complexes that interact, in turn, with target cells (Friend et al., 1996). Considerable effort has been made to characterize the structure of these complexes to identify characteristics that correspond to most efficient intracellular delivery of plasmid and subsequent gene expression. Most of these investigations have focused on the morphological features of the complexes as determined by various microscopic techniques, including metal shadowing electron microscopy (EM) (Gershon et al., 1993; Zabner et al., 1995), freeze-fracture EM (Sternberg et al., 1994), cryo-transmission EM (Gustafsson et al., 1995), and atomic force microscopy (Wheeler et al., 1996). Little consensus has been reached on relating structures observed to transfection activity.

Less attention has been paid to the relations between the fusogenic and motional properties of DNA-cationic lipid complexes and transfection potency. Fusion events are clearly integral to the formation of the complexes themselves as well as to the subsequent intracellular delivery of DNA. The motional properties of lipids as detected by NMR techniques can give insight into local structure that can be correlated with fusogenic and transfection behavior. In this work, we examine the structural, motional, and fusogenic properties of DOTMA/DOPE LUVs on the addition of pCMV5 plasmid, and correlate this information with transfection potential. It is shown that plasmid DNA is a highly potent promoter of fusion between DOTMA/DOPE (1:1) LUVs, forming structures characterized by narrow ^{31}P NMR signals characteristic of rapid, isotropic motional averaging. It is suggested that the lipid structures giving rise to this behavior may correspond to nonbilayer lipid arrangements, which facilitate the fusion events associated with transfection by DNA-cationic lipid complexes.

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MATERIALS AND METHODS

Lipids and chemicals

1,2-Dioleoyl-3-phosphatidylcholine (DOPC), 1,2-dioleoyl-3-phosphatidylethanolamine (DOPE), 1,2-dioleoyl-3-phosphatidylserine (DOPS), *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-1,2-dioleoyl-*sn*-phosphatidylethanolamine (NBD-PE), and *N*-(lissamine rhodamine B sulfonyl)-1,2-dioleoyl-

sn-phosphatidylethanolamine (Rh-PE) were obtained from Avanti Polar Lipids (Alabaster, AL). 3-(*N,N*-Dimethylamino)-1,2-propanediol, potassium hydride, and iodomethane (methyl iodide) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Magnesium sulfate ($MgSO_4$), β-mercaptoethanol, potassium acetate, potassium chloride, sodium chloride, anhydrous sodium sulfate, sodium phosphate, sodium hydroxide, glacial acetic acid, and citric acid were obtained from Fisher Scientific (Fair Lawn, NJ). Ampicillin, bovine serum albumin (BSA), 1-bromo-*cis*-9-octadecene (oleyl bromide), EDTA, HEPES, lithium chloride, lysozyme, polyethyleneglycol (PEG 8000), ribonuclease A (RNase), *t*-octylphenoxypolyethoxyethanol (Triton X-100), sodium dodecyl sulfate, and tris(hydroxymethyl)aminomethane (Tris) were obtained from Sigma Chemical Co. (St. Louis, MO). ^{14}C-labeled cholesteryl hexadecyl ether ([^{14}C]CHOLHEX) was purchased from Dupont NEN Products (Boston, MA). Chlorophenol red galactopyranoside (CPRG) was purchased from Boehringer Mannheim (Germany). Deuterium oxide was obtained from MSD Isotopes (Montreal, ON). All organic solvents were purchased from Fisher Scientific (Nepeau, ON). Freon 22 was obtained from Allied Chemical Ltd. (Mississauga, ON). Silica gel 60 (particle size 63–200 μm, 70–230 mesh) was purchased from VWR Scientific (Edmonton, AL). Agarose was purchased from Bio-Rad (Richmond, CA). Glycerol and glucose were obtained from BDH (Toronto, ON). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, and *Eco*RI were purchased from Gibco BRL (Burlington, ON). Baby hamster kidney (BHK) cells (BHK 21) were obtained from the American Tissue Culture Collection (ATCC CCL-10). Distilled water was purified from a Corning Mega-Pure MP-4S system.

Synthesis of DOTMA

DOTMA was synthesized as described previously (Felgner et al., 1987), by the following modified procedure. A mixture of 3-(dimethylamino)-1,2-propanediol (0.92 g, 1.49 mmol), potassium hydride (0.36 g, 3.14 mmol), and oleyl bromide (1.50 g, 4.48 mmol) in xylenes (20 ml) was stirred at room temperature and reduced pressure (30 mm Hg) for 30 min. After the mixture was heated to 50°C with a condenser and continuously stirred for an additional 15 min, the mixture was heated to reflux under continuous nitrogen flow at atmospheric pressure for 4 h. After the mixture was cooled and hexane (150 ml) was added, distilled water (150 ml) was added dropwise to the mixture. The reaction mixture was then extracted until neutrality was reached. After the organic layer was dried with anhydrous sodium sulfate, the crude intermediate product was concentrated under rotary evaporation and was purified with a silica gel column, with stepwise elution of 2:1 diethyl ether/hexane and then 4:2:1 diethyl ether/hexane/ethanol. The intermediate product, 2,3-dioleyloxy-1-(dimethylamino)propane, was obtained as a colorless oil (0.55 g, 60% yield; TLC, R_f = 0.40 with 4:2:1 diethyl ether/hexane/ethanol), and the structure was confirmed by 200 MHz ^{1}H NMR. The final product was prepared by stirring the intermediate product (0.42 g, 0.67 mmol) with methyl iodide (0.68 g, 4.79 mmol) in dichloromethane (40 ml) for 20 h in the dark. After rotary evaporation, the residue was dissolved in 60 ml dichloromethane and was repeatedly extracted with aliquots of 40 ml 1.0 M NaCl until the iodide form of DOTMA (TLC, R_f = 0.35 with 6:1 dichloromethane/methanol) was converted into the chloride form of DOTMA (TLC, R_f = 0.22 with 6:1 dichloromethane/methanol). Column chromatography on silica gel and eluting with 1:1 hexane/ethanol and then 6:1 dichloromethane/methanol gave 0.30 g (67% yield) of pure product. The structure of DOTMA was confirmed by 200 MHz ^{1}H NMR and low-resolution-power liquid secondary ion mass spectroscopy (Kratos Concept II H) with a parent ion mass of 635 atomic mass units.

Plasmid

Plasmid DNA (pCMV5) (Andersson et al., 1989) was grown in *Escherichia coli* (DH5α) and was selected by resistance to ampicillin. pCMV5 was isolated by alkali lysis and purified by polyethylene glycol (PEG) precipitation (Sambrook et al., 1989). Briefly, 3.0 liters of saturated bacterial cultures was collected by centrifugation and resuspended in 108 ml of 50 mM glucose, 25 mM Tris-Cl (pH 8.0), 10 mM EDTA. After the addition of a tip-in spatula of solid lysozyme, and then 240 ml of freshly prepared 0.20 M NaOH/1% sodium dodecyl sulfate with gentle mixing for 15 min, 180 ml of ice-cold 5.0 M potassium acetate/glacial acetic acid/distilled water was distributed evenly in the lysate, which was then cooled in ice for 15 min, centrifuged at 5000 rpm for 30 min, and filtered through a layer of cheesecloth. An equal volume of isopropanol was added to the supernatant for precipitation. After centrifugation, the pellet was rinsed with 70% ethanol and resuspended in 18 ml TE (10 mM Tris-Cl, pH 8.0, 1 mM EDTA). An equal volume of ice-cold 5.0 M LiCl was then added for precipitation; and an equal volume of isopropanol was added to the resulting supernatant for reprecipitation. The resulting pellet was washed with 70% ethanol and was resuspended in 3.0 ml TE with 100 μl DNase-free pancreatic RNase (10 mg/ml) for 2 h at room temperature. Three milliliters of 1.6 M NaCl with 13% (w/v) PEG 8000 was added, and the plasmid was recovered by centrifugation at 11,000 × *g* for 5 min. The pellet was extracted once with phenol, once with phenol/chloroform, and once with chloroform. The aqueous layer was treated with 600 μl 3.0 M sodium acetate and two volumes of ethanol. The precipitated plasmid was washed with 70% ethanol and was resuspended in distilled water. The purity of pCMV5 was confirmed by 1% agarose gel electrophoresis with restriction endonuclease *Eco*RI digest. Because one nucleotide contains only one phosphate group, the concentration of pCMV5 was determined by using standard phosphorus assays (Fiske and Subbarow, 1925) and was expressed as the phosphorus concentration of pCMV5. A similar procedure was used for the preparation of pCMVβgal.

