Characterization of Lipid DNA Interactions. I. Destabilization of Bound Lipids and DNA Dissociation

Pierrot Harvie, Frances M. P. Wong, and Marcel B. Bally
Division of Medical Oncology, Department of Advanced Therapeutics, British Columbia Cancer Agency, Vancouver, British Columbia V5Z 4E6, Canada

ABSTRACT We have recently described a method for preparing lipid-based DNA particles (LDPs) that form spontaneously when detergent-solubilized cationic lipids are mixed with DNA. LDPs have the potential to be developed as carriers for use in gene therapy. More importantly, the lipid-DNA interactions that give rise to particle formation can be studied to gain a better understanding of factors that govern lipid binding and lipid dissociation. In this study the stability of lipid-DNA interactions was evaluated by measurement of DNA protection (binding of the DNA intercalating dye TO-PRO-1 and sensitivity to DNase I) and membrane destabilization (lipid mixing reactions measured by fluorescence resonance energy transfer techniques) after the addition of anionic liposomes. Lipid-based DNA transfer systems were prepared with pInexCAT v.2.0, a 4.49-kb plasmid expression vector that contains the marker gene for chloramphenicol acetyltransferase (CAT). LDPs were prepared using *N*-*N*-dioleoyl-*N,N*-dimethylammonium chloride (DODAC) and either 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) or 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE). For comparison, liposome/DNA aggregates (LDAs) were also prepared by using preformed DODAC/DOPE (1:1 mole ratio) and DODAC/DOPC (1:1 mole ratio) liposomes. The addition of anionic liposomes to the lipid-based DNA formulations initiated rapid membrane destabilization as measured by the resonance energy transfer lipid-mixing assay. It is suggested that lipid mixing is a reflection of processes (contact, dehydration, packing defects) that lead to formulation disassembly and DNA release. This destabilization reaction was associated with an increase in DNA sensitivity to DNase I, and anionic membrane-mediated destabilization was not dependent on the incorporation of DOPE. These results are interpreted in terms of factors that regulate the disassembly of lipid-based DNA formulations.

INTRODUCTION

It has been argued that synthetic lipid-based vectors can be designed to transfer DNA, typically a plasmid expression vector, in a manner that is comparable to that of viruses. For expression to be achieved, lipid-based gene transfer formulations and viruses must share common attributes that facilitate cell binding, cellular uptake, membrane fusion/disruption, DNA release into the cytoplasm, as well as DNA transfer to the nucleus (Chowdhury et al., 1993; Wagner et al., 1992). There are important differences, however, particularly considering that synthetic DNA delivery systems do not typically incorporate the DNA-binding proteins required for nuclear localization and transcription factors important in the regulation of gene expression. Although improvements in the design of synthetic DNA vectors may require the incorporation of such proteins (Boulikas, 1996; Cristiano and Curiel, 1996), it is important to remember that the primary advantage of lipid-based DNA transfer technology is its simplicity. Because in vitro studies have established that synthetic DNA delivery systems can efficiently transfer plasmid DNA into cells (Reimer et al., 1997), it can be suggested that low levels of transgene expression are due, in part, to problems associated with intracellular processing of the lipid-based DNA formulations.

It is reasonably well established that formation of lipid-based DNA delivery systems is a consequence of a self-assembly process triggered by electrostatic interactions between cationic lipids and DNA (Lasic and Templeton, 1996). As first demonstrated by Felgner et al. (1987), cationic liposomes can be added to plasmid DNA, generating a complex that forms via electrostatic interactions (Felgner and Ringold, 1989). This complex, essentially a liposome-DNA aggregate (LDA), is effective at delivering plasmid DNA to cells and can facilitate transfection of many cell types in vitro and in vivo (Lasic and Templeton, 1996). The addition of DNA to preformed cationic liposomes, however, triggers significant structural changes in the liposomes as well as the DNA (Fig. 1). The resulting formulation is heterogeneous, and it has proved difficult to define factors that control the liposome-DNA aggregation reaction. For this reason, investigators have been motivated to develop novel lipid-based formulations that do not involve preformed liposomes. We have demonstrated that hydrophobic lipid-DNA complexes can be prepared by mixing DNA with cationic lipids in monomeric or micellar form (Reimer et al., 1995; Wong et al., 1996). This complex can be used as a well-defined intermediate in the preparation of lipid-DNA particles (LDPs), which form as a consequence of a self-assembling process (Fig. 1) (Bally et al., 1997; Zhang et al., 1997). In the presence of an appropriate nonionic detergent, mixed micelles containing cationic lipids interact with DNA

Received for publication 10 November 1997 and in final form 1 May 1998.