Preparation of LUVs

Mixtures of lipids dispensed in chloroform were dried under a stream of nitrogen gas with continuous vortex mixing. The residual solvent was removed under high vacuum for 2 h. The resulting lipid films were hydrated with appropriate buffers and then freeze-thawed five times to produce homogeneous multilamellar vesicle systems (MLVs). Large unilamellar vesicle systems (LUVs) were obtained by extruding MLVs 10 times through two 100-nm pore size polycarbonate filters (Costar Nuclepore polycarbonate membrane) under nitrogen at a pressure of 300–400 psi (Mayer et al., 1986). The size of LUVs was checked with a Nicomp model 270 submicron particle sizer, using quasielastic light scattering techniques. Phosphorus assays were used to quantify phospholipid concentration (Fiske and Subbarow, 1925).

Lipid-mixing fusion assay

Fusion was determined by the decrease in resonance energy transfer (RET) resulting from fluorescent probe dilution (Struck et al., 1981). The exchange of labeled lipids between two populations of vesicles, even in aggregated systems, has been reported to be negligible (Hoekstra, 1982). LUVs of the desired composition were prepared in 20 mM HEPES (pH 7.4) and were diluted to 10 mM total lipid. Fluorescently labeled LUVs of DOTMA/DOPE (1:1) or DOTMA/DOPC (1:1) containing 0.5 mol% each of NBD-PE and Rh-PE were prepared similarly. For the fusion assays used to study the effects of NaCl, citrate, and pCMV5 on DOTMA/DOPE (1:1) and DOTMA/DOPC (1:1) LUVs, a mixture of labeled and nonlabeled LUVs (1:9) was prepared. Then 100 μl of the mixed LUVs was added to 1850 μl of 20 mM HEPES (pH 7.4) in a cuvette with continuous stirring, and the fluorescence intensity (*F*) was monitored over time. Fusion was induced by the addition of 50 μl of appropriate anions at 30 s. Excitation and emission wavelengths were 445 nm and 535 nm, respectively, and a 530-nm emission cutoff filter was used. A blank assay with 50 μl of 20 mM HEPES (pH 7.4) was used as a reference for zero fluorescence (F_o). The maximum fluorescence intensity (F_{max}) was measured by the addition of 40 μl 4% Triton X-100. A mock complete mixing system of LUVs containing 0.05 mol% each of fluorescent probes was also prepared. The

fluorescence intensity before (F_b) and after (F_a) the addition of 40 μl 4% Triton X-100 in this mock sample was taken into consideration when the percent change in fluorescence (% $\Delta F/\Delta F_{max}$) was calculated for each point in the fluorescence time course.

$$\% \Delta F/\Delta F_{max} = \{[(F - F_o)/(F_{max} - F_o)]100\}(F_b/F_a) \quad (1)$$

For the study of fusion of DOTMA/DOPE or DOTMA/DOPC LUVs with DOPS systems, 10 μl of fluorescently labeled DOTMA/DOPE (1:1) or DOTMA/DOPC (1:1) LUVs (10 mM total lipid) was added to 1940 μl of 20 mM HEPES (pH 7.4) in a cuvette with continuous stirring. At 30 s, 50 μl of the appropriate composition of nonlabeled DOPS/DOPE or DOPS/DOPC LUVs (10 mM total lipid) was added to induce fusion. Mock samples for different compositions containing 0.083 mol% each of fluorescent probes were prepared. An assay of 50 μl of 20 mM HEPES (pH 7.4) was used for zero fluorescence (F_{nu}), and the fluorescence of the appropriate mock samples was taken to be the maximum fluorescence (F_{max}). The percentage change in fluorescence was then calculated as

$$\% \Delta F/\Delta F_{max} = [(F - F_{nu})/(F_{max} - F_{nu})]100 \quad (2)$$

for each point in the fluorescence time course. For studying the interaction between the DNA-lipid complexes and anionic model membrane system, 10 μl of fluorescently labeled DOTMA/DOPE (1:1) or DOTMA/DOPC (1:1) LUVs (10 mM total lipid) was added to 1890 μl of 20 mM HEPES (pH 7.4) in a cuvette with continuous stirring. An aliquot of 50 μl of serial diluted pCMV5 stocks was added at 30 s in each assay. At 90 s, 50 μl of 10 mM nonlabeled DOPS/DOPE (1:1) or DOPS/DOPC (1:1) LUVs was added. The data processed from this experiment was right-shifted for 60 s (i.e., time 0 was taken to be the 60-s time point). The fluorescence intensity obtained after the addition of pCMV5 to the labeled LUVs was taken as the reference for each assay (F_{nu}). The maximum fluorescence intensity (F_{max}) was measured by the addition of 25 μl 4% Triton X-100. A mock sample of LUVs containing 0.083 mol% each of fluorescent probes was used, and the % $\Delta F/\Delta F_{max}$ was calculated as

$$\% \Delta F/\Delta F_{max} = \{[(F - F_{nu})/(F_{max} - F_{nu})]100\}(F_b/F_a) \quad (3)$$

for each point in the fluorescence time course. All fusion assays were performed at 25°C.

^{31}P NMR spectroscopy

Solid-state broad-band decoupling ^{31}P NMR spectra were recorded at 81.02 MHz on a Bruker MSL 200 spectrometer, using a 4.0-μs pulse and a 1.5-s repeat time. The free induction decay (FID) was accumulated over 1500–2000 scans and was Fourier transformed with 50-Hz line broadening. For studies showing the effect of DOTMA on DOPE, various compositions of freeze-thawed DOTMA/DOPE (50 μmol, 33 mM DOPE) MLVs were hydrated in 20 mM HEPES/D_2O (pH 7.4). The temperature was controlled with a Bruker variable temperature 1000 unit at settings with increments of 10°C. The temperature under 40°C was controlled with a liquid nitrogen flow system; the temperature above 40°C was maintained with a nitrogen gas flow system. For the experiments showing the effects of different anions on DOTMA/DOPE or DOTMA/DOPC systems, samples of DOTMA/DOPE (1:1) or DOTMA/DOPC (1:1) (50 μmol each, 100 mM total lipid) LUVs in 20 mM HEPES/D_2O (pH 7.4) were prepared as described earlier. Then the sequential addition of appropriate amounts of different anions to each sample was used to induce changes in lipid organization. Temperature was maintained at 25°C with a liquid nitrogen flow system. The FIDs of the resuspended pellet and supernatant of DNA-cationic lipid complexes were accumulated over 10,000–20,000 scans. For the separation of DNA-cationic lipid complexes, the appropriate amount of plasmid DNA was added to a population of LUVs prepared in 20 mM HEPES. The mixture was centrifuged at 11,000 × g in a Sorvall RC-5B superspeed centrifuge for 15 min. The pellet was twice washed with distilled water and centrifuged before resuspending in 20 mM HEPES/D_2O. All ^{31}P NMR spectra were locked with D_2O. A mixture of phosphoric acid/D_2O was used as the reference for chemical shifts in all ^{31}P NMR spectra.

Freeze-fracture electron microscopy

Samples of DOTMA/DOPE (1:1) and DOTMA/DOPC (1:1) (30 mM total lipid) LUVs in 20 mM HEPES (pH 7.4) were prepared as described earlier. Fifty-milliliter aliquots of lipid sample were titrated with the appropriate amount of NaCl, citrate, or pCMV5. After incubation at room temperature for 15 min, 33.5 μl of glycerol was added as a cryoprotectant to make a final glycerol level of 25% by volume in each sample. The final concentration of total lipid in each sample was 11 mM (0.75 μmol each of DOTMA and DOPE or DOPC). After physical mixing and incubating for another 15 min, a 2.0-μl droplet of the sample was pipetted onto a flat-top gold support disc, which was then plunged into liquid nitrogen-cooled liquid freon 22 (monochlorodifluoromethane; freezing point −160°C). After 5 s, the sample was transferred to a specimen table immersed in liquid nitrogen before insertion into the freeze-fracture apparatus (Balzers; BAF400D). Fracturing was performed at −110°C with a vacuum of 10^{-6} to 10^{-7} torr. Immediately after the fracturing, a 2-nm coating of platinum-carbon at an angle of 45° and then a 20 nm coating of carbon at an angle of 90° were applied. The gold cup was then warmed to room temperature and was submerged in distilled water for replica removal. Replicas were cleaned overnight in commercial bleach solution and were rinsed two times with distilled water before mounting on a grid by using a platinum transfer loop. The dried replicas were examined under a transmission electron microscope (Jeol; JEM-1200EX).

Separation and quantification of DNA/cationic lipid complexes

DNA-cationic lipid complexes were formed by incubating appropriate amounts of preformed cationic vesicles with 20 μg pCMVβgal to obtain the desired charge ratio (±) in 250 μl distilled water. Trace amounts of [^{3}H]pCMVβgal and [^{14}C]CHOL-HEX were used as markers. The resulting mixture was incubated at room temperature for 15 min and was then centrifuged at 11,000 × g in a Sorvall MC-12V microcentrifuge for 15 min. The supernatant was pipetted out for storage, and the pellet was resuspended in 250 μl distilled water. Mock samples for each charge ratio without centrifugation were also prepared. Aliquots of 50 μl from samples of the pellet and the supernatant were counted for radioactivity and were compared with mock samples for the percentage of [^{3}H]DNA and [^{14}C]lipid recovery, respectively.

In vitro DNA transfection on BHK cells

All DNA transfection procedure was carried out in a laminar flow hood (Forma Scientific). DNA-cationic lipid complexes were prepared as described above, except that 0.5 μg of pCMVβgal was used for each transfection preparation. Appropriate amounts of vesicles were used for each charge ratio. Triplicates of transfection samples were prepared simultaneously. Standards of β-galactosidase were prepared by twofold serial dilutions of 200 milliunits of β-galactosidase with 0.5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) (pH 8.0). A unit of β-galactosidase will hydrolyze 1.0 μmol of *o*-nitrophenyl-β-D-galactoside to *o*-nitrophenol and D-galactose per minute at pH 7.3 at 37°C. A similar transfection protocol has been published elsewhere (Felgner et al., 1994). First, BHK cells (BHK 21) cultured in DMEM with 10% FBS and 100 units of penicillin and 100 μg streptomycin as antibiotics were plated onto a 96-well microtiter plate with 2 × 10^4 cells per well. The plate was then incubated for 20 h at 37°C with 5% carbon dioxide. Wells for standards were not plated. For each well, appropriate amounts of DNA-lipid complexes (preformed for 30 min) were diluted with DMEM/FBS. Aliquots of 100 μl of DNA-lipid complexes were plated and the resulting plate was incubated at 37°C, 5% CO_2 for 4 h. After the removal of the transfected media, 100 μl of DMEM/FBS was added, and cells were further incubated

for 20 h at 37°C, 5% CO_2. Cells were lysed by adding 50 μl of lysis buffer containing 0.1% Triton X-100 in 250 mM phosphate buffer (pH 8.0) and were left at −70°C for ~30 min to ensure complete lysis. After thawing, 50 μl of PBS containing 0.5% BSA or 50 μl of the appropriate β-galactosidase standard was added. Color development was induced by adding 150 μl of substrate buffer containing chlorophenol red galactopyranoside (CPRG) (1 mg/ml), 60 mM Na_2HPO_4, 1 M $MgSO_4$, 10 mM KCl, and 50 mM β-mercaptoethanol, and was measured at 540 nm with a microplate EL-309 autoreader (Bio-Tek Instruments). For the transfection of samples from the resuspended pellet and the supernatant of DNA-lipid complexes after centrifugation at 11,000 × *g*, trace amounts of [^{3}H]pCMVβgal and [^{14}C]CHOL-HEX were included for quantification.

RESULTS

DOTMA can stabilize DOPE into a bilayer organization

The effect of DOTMA on the structural behavior of DOPE in aqueous dispersions of mixtures of DOTMA and DOPE was investigated by ^{31}P NMR techniques. DOTMA/DOPE dispersions with increasing DOTMA content (from 0% to 80%) were prepared in 20 mM HEPES buffer (pH 7.4). For each sample, ^{31}P NMR spectra were recorded from 10°C to 70°C in increments of 10°C. As shown in Fig. 1, at both 20°C and 40°C, aqueous dispersions of pure DOPE exhibit a typical hexagonal (H_{II}) phase ^{31}P NMR signal (Cullis and de Kruijff, 1979), which is characterized by a high field shoulder and a low field peak, where the separation between the shoulder and peak is about half of that observed for the bilayer ^{31}P NMR signal. As the DOTMA content increases, an increased bilayer ^{31}P NMR component, characterized by a high field peak and a low field shoulder, is observed. DOPE was partially stabilized into a bilayer organization at temperatures up to 20°C in the presence of 20% DOTMA, whereas at 50% DOTMA content, DOPE was stabilized into a bilayer organization up to 60°C. With an 80% DOTMA content, bilayer organization was observed over the entire temperature range studied. Thus for a DOTMA/DOPE (1:1) system, which is the composition of the commercially available Lipofectin reagent, DOPE is stabilized into a bilayer organization at both room and physiological temperatures. The ability of DOTMA to stabilize DOPE into a bilayer organization is similar to the ability of lipids such as phosphatidylcholine (PC) as well as anionic phospholipids such as phosphatidylserine (PS) to stabilize PE into a bilayer organization (Cullis and de Kruijff, 1979).

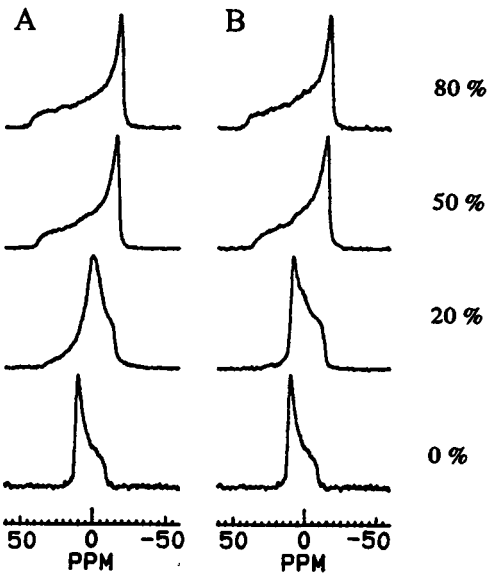

FIGURE 1 ^{31}P NMR spectra of freeze-thawed DOTMA/DOPE MLVs in 20 mM HEPES (pH 7.4) with increasing DOTMA content (0%, 20%, 50%, and 80%) at (*A*) 20°C and (*B*) 40°C. Sample preparation and NMR parameters are described in Materials and Methods.

pCMV5 can trigger membrane fusion between DOTMA/DOPE LUVs

The ability of equimolar amounts of DOTMA to stabilize DOPE into a bilayer organization allows stable large unilamellar vesicles to be made. The addition of plasmid DNA results in formation of the DNA-lipid complexes used for transfection. It is of interest to examine the fusogenic behavior of DOTMA/DOPE (1:1) LUVs in response to the presence of plasmid DNA. This was done with the lipid mixing assay detailed in Materials and Methods. DOTMA/DOPE (1:1) LUVs containing 0.5 mol% each of NBD-PE and Rh-PE and nonlabeled 1:1 DOTMA/DOPE vesicles were prepared. When the labeled vesicles fuse with nonlabeled vesicles, dilution of the fluorescent probes results in an increase in NBD-PE fluorescence. Fusion between DOTMA/DOPE LUVs on the addition of pCMV5 plasmid is shown in Fig. 2 *A*. The number beside the fluorescence profile is the cation-to-anion charge ratio. For the titration of pCMV5, the charge ratio is determined as moles of DOTMA/moles of phosphorus in the plasmid. As the pCMV5 content increases, increased fusion is observed.