Address reprint requests to Dr. Pierrot Harvie, Division of Medical Oncology, Department of Advanced Therapeutics, British Columbia Cancer Agency, 600 West 10th Avenue, Vancouver, BC V5Z 4E6, Canada. Tel.: 604-877-6098, ext. 3155; Fax: 604-877-6011; E-mail: pharvie@bccancer.bc.ca.

0006-3495/98/08/1040/12 $2.00

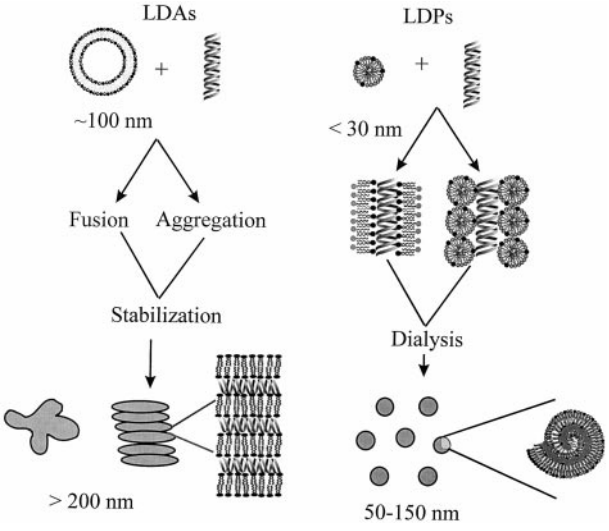

FIGURE 1 Schematic description of intermediates that may be involved in liposome/DNA aggregate (LDA) or lipid/DNA particle (LDP) formation.

to spontaneously form small particles (<200 nm) (Zhang et al., 1997).

The structures that have been proposed for lipid-based DNA formulations are as varied as the procedures used to prepare them (Templeton et al., 1997). For simplicity, it is perhaps easiest to imagine that these formulations are complex mixed lipid micelles in which associated DNA is protected by bound lipids. In this context micelles are defined, as suggested by Tanford (1991), as "any water-soluble aggregate spontaneously and reversibly formed from amphiphiles." It is not clear whether bound lipids are organized in a bilayer structure or whether a hydrophobic complex of bound lipids and DNA is surrounded by a lipid monolayer. It is important to elucidate the role of lipid constituents in the self-assembly process that leads to formation of these complex micelles, particularly because this information will be required to develop rationally designed formulations for therapeutic applications. It is equally important that we gain better insight into the disassembly process that results in the release of DNA from the macromolecular structure, a requirement following cell uptake.

The development of methods that model intracellular processing events has been useful in terms of assigning definitive roles of individual lipid components, as well as increasing our understanding of how macromolecular structure may influence these roles. For this reason studies completed in the laboratory of Frank Szoka (Xu and Szoka, 1996) are of particular interest. These investigators demonstrated, using preformed cationic liposome-DNA aggregates, that anionic membranes promote liposome-DNA aggregate dissociation. It was postulated that after endocytosis of cationic liposomes/nucleic acid aggregates, cationic lipids can induce a flip-flop of anionic lipids from the cytoplasmic face of the endosomal membrane, resulting in destabilization of the complex and release of the nucleic acid (Xu and Szoka, 1996; Zelphati and Szoka, 1996). We suggest here that this assay can be used as a tool for characterization of lipid-DNA interactions and the factors that bring about the disassembly of lipid-based DNA formulations. The studies described here focus on the disassembly process in an effort to establish how associated lipids and macromolecular structure influence micellar destabilization.

MATERIALS AND METHODS

Materials

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dimyristoyl-*sn*-glycero-3-phospho-L-serine (DOPS), 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG), L-α-phosphatidylethanolamine-*N*-(4-nitrobenzo-2-oxa-1,3-diazole) (NBD-PE), and L-α-phosphatidylethanolamine-*N*-(lissamine rhodamine B sulfonyl) (Rh-PE) were purchased from Avanti Polar Lipids (Alabaster, AL). Steven Ansell from Inex Pharmaceuticals Corp. (Vancouver, BC) synthesized *N*-*N*-dioleoyl-*N*,*N*-dimethylammonium chloride (DODAC). TO-PRO-1 was purchased from Molecular Probes (Eugene, OR). *N*-Octyl-β-D-glucopyranoside (OGP) and bovine pancreas DNase I were purchased from Sigma Chemical Company (St. Louis, MO). Dialysis membranes (SPECTRA/POR, MW cutoff 12,000–14,000) were purchased from Fisher Scientific (Ottawa, ON). All other chemicals used were reagent grade, and all solvents used were high-performance liquid chromatography grade. The B16/BL6 murine melanoma cell line was obtained from NCI Tumor Repository 12-102-54 (Bethesda, MD).