A comparison between plasmid-induced fusion and that induced by other anions is provided in Fig. 2, *B* and *C*. For the titration of citrate and NaCl, the number beside each fluorescence profile is the respective anion concentration. To achieve 80% fusion at 3.5 min after the addition of anions, a phosphorus concentration of pCMV5 of 0.063 mM (charge ratio = 3.0) is required; whereas 0.93 mM citrate carboxyl (charge ratio = 0.20) and 800 mM NaCl (charge ratio = 2.3×10^{-4}) are required. Thus, on a per-molecule basis, pCMV5 is ~5.0×10^4-fold more effective at inducing membrane fusion between 1:1 DOTMA/DOPE LUVs than citrate, which in turn is about 2.7×10^3-fold more effective than NaCl.

Addition of pCMV5 to DOTMA/DOPE (1:1) LUVs causes the formation of large lipid structures characterized by isotropic motional averaging

The structural characteristics of the DNA-cationic lipid complexes are of obvious interest. ^{31}P NMR was used to identify the behavior of DOPE in the complexes; and

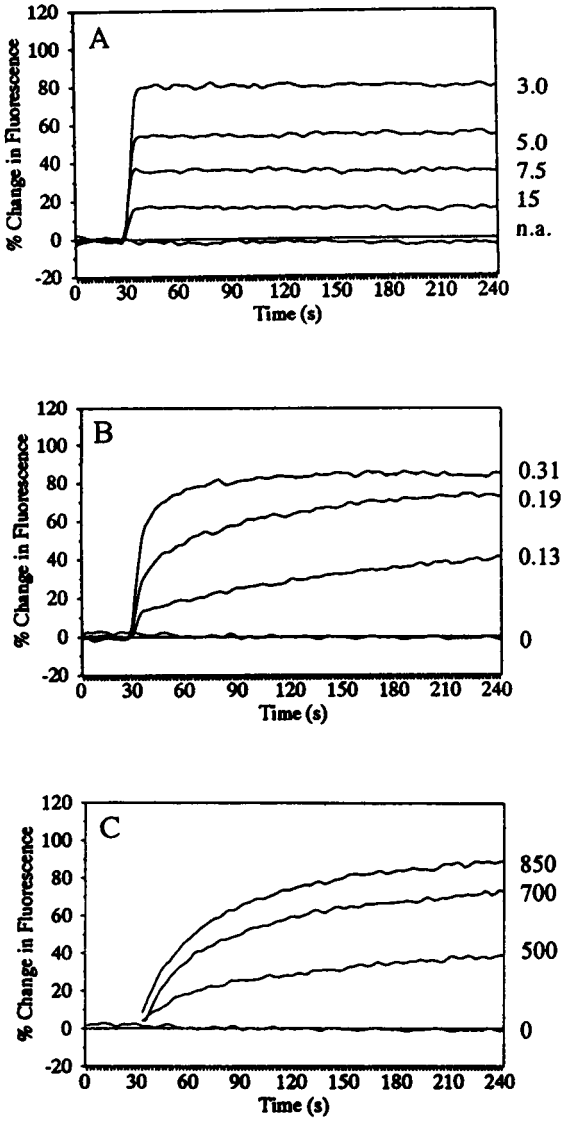

FIGURE 2 Effect of (*A*) pCMV5 plasmid, (*B*) citrate, and (*C*) NaCl on fusion of DOTMA/DOPE (1:1) vesicles in 20 mM HEPES (pH 7.4) at 25°C. Vesicles were prepared with and without 0.5 mol% each of NBD-PE and Rh-PE. The labeled and unlabeled vesicles were mixed in a 1:9 ratio and diluted to 0.38 mM total lipid. Different anions were added at 30 s. The concentration of citrate and NaCl (mM), and the charge ratio of DNA-cationic lipid complexes are indicated. The system without DNA is indicated by "n.a." The charge ratio is defined as moles of DOTMA/moles of phosphorus in pCMV5.

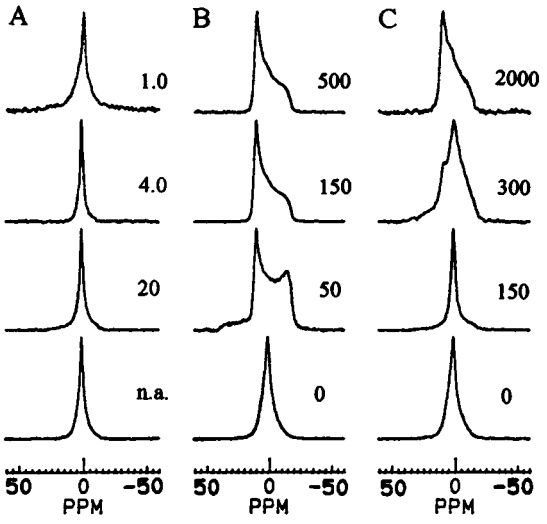

FIGURE 3 ^{31}P NMR spectra of DOTMA/DOPE (1:1) LUVs with sequential addition of pCMV5 plasmid (*A*), citrate (*B*), and NaCl (*C*) in HEPES (10–20 mM; pH 7.4) at 25°C. The charge ratio of DNA-cationic lipid complexes and the concentration of citrate and NaCl (mM) are indicated. The system without pCMV5 is indicated by "n.a." The charge ratio is defined as moles of DOTMA/moles of phosphorus in pCMV5.

freeze-fracture electron microscopy was used to examine local structure. As shown in Fig. 3, DOTMA/DOPE (1:1) LUVs at 25°C exhibit a narrow "isotropic" ^{31}P NMR signal with a peak width at half-height of ~7.0 ppm. The addition of pCMV5 to a charge ratio of 1.0 does not cause significant broadening (Fig. 3 *A*). An additional broad ^{31}P NMR signal with a half-width of ~100 ppm was detected beneath this broad isotropic signal (data not shown). Two lines of evidence indicate that this broad line originated primarily from plasmid phosphorus. First, the intensity of the narrow ^{31}P NMR component was not significantly affected by the addition of plasmid DNA. At the 1.0 charge ratio, for example, the intensity of the narrow component was ~92% of that observed before the addition of plasmid. Second, the addition of pCMV5 plasmid to pure DOTMA LUVs (charge ratio 1.0) resulted in a broad spectral feature with a width of ~100 ppm (data not shown). Thus the observed isotropic ^{31}P NMR signal and particle sizes of microns or larger from quasielastic light scattering (QELS) measurement indicate large systems with rapid isotropic motion of the phospholipid. This can correspond to a variety of lipid structures, including small bilayer vesicles or nonbilayer structures such as cubic phases (Cullis et al., 1985).

To visualize the structures formed on the addition of plasmid to DOTMA/DOPE (1:1) LUVs, freeze-fracture EM studies were performed. In the absence of plasmid, vesicles with an average size of 100 nm were observed (Fig. 4 *A*). When pCMV5 plasmid was added to achieve a ± charge ratio of 600, the majority of the vesicles remained in dispersion (data not shown). At a charge ratio of 20, most vesicles aggregated into small clusters, with little evidence of fusion (Fig. 4 *B*). This is consistent with the 20% lipid mixing observed employing the fusion assay (Fig. 2 *A*). At a charge ratio of 4.0, similar aggregated vesicles fuse into complexes with sizes ranging from 200 to 1000 nm (Fig. 4 *C*). Structures that resemble lipidic particles (Verkleij et al., 1979) arranged in a row were occasionally observed (*arrow 1*). The observation of large complexes correlates with the results of the fluorescent fusion assay, in which 80% fusion was observed at a charge ratio of 3.0 (Fig. 2 *A*). At charge ratios of 1.0 and lower, similar aggregates of fused lipid assemblies were observed (data not shown).

The structural behavior of DOTMA/DOPE LUVs in the presence of other anions was also examined by ^{31}P NMR.

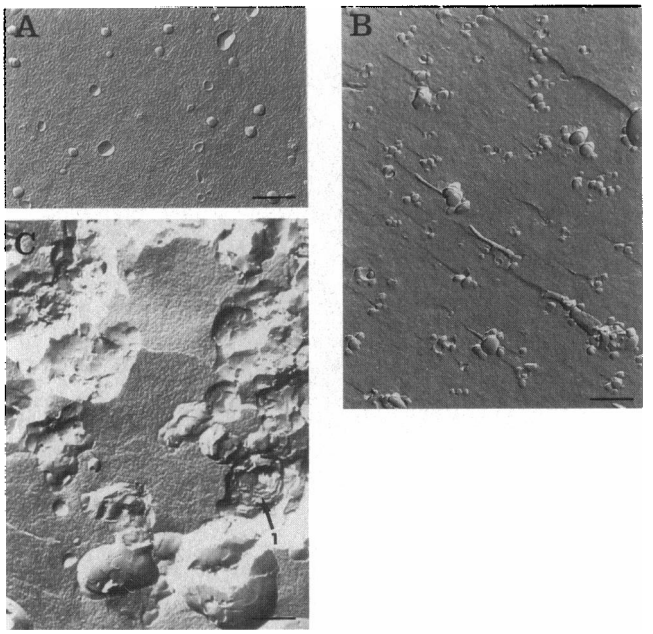

FIGURE 4 Freeze fracture electron micrographs of DOTMA/DOPE (1:1) LUVs in HEPES buffer (10–20 mM, pH 7.4) with different amounts of pCMV5. DOTMA/DOPE (1:1) LUVs (*A*) in the absence of pCMV5; (*B*) in the presence of pCMV5 (charge ratio of 20); and (*C*) in the presence of pCMV5 (charge ratio of 4.0) are shown. The bar on all electron micrographs represents 200 nm and the shadow direction is running from bottom to top. The original magnification was 20,000×.

Titration of DOTMA/DOPE LUVs with chloride and citrate was performed at pH 7.4, and the respective ^{31}P NMR spectra are shown in Fig. 3, *B* and *C*. At a high NaCl concentration of 300 mM, a mixture of bilayer, isotropic, and H_{II} signals was observed (Fig. 3 *C*). The size of the system increased from ~100 nm to ~600 nm, as detected by QELS. The further addition of NaCl (0.7 to 2.0 M) induced formation of the H_{II} phase. In contrast, mixtures of bilayer and H_{II} signals were observed in the range of 10–50 mM citrate (Fig. 3 *B*). Further addition of citrate induces complete H_{II} structural organization in the DOTMA/DOPE LUVs.

Addition of pCMV5 to DOTMA/DOPC (1:1) LUVs causes formation of large bilayer lipid structures

Previous studies have shown that the DOPE "helper" lipid is essential for efficient transfection employing DNA-cationic lipid complexes (Leventis and Silvius, 1990; Farhood et al., 1995). This likely arises from the well-known preference of DOPE for nonbilayer structures, some of which may play a direct role in membrane fusion (Cullis et al., 1985; Litzinger and Huang, 1992). It is therefore of interest to examine the structural and fusogenic properties of DNA-cationic lipid complexes in which DOPE is replaced with the bilayer forming lipid, DOPC. As shown in Fig. 5 *A*, considerable fusion was observed when pCMV5 plasmid was added to DOTMA/DOPC (1:1) LUVs. Compared with DOTMA/

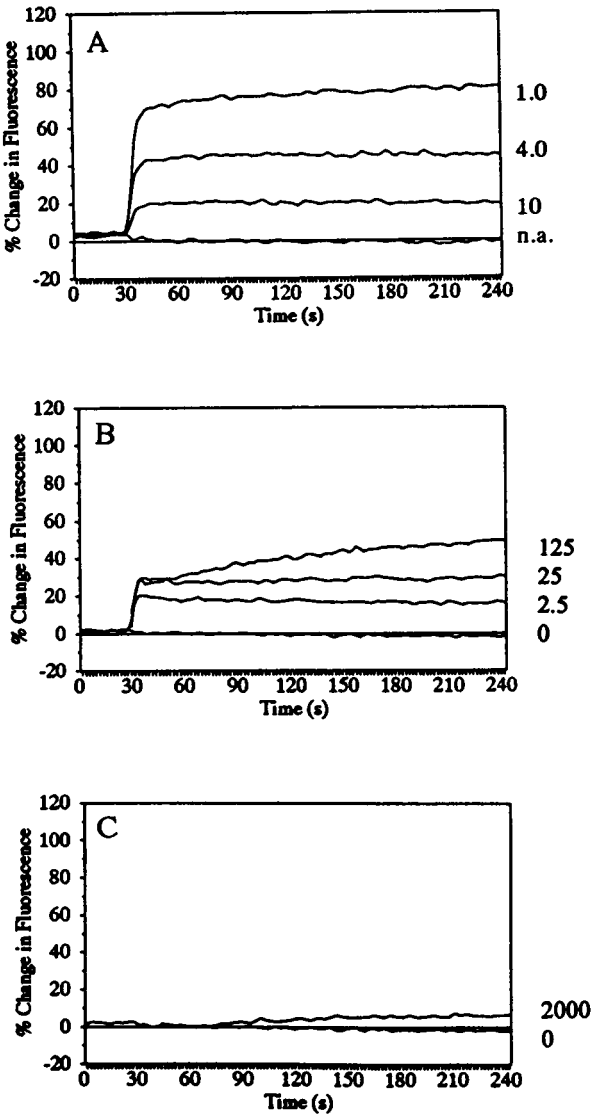

FIGURE 5 Effect of (*A*) pCMV5 plasmid, (*B*) citrate, and (*C*) NaCl on fusion of DOTMA/DOPC (1:1) vesicles in 20 mM HEPES (pH 7.4) at 25°C. Vesicles were prepared with and without 0.5 mol% each of NBD-PE and Rh-PE. The labeled and unlabeled vesicles were mixed in a 1:9 ratio and diluted to 0.50 mM total lipid. Different anions were added at 30 s. The concentration of citrate and NaCl (mM), and the charge ratio of DNA-cationic lipid complexes are indicated. The system without pCMV5 is indicated by "n.a." The charge ratio is defined as moles of DOTMA/moles of phosphorus in pCMV5.

DOPE (1:1) LUVs, DOTMA/DOPC (1:1) LUVs require ~1.5–2.0-fold more pCMV5 to obtain the same level of lipid mixing. In ^{31}P NMR studies, when pCMV5 was added to DOTMA/DOPC (1:1) LUVs, an isotropic signal was maintained at charge ratios greater than 8.3; however, at higher pCMV5 content (lower ± charge ratios), mixtures of isotropic and bilayer signals were detected (Fig. 6 *A*). Again, quasielastic light scattering studies revealed a size increase to microns or larger at high pCMV5 levels (charge ratio of 2.2). Freeze-fracture EM studies of these complexes revealed behavior similar to that of the DOPE-containing

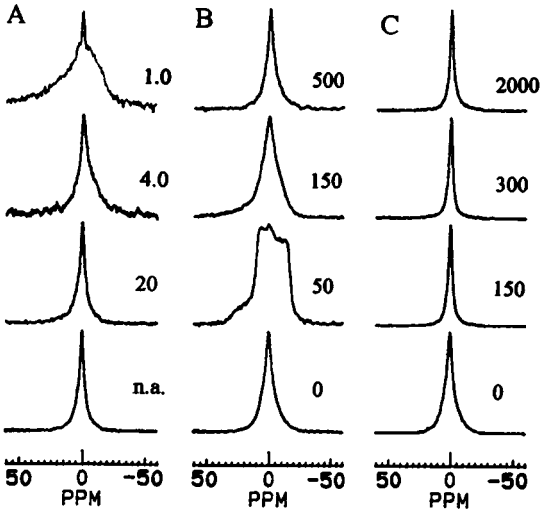

FIGURE 6 ^{31}P NMR spectra of DOTMA/DOPC (1:1) LUVs with the sequential addition of pCMV5 plasmid (*A*), citrate (*B*), and NaCl (*C*) in HEPES (10–20 mM; pH 7.4) at 25°C. The charge ratio of DNA-cationic lipid complexes and the concentration of citrate and NaCl (mM) are indicated. The system without pCMV5 is indicated by "n.a." The charge ratio is defined as moles of DOTMA/moles of phosphorus in pCMV5.

systems; however, in the large fused systems obtained at low charge ratios, structures that could correspond to lipidic particles were not observed (Fig. 7).