The plasmid pInexCAT v2.0, containing the *Escherichia coli* chloramphenicol acetyltransferase (CAT) gene under the control of the CMV promoter, was constructed and provided by Roger Graham of Inex Pharmaceuticals. Briefly, the CAT gene, containing the alfalfa mosaic virus (AMV) translational enhancer, was cleaved from pCMV4CAT plasmid (generously provided by K. Brigham, Toronto), using *Not*I restriction endonuclease. Similarly, the *Not*I fragment of pCMVβ obtained from Clontech (Palo Alto, CA) was removed, and the CAT gene was inserted into this site. The resulting plasmid, pInexCAT v2.0, is 4490 bp and includes the CMV promoter, the SV40 intron for processing the message, the AMV enhancer, and the SV40 polyadenylation signals. The plasmid was isolated by standard molecular techniques (Sambrook et al., 1989) and purified with a Qiagen Plasmid Purification Kit (Qiagen, Chatsworth, CA). The nucleic acid concentration was measured by UV absorption at 260 nm and verified by electrophoresis on 0.8% agarose gels.

Methods

Preparation and characterization of lipid/DNA particles

Lipid/DNA particles were generated by the method of Zhang et al. (1997), as modified by Wong et al. (manuscript submitted for publication). The lipid mixtures (DODAC:DOPE 50:50 mol% or DODAC:DOPC 50:50 mol%) were dissolved in chloroform:methanol (1:1) in a borosilicate glass tube. Solvent was evaporated under N_2 to obtain a thin lipid film. Residual solvents were removed under vacuum for at least 1 h at a pressure of <50 mTorr in a freeze-dry system (LabConco, Kansas City, MO). The lipid films were solubilized in 35 mM OGP such that the final lipid concentration was 1.7 mM. Plasmid DNA (pInexCAT v2.0) in an equal volume of 35 mM OGP was added to the mixed lipid-detergent micelles such that the desired charge ratio (cationic lipid to anionic nucleotide phosphate ratio) was achieved. Particles form spontaneously, i.e., the sample becomes cloudy. The mixture was then dialyzed for 72 h against sterile water with changes every 12 h. Particle size distribution and homogeneity were evaluated after dialysis by quasielastic light scattering (QELS), using a Nicomp 270 submicron particle sizer (Pacific Scientific, Santa Barbara, CA) operating at 632.8 nm. LDPs typically exhibited a mean diameter of 100–200 nm.

Preparation of liposome/DNA aggregates

Cationic liposomes were prepared by the method of Hope et al. (1986). Briefly, lipid films (DODAC:DOPE 50:50 mol% or DODAC:DOPC 50:50 mol%) were prepared as described above and hydrated in sterile water to achieve a final lipid concentration of 40 mM. Multilamellar vesicles (MLVs) were prepared by mechanical agitation and were subsequently subjected to five freeze-thaw cycles before the sample was extruded 10 times through two stacked 0.1-μm pore size polycarbonate membranes (Nuclepore, Cambridge, MA). Extrusion was done with an Extruder (Lipex Biomembranes, Vancouver, BC) operating at room temperature at pressures of $<$100 psi. Vesicle size distribution and homogeneity were evaluated by QELS. Typically, this procedure generated liposomes with mean diameters of 80–100 nm. Liposome/DNA aggregates were prepared on ice by adding 20 μg of DNA (pInexCAT v.2.0) to 200 nmol of lipids. The final charge ratio of these aggregates was 1.62:1 (+:−). After DNA addition, the mean particle size of the LDAs was typically 400-1000 nm.

Anionic liposome preparation

Liposomes containing anionic lipids were prepared according the method of Hope et al. (1986). Lipid films were prepared from chloroform solutions containing DOPS:DOPE:DOPC (1:2:1 mole ratio), DOPS:DOPC (1:1 mole ratio), or DMPG:DOPC (1:1 mol ratio) as described above and hydrated in 20 mM HEPES buffer (pH 7.4) to form multilamellar vesicles (MLVs) at 50 mM lipid concentration. The lipid mixture was subjected to five freeze-thaw cycles, and finally MLVs were extruded 10 times though two stacked 1.0-μm pore size polycarbonate membranes. Vesicle size distribution and homogeneity were evaluated by QELS, and the resulting liposomes exhibited a mean diameter between 200 and 400 nm.