The effects of citrate and chloride on the structural and fusogenic behavior of DOTMA/DOPC (1:1) LUVs were also examined. As shown in Fig. 5 *B*, $\sim 10^3$ times as much citrate in DOTMA/DOPC LUVs is required to induce the same level of lipid mixing as for DOTMA/DOPE LUVs. No significant lipid mixing was observed at NaCl concentrations of up to 2.0 M (Fig. 5 *C*), in agreement with the observation of an isotropic ^{31}P NMR signal (Fig. 6 *C*), with no significant increase in the size of vesicles, as detected by QELS and freeze-fracture techniques (data not shown). However, when citrate was added, unusual behavior was observed. A mixture of bilayer, isotropic, and H_{II} ^{31}P NMR signals was detected at 10–100 mM citrate (Fig. 6 *B*), and at 150 mM or higher concentrations of citrate, an isotropic lineshape was observed. The size of the DOTMA/DOPC (1:1) system in the presence of $\geq$150 mM citrate is at least 100-fold larger than that of the untreated vesicles as detected by QELS, again suggesting the formation of cubic or other nonbilayer structures giving rise to isotropic NMR spectra.

DOTMA/DOPE LUVs fuse with anionic vesicles and pCMV5 inhibits such fusion

For transfection to occur, the DNA-cationic lipid complexes must first fuse with the target cell membrane, which bears a negative charge due to sialic acid residues in the glycocalyx. As a simple model for this interaction, fusion between DOTMA/DOPE (1:1) LUVs or DNA-cationic lipid complexes, and anionic LUVs containing phosphatidylserine

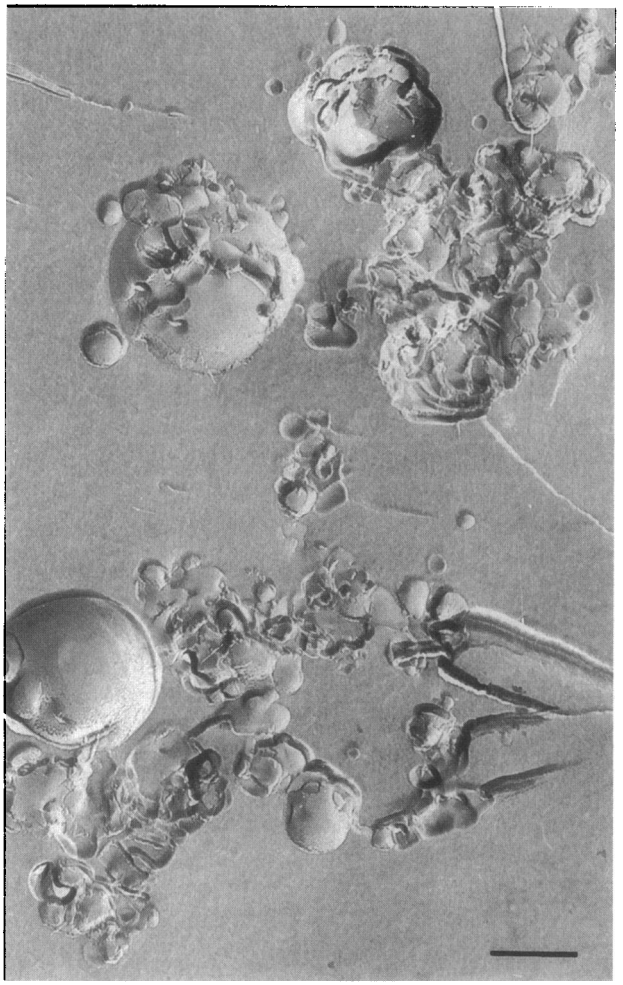

FIGURE 7 Freeze fracture electron micrographs of DOTMA/DOPC (1:1) LUVs in HEPES buffer (20 mM, pH 7.4) in the presence of pCMV5 at a charge ratio of 4.0. The bar on the electron micrograph represents 200 nm and the shadow direction is running from bottom to top. The original magnification was 20,000×.

(PS) was examined. As shown in Fig. 8, no fusion was observed when DOTMA/DOPE LUVs were incubated with DOPC LUVs. As the DOPS content was increased to 2 mol% in the DOPS/DOPC LUVs, the amount of fusion with DOTMA/DOPE LUVs increased considerably. When pure DOPS LUVs were used, 65% fusion at 3.5 min after addition was observed. If DOPE was substituted for DOPC in the DOPS-containing vesicles, the rate and extent of fusion increased markedly (data not shown).

The influence of pCMV5 on fusion between cationic and anionic LUVs is illustrated in Fig. 9. In these experiments, the fluorescently labeled DOTMA/DOPE LUVs were first mixed with pCMV5, and the complexes were then incubated with nonlabeled DOPS/DOPC (1:1) vesicles. When no pCMV5 plasmid was present, 60% fusion was observed; however, at higher pCMV5 levels corresponding to charge ratios of 0.91 or smaller, fusion was markedly inhibited. Fusion of the same complexes with DOPS/DOPE (1:1) LUVs showed similar behavior. In the absence of plasmid,

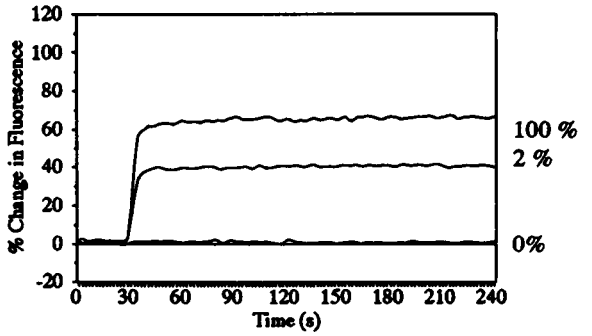

FIGURE 8 Fusion of DOTMA/DOPE (1:1) LUVs with various compositions of DOPS/DOPC LUVs in 20 mM HEPES (pH 7.4) at 25°C. The percentage of DOPS content in each vesicle system is indicated. Fusion assays were carried out as described in Materials and Methods.

80% fusion was observed; whereas the presence of pCMV5 at a charge ratio of 1.0 reduced the fusion index to 60%, and only 5% fusion was observed at a charge ratio of 0.83 (data not shown).

Complexes formed by the addition of plasmid DNA to cationic LUVs can be separated into two fractions by centrifugation

In an attempt to understand the origin of the isotropic ^{31}P NMR resonance observed for pCMV5-DOTMA/DOPE (1:1) at a charge ratio of 1.0 (Fig. 3), the complexes were separated into two fractions by centrifugation. Briefly, it was reasoned that the dispersion may consist of a dense DNA-rich fraction and vesicles with little associated DNA that make a dominant contribution to the narrow ^{31}P NMR signal. As shown in Fig. 10, centrifugation of the pCMVβgal containing complexes at 11,000 $\times$ g for 15 min resulted in a proportion in the pellet that was sensitive to the charge ratio. The maximum amount of lipid and DNA in the pellet fraction appears in the region with a charge ratio of

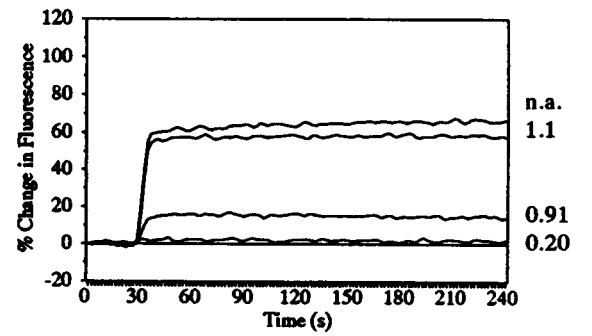

FIGURE 9 Fusion of pCMV5-DOTMA/DOPE (1:1) complexes with DOPS/DOPC (1:1) LUVs in 20 mM HEPES (pH 7.4) at 25°C. A 0.024 mM DOTMA concentration was used in each assay. Fusion assays were carried out as described in Materials and Methods. The charge ratio of each system is indicated and is defined as moles of DOTMA/moles of phosphorus in pCMV5. The system without pCMV5 is indicated by "n.a."