In vitro cell transfection

B16/BL6 melanoma cells were plated at 6×10^3 cells/well in a 96-well plate containing Dulbecco's minimum essential medium supplemented with 10% fetal bovine serum (FBS) and incubated overnight at 37°C in 5% CO_2. At the time of transfection, medium was removed and 100 μl of LDPs or LDAs containing 2 μg of pInexCAT v2.0 in Dulbecco's minimum essential medium with 10% FBS was added to each well. Plates were incubated at 37°C for 4 h. Medium was removed and replaced by 100 μl of fresh medium for a further 48 h. Chloramphenicol acetyltransferase activity was then evaluated as described previously by Seed and Sheen (1988). Cells were washed extensively and transferred to Eppendorf microcentrifuge tubes. Samples were then subjected to three cycles of freeze/thaw and centrifuged at $8000 \times g$ in an Eppendorf microcentrifuge for 10 min at room temperature; the supernatants were recovered and heat-inactivated for 15 min at 65°C. Samples were centrifuged for 10 min at $8000 \times g$, and 55 μl of the supernatant from each sample was evaluated for CAT activity. To each sample, 50 μl (250,000 dpm) of ^{14}C-chloramphenicol (NEN-Dupont, Boston, MA) and 25 μl *N*-butyryl Co-A (5 mg/ml) were added and incubated at 37°C for 2 h. Mixed xylenes (Aldrich Chemical Co., Milwaukee, WI) (300 μl) were added to each tube and vortexed vigorously for 30 s, followed by centrifugation for 3 min at $8000 \times g$. The upper phase was transferred to a fresh microfuge tube, and 750 μl buffer (15 mM Tris-HCl, pH ~8.0, 60 mM KCl, 15 mM NaCl, 5 mM EDTA; pH 8.0) was added to each sample, vortexed, and centrifuged again. For each sample, 100 μl of the resulting upper phase was sampled, 5 ml Picofluor scintillant (Packard Instrument Co., Meriden, CT) was added, and radioactivity (^{14}C) was determined in a Canberra-Packard scintillation counter (1900 TR Tri Carb). CAT units were determined by comparison to a standard curve. Protein concentration was measured using the bicinchoninic acid (BCA) protein assay from Sigma (St. Louis, MO). Data were expressed as CAT mUnits/μg protein and represented the mean of a minimum of six different measurements ± SE.

Lipid mixing assay

Anionic MLVs containing 0.5 mol% NBD-PE and 0.5 mol% Rh-PE were prepared using the procedures described above. These liposomes were used to assess lipid mixing after the addition of anionic liposomes to the lipid-based DNA formulations according to the well-established fluorescence resonance energy transfer assay (Struck et al., 1981). Typically, 40 nmol of fluorescent anionic liposomes was mixed with 40 nmol of LDPs or LDAs in a quartz cuvette containing water or 150 mM NaCl or 10% FBS in 150 mM NaCl in a final volume of 500 μl. Fluorescence intensity (I_0) was measured with an excitation wavelength of 465 nm (slit width 5.0) and an emission wavelength of 517 nm (slit width 5.0) (Holland et al., 1996). After a 5-min time course, Triton X-100 was added to achieve a final concentration of 0.1%. This resulted in solubilization of the lipid structures, fostering optimal lipid mixing, and defined the maximum fluorescence intensity (I_{final}). The fluorescence intensity at 5 min (I_5) was used to calculate the percentage of lipid mixing $(I_5 - I_o/I_o - I_{final}) \times 100$. As a negative control, vesicles composed of DOPC alone were mixed with anionic MLVs according to the same experimental condition described above. For the positive control, fluorescent anionic liposomes were mixed with nonflorescent anionic liposomes followed by calcium addition. Briefly, 40 nmol of fluorescent anionic liposomes was mixed with 40 nmol of nonfluorescent anionic liposomes in a quartz cuvette containing water in a final volume of 500 μl. Lipid mixing was induced by the addition of 2.5 μl of 1 M $CaCl_2$. The percentage of lipid mixing was calculated as previously described.

TO-PRO-1 accessibility

The DNA intercalating dye TO-PRO-1 fluoresces when bound to DNA. It has been used to assess the accessibility of DNA to small molecules and to indicate DNA condensation (Hirons et al., 1994). For the studies described here, 15 μl of LDPs or LDAs (26 nmol of lipids) containing 2 μg of DNA was added to a quartz cuvette containing water or 150 mM NaCl or 10% FBS in 150 mM NaCl in a final volume of 500 μl. TO-PRO-1 (1 μl) was added to this sample to achieve a final concentration of 1×10^{-6} M. Fluorescence intensity (I) was measured at room temperature with a Luminescence Spectrometer 50B (Perkin-Elmer, Norwalk, CT) with an excitation wavelength of 509 nm (slit width 2.5) and an emission wavelength of 533 nm (slit width 5.0). All TO-PRO-1-containing samples were maintained in the dark to minimize photobleaching. To evaluate destabilization of LDPs or LDAs, a 10-fold excess of anionic liposomal lipid was added, and changes in fluorescence intensity were measured at room temperature at specific time points (1, 2, 5, 15, 30, 60, and 120 min). At the end of the time course, OGP (50 μl) was added for the 100 mM final concentration, allowing maximum TO-PRO-1 intercalation and thus defining the maximum fluorescence intensity (I_{OGP}). Percentage of dye exclusion was calculated as $(I_{OGP} - I_{OGP} - I)/I_{OGP}) \times 100$. When free DNA was mixed with anionic liposomes followed by TO-PRO-1 addition, the fluorescence reading was equivalent to that obtained in the absence of anionic liposomes. Moreover, in the presence of all lipid components and OGP, I_{OGP} was the same as that obtained using an equivalent amount of free DNA in the absence of added lipids.

DNase I stability assay

LDPs and LDAs containing 1 μg of DNA were incubated with 2.33 units of DNase I at 37°C for 20 min in 50 mM Tris-HCl (pH 8.0), 0.1 mM $MgSO_4$, and 0.1 mM dithiothreitol in a final volume of 120 μl. To stop the enzymatic reaction, 5 μl of 500 mM EDTA was added. Subsequently, 3 μl of 1 M OGP was used to dissociate the lipid/DNA complexes, and the DNA was isolated by phenol extraction. The extracted DNA was precipitated with one-tenth volume of 3 M sodium acetate (pH 5.2) and three volumes of absolute ethanol at −20°C for 2 h, and recovered after centrifugation at $12,000 \times g$ for 30 min at 4°C. The DNA pellet was hydrated in 10 μl sterile water before loading onto a 0.8% agarose gel. Electrophoresis was carried out in TBE buffer (89 mM Tris-borate, 2 mM EDTA,

pH 8.0) according to standard techniques (Sambrook et al., 1989). DNA was visualized by staining the gel in ethidium bromide before photography with UV transillumination.

For studies involving anionic liposome-mediated destabilization, the conditions used were identical to those described for the TO-PRO-1 assay. LDPs or LDAs in water or in 150 mM NaCl were incubated at room temperature in the presence or absence of anionic liposomes for 30 min. Subsequently, DNase I was added and the samples were incubated for an additional 30 min at room temperature. DNA was isolated and analyzed after electrophoresis as described above.

Statistics

The difference between groups was examined using a *t*-test for independent samples. A difference at $p < 0.05$ was considered to be statistically significant (p values are given in the table legends).

RESULTS

We have described a procedure for the preparation of lipid-based DNA systems that form as a consequence of a self-assembly process dependent on the formation of a hydrophobic cationic lipid-DNA complex intermediate (Bally et al., 1997; Zhang et al., 1997). This procedure is unlike those involving preformed cationic liposomes (Fig. 1). However, as shown in Fig. 2, the level of transgene expression obtained after transfection of B16/BL6 melanoma cells with pInexCATv2.0 formulated using DODAC:DOPE LDAs (preformed cationic liposome method) or DODAC:DOPE LDPs (hydrophobic cationic lipid-DNA complex method) is not significantly different. Optimal in vitro transfection activity of these systems was dependent on the use of phosphatidylethanolamine. It has been argued that DOPE is required for transfection because of its ability to adopt nonbilayer structures and promote membrane fusion (Farhood et al., 1995). Alternatively, we have demonstrated that phosphatidylethanolamine promotes destabilization of cationic lipid-DNA complexes (Wong et al., 1996, and manuscript submitted for publication). The studies summarized here were initiated on the basis that headgroup interactions are important in controlling destabilization or disassembly of lipid-based DNA formulations. To characterize destabilization of these lipid-based DNA formulations, we have used an approach similar to that described by Xu and Szoka (1996), in which anionic liposomes were used to promote destabilization of formulations prepared using preformed cationic liposomes. We have employed several assays to assess destabilization, including: 1) lipid mixing between cationic lipid-DNA formulations and anionic liposomes, 2) DNA intercalating dye binding (TO-PRO-1), and 3) sensitivity of DNA to DNase I-mediated hydrolysis.

Lipid mixing was measured using a fluorescence resonance energy transfer (RET) assay, which is dependent on the dilution of fluorescent lipid probes in model membranes. Lipid mixing is a process that can be achieved as a consequence of membrane fusion, membrane destabilization, and/or fluorescent lipid transfer in the presence of unlabeled membranes. Three different formulations of anionic liposomes were used to characterize the RET lipid mixing reaction induced comixing with the lipid-based DNA formulations (LDPs or LDAs). As a membrane fusion positive control, lipid mixing between labeled and unlabeled anionic liposomes was evaluated after the addition of 5 mM $CaCl_2$. The results, shown in Fig. 3 *A*, indicate that Ca^{2+}-induced increases in fluorescence (attributed to lipid dilution) are observed only with anionic liposomes prepared with DOPE (i.e., DOPS:DOPE:DOPC anionic liposomes). No lipid mixing was observed when Ca^{2+} was added to DOPS:DOPC (1:1) or DMPG:DOPC (1:1) liposomes.