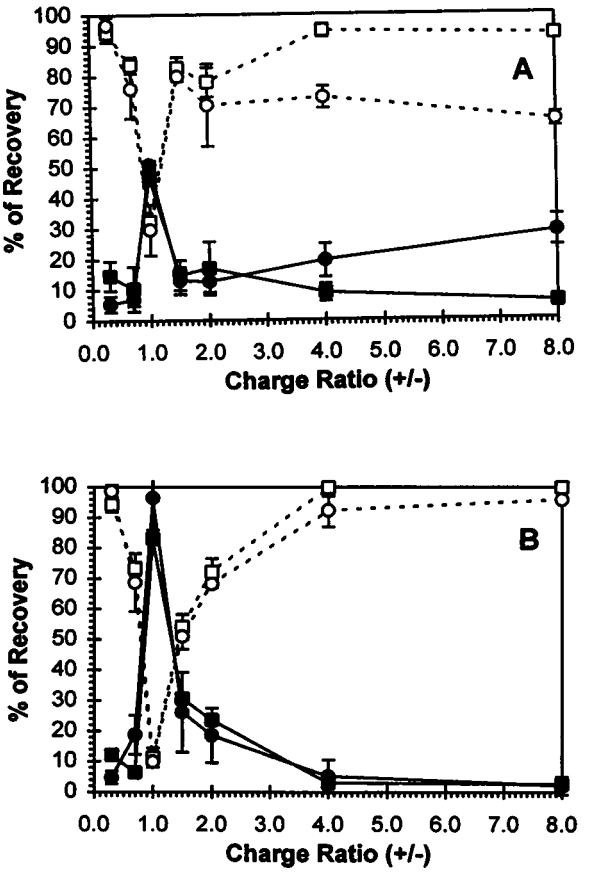

FIGURE 10 DNA and lipid recovery in supernatant and pellet fractions after centrifugation of DNA-cationic lipid complexes. Trace amounts of ^{3}H pCMVβgal and ^{14}C CHOL-HEX, and 20 μg pCMVβgal were used for both pCMVβgal-DOTMA/DOPE (*A*) and pCMVβgal-DOTMA/DOPC (*B*). DNA (○, ●) and lipid (□, ■) recovery in the supernatant (– – –) and the resuspended pellet fractions (——) are shown. The charge ratio is defined as moles of DOTMA/moles of phosphorus in pCMVβgal.

1.0. Contrary to expectation, there was no evidence for a DNA-rich pellet; the lipid and DNA were separated in approximately equal proportions into the pellet and supernatant. It is therefore probable that the pellet and supernatant fractions differ in size rather than in density, with the larger complexes contributing the pellet fraction. Notably, when DOPC was substituted for DOPE, the pellet fraction was increased to over 90% of the lipid and plasmid as compared to 50% for the DOPE-containing system.

The ^{31}P NMR characteristics of the pellet and supernatant at a charge ratio of 1.0 for both DOPE- and DOPC-containing complexes are shown in Fig. 11. The supernatant ^{31}P NMR peak remains narrow in both cases. As may be expected, the ^{31}P NMR resonance for the pellet fractions is broader; however, the resonance observed for the pellet obtained from the DOPE-containing system is again substantially narrower than the bilayer lineshape derived from the DOPC-containing system. Similar ^{31}P NMR phenomena were observed for both pCMV5- and pCMVβgal-containing complexes (data not shown).

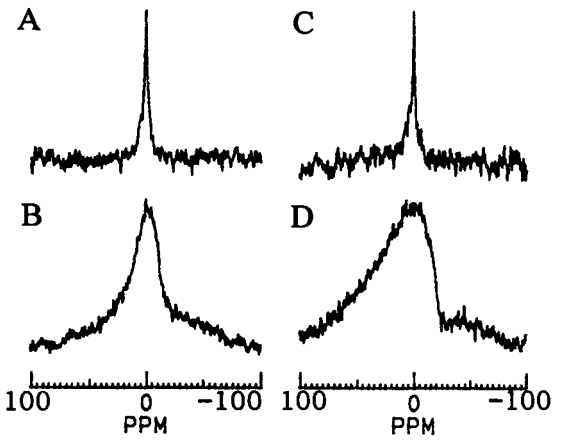

FIGURE 11 ^{31}P NMR spectra of pCMV5-cationic lipid complexes at a charge ratio of 1.0 in HEPES buffer (20 mM; pH 7.4) at 25°C. ^{31}P NMR spectra are shown for the supernatant (*A*, *C*) and the pellet fractions (*B*, *D*) of pCMV5-DOTMA/DOPE complexes (*A*, *B*) and pCMV5-DOTMA/DOPC complexes (*C*, *D*) after centrifugation for 15 min at 11,000 × *g*. The charge ratio is defined as moles of DOTMA/moles of phosphorus in pCMV5.

The transfection potency of pCMVβgal-DOTMA/DOPE (1:1) complexes correlate with the pellet fraction

The relation between the structural and motional properties of the DNA-cationic lipid complexes and their ability to transfect target cells is of obvious importance. The transfection of BHK cells employing pCMVβgal-DOTMA/DOPE (1:1) complexes at various charge ratios is illustrated in Fig. 12. It may be observed that maximum transfection is observed at a charge ratio of 1.0, in agreement with previous studies (Gao and Huang, 1991; Felgner et al., 1994). No transfection was observed at charge ratios below 1.0; and the levels of transfection decrease to background levels at charge ratios greater than 4.0. The importance of DOPE as a "helper" lipid is clear, again in agreement with previous studies (Felgner et al., 1987, 1994; Farhood et al., 1995), as no significant transfection is observed when DOPC is substituted for DOPE. No transfection was observed in the absence of plasmid or lipid.

The next set of transfection studies examined the relative transfection potencies of the pellet and supernatant fractions. Remarkably, as shown in Fig. 13, the pellet fractions containing large complexes exhibited significantly greater transfection potency than the unfractionated complex or the supernatant fractions. Interestingly, the reduction in transfection potency of the unfractionated complexes at higher charge ratios (Fig. 12) corresponds approximately to the amount of material that can be pelleted by centrifugation (Fig. 10).

DISCUSSION

This investigation was focused on characterizing the structural, motional, and fusogenic properties of DOTMA/DOPE systems in isolation and in the presence of plasmid DNA. There are three major points of interest in the results obtained. These concern the ability of plasmid DNA and other multivalent anions to induce fusion between cationic LUVs, the structural and motional features of the complexes formed, and the relation between these features and transfection potential. We discuss these areas in turn.

The ability to form DOTMA/DOPE LUVs relies on the ability of the cationic lipid DOTMA to stabilize DOPE into a bilayer organization, which is similar to the ability of

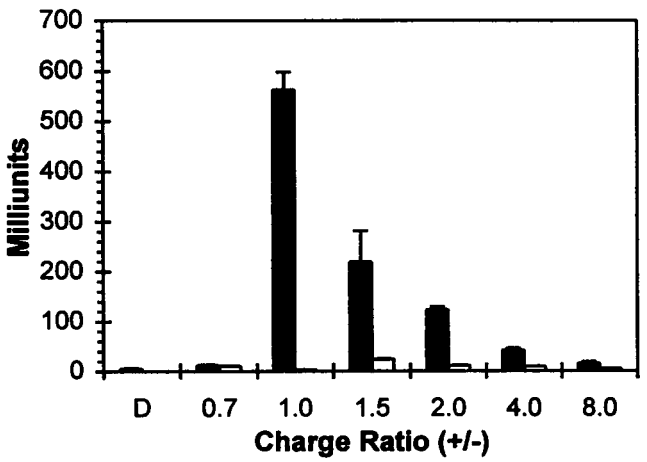

FIGURE 12 Transfection of BHK cells by pCMVβgal-cationic lipid complexes at various charge ratios. Triplicate results are shown for the transfection of pCMVβgal-DOTMA/DOPE (1:1) complexes (■) and pCMVβgal-DOTMA/DOPC (1:1) complexes (□) on BHK cells, as determined by the β-galactosidase assay described in Materials and Methods. The charge ratio is defined as moles of DOTMA/moles of phosphorus in pCMVβgal. Transfection by "naked" pCMVβgal is indicated by column D.