In contrast, results obtained when LDPs or LDAs were mixed with anionic liposomes (Fig. 3 *B* and Table 1) are not dependent on the presence of DOPE. As shown in Fig. 3 *B*, increased fluorescence indicative of fluorescent lipid dilution is observed when DODAC:DOPE-formulated LDPs or LDAs are mixed with DOPS:DOPC liposomes. A rapid

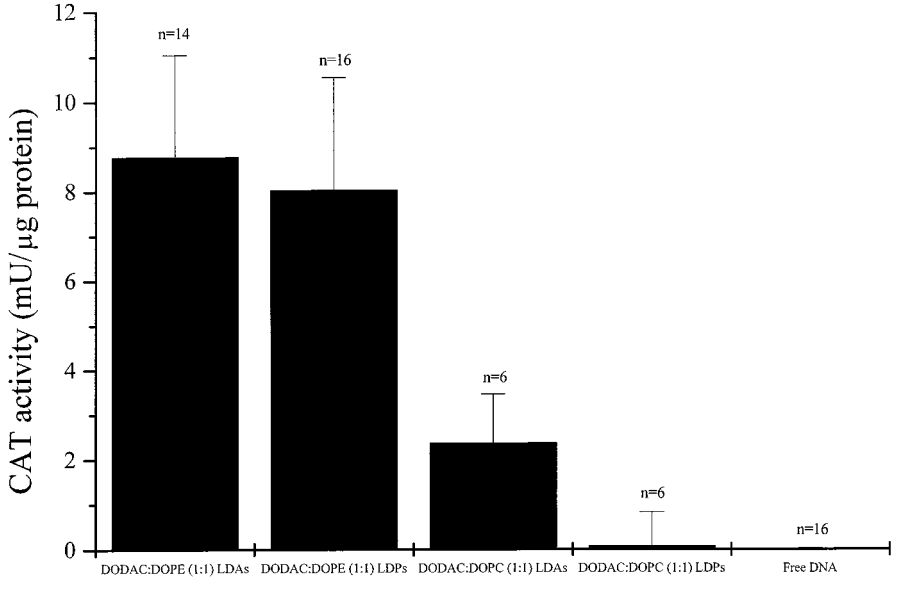

FIGURE 2 Chloramphenicol acetyltransferase (CAT) activity (mUnits/μg protein) after transfection of Bl6/BL6 melanoma cells with liposome/DNA aggregates (LDAs) or lipid/DNA particles (LDPs) composed of DODAC:DOPE or DODAC:DOPC. Each well contained 2 μg DNA (pInexCAT v2.0) per well. Cells were cultured and CAT activity was assayed according to procedures described in Materials and Methods. Values represent the mean $\pm$ SE.

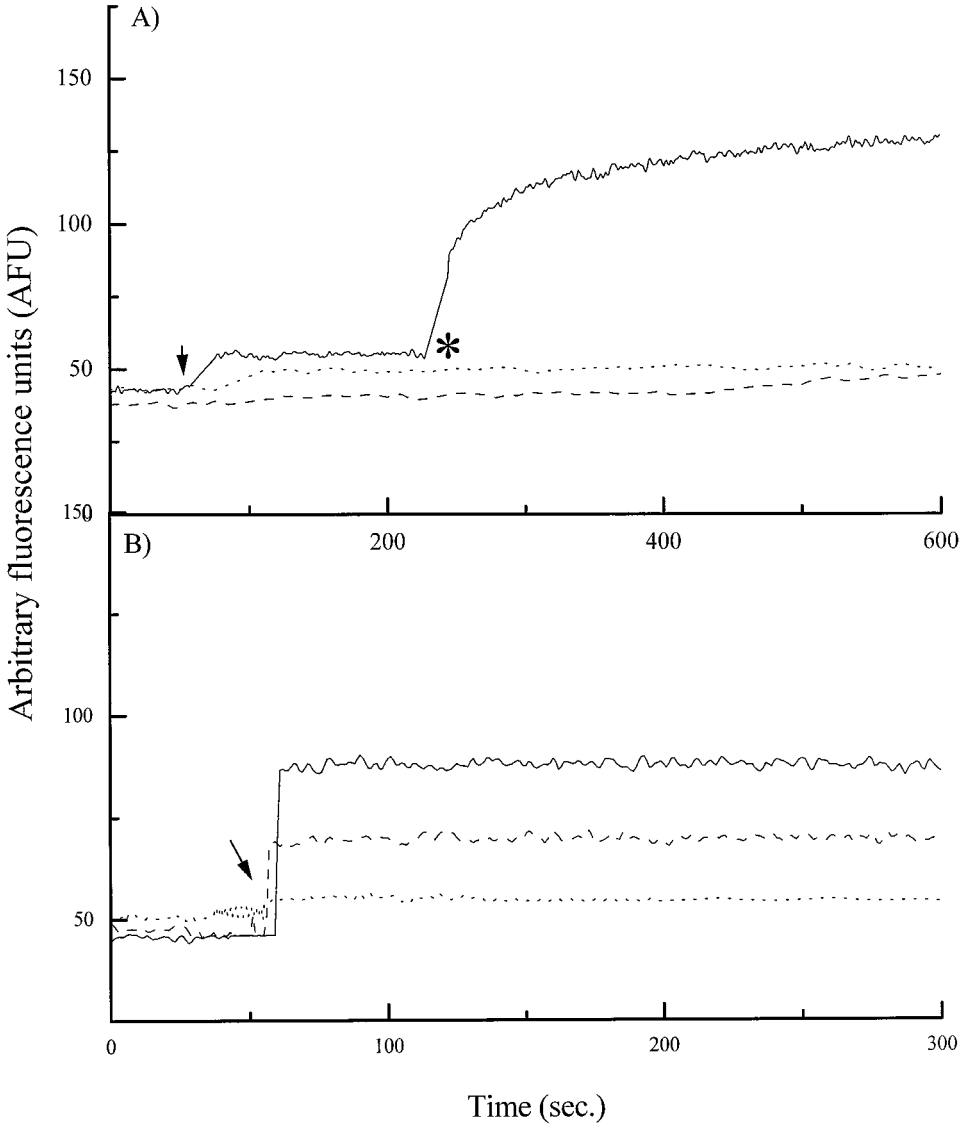

FIGURE 3 Lipid mixing assay. (*A*) Effect of 5 mM $CaCl_2$ on lipids mixing of anionic liposomes composed of DOPS:DOPE:DOPC (1:2:1) (——), DOPS:DOPC (1:1) (– – –), and DMPG:DOPC (·····). (*B*) Lipid mixing of DODAC:DOPE (1:1) lipid-DNA particles (——), DODAC:DOPE (1:1) liposome/DNA aggregates (– – –), and DOPC liposome alone (·····) after the addition of DOPS:DOPC (1:1) anionic liposomes. In *A* the arrow indicates unlabeled anionic liposome addition, and * indicates 5 mM $CaCl_2$ addition. In *B* the arrow indicates DOPS:DOPC (1:1) addition.

increase in the fluorescence is observed for both formulations; however, the magnitude of the change observed is approximately twofold greater for the LDP formulations. Results summarized in Table 1, in which the percentage of lipid mixing is measured relative to the maximum level of fluorescence obtained after the addition of detergent (Triton X-100), demonstrate that 1) lipid mixing observed after the addition of anionic liposomes to lipid-based DNA formulations was not dependent on the incorporation of DOPE into the anionic liposomes, a result that contrasts with those obtained for Ca^{2+}-induced fusion of DOPS liposomes; 2) although lipid mixing observed after the addition of anionic

TABLE 1 Percentage of lipid mixing in water after anionic MLV addition to the lipid-DNA particles or liposome/DNA aggregates

	DOPS:DOPE:DOPC (1:2:1)	DOPS:DOPC (1:1)	DOPC:DMPG (1:1)
DODAC:DOPE (1:1) LDPs	74.40 ± 5.60	81.55 ± 1.65[§]	52.37 ± 0.74[§]
DODAC:DOPC (1:1) LDPs	49.57 ± 11.35	48.13 ± 0.93	34.49 ± 4.37
DODAC:DOPE: (1:1) LDAs	38.08 ± 10.77*	39.18 ± 6.73*	29.11 ± 0.23*
DODAC:DOPC (1:1) LDAs	30.30 ± 5.40	24.97 ± 0.52[#]	27.91 ± 1.56

Values represent the mean from three different experiments ± SE.
*$p < 0.05$ when compared to DODAC:DOPE LDPs.
[#]$p < 0.05$ when compared to DODAC:DOPC LDPs.
[§]$p < 0.05$ when compared to DODAC:DOPC LDPs.
The percentage of lipid mixing has been calculated as (fluorescence intensity 5 min after anionic liposomes − intensity at t_o)/(intensity after 0.1% Triton − intensity at t_o) × 100.

liposomes to lipid-based DNA formulations was not dependent on incorporation of DOPE into the DNA formulation, the percentage of lipid mixing could be as much as twofold greater for the DOPE-containing formulations; and 3) significantly ($p < 0.05$) more lipid mixing was observed when LDPs were used, in comparison to LDAs.