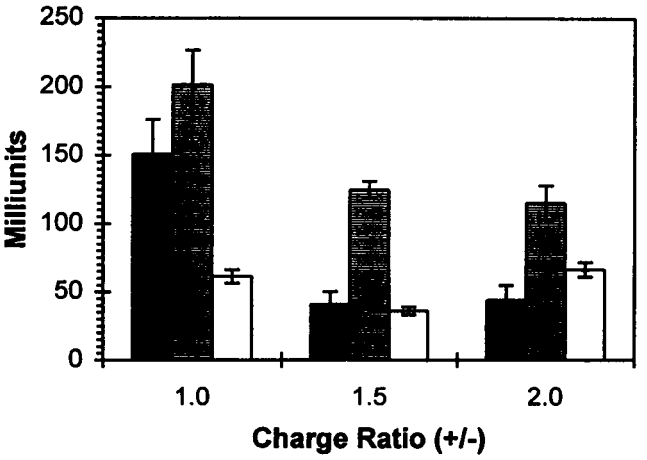

FIGURE 13 Transfection of BHK cells by fractionated and unfractionated DNA-cationic lipid complexes obtained by centrifugation. Triplicate results are shown for the transfection of the unfractionated pCMVβgal-DOTMA/DOPE (1:1) complexes (■), the pellet fractions (▤), and the supernatant fractions (□) at charge ratios (±) of 1.0, 1.5, and 2.0, as determined by the β-galactosidase assay described in Materials and Methods. No transfection was observed in the presence of "naked" pCMVβgal.

anionic phospholipids, such as phosphatidylserine (PS), to stabilize DOPE into the bilayer phase (Cullis and de Kruijff, 1979). Furthermore, the ability of polyanions such as plasmid DNA to trigger fusion between DOTMA/DOPE (1:1) LUVs is analogous to the ability of mono- and divalent cations to trigger fusion between PS/PE vesicles, as demonstrated extensively elsewhere (Tilcock and Cullis, 1981; Bally et al., 1983; Hope et al., 1983; Düzgünes and Papahadjopoulos, 1983; Düzgünes et al., 1987). The mechanism whereby divalent cations such as Ca^{2+} can induce fusion in these systems has been shown to arise primarily from effects related to the neutralization of the surface charge due to the anionic lipid rather than lateral segregation of the anionic species into local domains, although lateral segregation can occur in certain situations (Tilcock et al., 1984). Neutralization of the surface charge leads to a reduced effective headgroup size of the charged lipid species and correspondingly reduced ability to stabilize the bilayer organization. In addition, this reduced surface charge leads to reduced intervesicular electrostatic repulsion, thus promoting the aggregation step required for fusion. It is likely, but not proven, that the ability of plasmid to induce fusion in the DOTMA/DOPE LUVs investigated here is also due to charge neutralization effects, as there is no evidence of lateral segregation of DOTMA. Two observations support this. First, the only situation giving rise to lateral phase separation of PS in PS/PE systems arises because of segregation of PS-Ca^{2+} complexes into crystalline domains. There was no evidence, as detected by freeze-fracture, for the presence of crystalline domains, which give rise to characteristic freeze-fracture morphology. Second, the ^{31}P NMR spectra arising from the DOTMA/DOPE vesicles in the presence of plasmid are not consistent with lateral segregation of the DOTMA component. Specifically, if the DOTMA component was appreciably laterally segregated, DOPE would be expected to revert to the H_{II} phase organization it adopts in isolation at high plasmid levels. This is not observed (Fig. 3 *A*). It should be noted that this behavior contrasts with the behavior of the DOTMA/DOPC systems in the presence of plasmid DNA. In this case, the ability of plasmid to cause fusion resulting in DOPC in the bilayer organization it adopts in isolation is fully consistent with sequestration of the DOTMA by the plasmid. This is also consistent with recent work by Mitrakos and Macdonald (1996), who describe the ability of polyadenylic acid to sequester cationic lipids in mixtures with PC.

Lipid mixing studies characterizing fusion of cationic vesicles induced by monovalent and multivalent anions have been reported previously (Rupert et al., 1985; Stamatatos et al., 1988; Düzgünes et al., 1989). This includes oligonucleotides (Jääskeläinen et al., 1994) as well as genomic DNA (Gershon et al., 1993). The studies presented here allow a comparison of the potency of plasmid DNA with anions of different valence. The high potency of pCMV5 plasmid DNA for fusing DOTMA/DOPE LUVs as compared to chloride or citrate anions can be attributed to the much tighter binding expected for multivalent anions to cationic surfaces, as well as an ability of the DNA polymer to cross-link vesicles, thus directly promoting aggregation. This aggregation potential is observed at low plasmid levels in DOTMA/DOPE systems (Fig. 4 *B*), where the formation of small clusters precedes the formation of highly fused complexes. Lipid mixing studies demonstrating fusion between cationic and anionic vesicles have also been reported previously (Stamatatos et al., 1988; Düzgünes et al., 1989; Bailey and Cullis, 1997). The main point of the studies presented here concerns the ability of high levels of plasmid DNA to inhibit fusion between DOTMA/DOPE systems and anionic (DOPS/DOPC) systems. This is fully consistent with charge repulsion effects inhibiting interactions between the DNA-cationic lipid complexes and negatively charged vesicles. Related effects have been observed for oligonucleotide-cationic lipid complexes (Jääskeläinen et al., 1994). Such effects are also consistent with the inhibition of transfection at high plasmid DNA-to-cationic lipid levels (Fig. 12), which presumably reflect reduced association with target cells.

The nature of the complexes formed on the addition of plasmid DNA to DOTMA/DOPE vesicles and the relation between this structure and transfection potential is of importance. Previous studies on the relation between the structure of cationic lipid-plasmid DNA complexes and transfection potential have focused primarily on morphology as detected by electron microscopy techniques. The work of Sternberg et al. (1994) suggests that transfection potency correlates with the presence of long strands of DNA encapsulated in tubules of lipid. Such structures were not observed in the freeze-fracture studies performed here, although lipidic particle structures associated with nonbilayer lipid structures were observed, as also reported by Sternberg et al. (1994). As indicated below, the presence of nonbilayer lipid structure is consistent with the ^{31}P NMR studies.

The most striking result of the studies presented here is that the more potent transfection systems contain lipid in large structures that allow significant isotropic motional averaging. The nonfractionated DOTMA/DOPE complexes formed with plasmid DNA exhibit a narrow isotropic ^{31}P NMR signal, whereas considerably broader bilayer ^{31}P NMR spectra are observed for the DOTMA/DOPC systems in the presence of similar levels of plasmid. The isotropic signal observed for the nonfractionated plasmid-DOTMA/DOPE complexes with high transfection ability cannot be attributed to small vesicular structures, as the fusion, light scattering, and freeze-fracture studies reveal the presence of large, highly fused, lipid aggregates. In the case of the system fractionated by centrifugation, the ^{31}P NMR resonance observed for the DOPE-containing system is broader but remains significantly narrower than that of nontransfecting counterparts containing DOPC. Isotropic ^{31}P NMR signals are characteristic of lipid dispersions that form nonbilayer structures such as cubic or other inverted phases (Cullis and de Kruijff, 1979; Ellens et al., 1989; Lindblom and Rilfors, 1989), and variants such as interlamellar attachment sites may play direct roles in membrane fusion phe-

nomena (Siegel et al., 1989). Such lipid organization could promote fusion or membrane destabilization after interaction with target cell membranes. The available evidence suggests that destabilization of the endosomal membrane is a dominant route for cationic liposome-mediated transfection (Farhood et al., 1995; Wrobel and Collins, 1995; Zabner et al., 1995; Friend et al., 1996; Xu and Szoka, 1996). The results presented here suggest that nonbilayer lipid structures could play a direct role in this process, thus facilitating the transfection event.

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