We and others have demonstrated that lipid mixing is induced when DNA is added to cationic liposomes (Stegmann and Legendre, 1997; Wasan et al., 1997), and we argue that the potential of liposome DNA aggregates to interact with anionic membranes will be reduced because of this. To address this issue further, the RET assay was completed in the presence of 150 mM NaCl or in 150 mM NaCl containing 10% FBS (Table 2). Lipid-based DNA formulations were mixed with DOPS:DOPC liposomes, and the percentage of lipid mixing was measured as described in Table 1. The percentage of lipid mixing observed with LDPs was significantly greater than that observed with LDAs, even in the presence of salt and serum proteins. Importantly, however, the level of lipid mixing was reduced significantly ($p < 0.05$), as the medium changed from water (Table 1), to 150 mM NaCl without serum, to 150 mM NaCl with 10% serum. For DODAC:DOPE LDPs, the percentage of lipid mixing decreased from 82% to 68% when the assay was completed in 150 mM NaCl instead of water. A further decrease in lipid mixing from 68% to 23% was observed when the assay was completed in 150 mM NaCl containing 10% serum.

Considering that transfection assays completed using these formulations (Fig. 2) were all carried out in the presence of serum-containing media, it is unclear whether observed changes in lipid mixing provide a relevant correlation to the potential of lipid-based DNA formulations to transfect cells. Furthermore, the results shown thus far suggest that anionic membrane-induced lipid mixing is not a consequence of membrane fusion (as compared to the Ca^{2+} model of membrane fusion) and that lipid mixing is not dependent on the use of DOPE in the DNA formulation. We believe, however, that the lipid-mixing assay does provide useful information about the factors that control disassembly of the lipid-based DNA formulations. Referring to the results obtained with DODAC:DOPE versus DODAC:DOPC LDPs, it can be concluded that PE-containing formulations are more readily destabilized by the addition of anionic liposomes than those formulations prepared using PC.

TABLE 2 Percentage of lipid mixing after the addition of DOPS:DOPC (1:1) anionic MLVs to the lipid DNA particles or liposome/DNA aggregates in the presence of 150 mM NaCl or 150 mM NaCl containing 10% fetal bovine serum

	150 mM NaCl	150 mM NaCl + FBS 10%
DODAC:DOPE (1:1) LDPs	67.77 ± 4.75*	22.98 ± 2.34§
DODAC:DOPC (1:1) LDPs	43.20 ± 1.12#	17.29 ± 2.31§
DODAC:DOPE (1:1) LDAs	30.26 ± 4.32	13.07 ± 2.89§
DODAC:DOPC (1:1) LDAs	30.79 ± 6.45	9.01 ± 0.40§

Values represent the mean from three different experiments ± SE.
*$p < 0.05$ when compared to DODAC:DOPE LDAs.
#$p < 0.05$ when compared to DODAC:DOPE LDPs.
§$p < 0.05$ when compared to the same formulation in 150 mM NaCl without FBS.
The percentage of lipid mixing has been calculated as (fluorescence intensity 5 min after anionic liposomes − intensity at t_o)/(intensity after 0.1% Triton − intensity at t_o) × 100.

Additional information about anionic liposome-mediated disassembly of lipid-based DNA formulations can be obtained by evaluating DNA exposure. Binding of TO-PRO-1 to DNA is believed to provide a measure of DNA protection, and such dye intercalation assays have been used to define whether DNA is in a condensed form (Hirons et al., 1994). The effect of anionic liposome addition on dye exclusion from DNA as measured using this dye-binding assay was evaluated; the results have been summarized in Fig. 4. The plasmid expression vector was well protected for all lipid-based DNA formulations evaluated. TO-PRO-1 binding was not detected, as judged by measuring TO-PRO-1 fluorescence induced by DNA binding. Dye exclusion indices of 95–99% were measured for these systems before anionic liposome addition. After the addition of anionic liposomes (DOPS:DOPC, DMPG:DOPC, or DOPS:DOPE:DOPC), rapid (<1 min) increases in TO-PRO-1 accessibility were observed. For DODAC:DOPE LDAs and LDPs, dye exclusion decreased to values of ~75% (Fig. 4, *A* and *C*). When DOPS-containing liposomes were used to destabilize these formulations, only minimal additional decreases in the percentage of dye exclusion were observed over the 2-h time course. In contrast, the addition of DMPG:DOPC liposomes caused a progressive increase in TO-PRO-1 binding, and at the end of a 2-h incubation the dye exclusion index was less than 50%. LDPs and LDAs prepared with DODAC:DOPC (Fig. 4, *B* and *D*) were also destabilized rapidly after the addition of the anionic liposomes and, based on these data, it can be suggested that the DOPC formulations were, in general, more sensitive to anionic liposome-mediated destabilization. This is particularly evident when these formulations were incubated with DOPS:DOPE:DOPC liposomes, for which dye exclusion dropped from greater than 95% to less than 45% after the 2-h incubation. Like the results obtained with DODAC:DOPE LDPs and LDAs, DMPG:DOPC-mediated increases in TO-PRO-1 binding were time dependent when the DNA was formulated using DODAC:DOPC.

The influence of added salts and serum proteins on TO-PRO-1 binding after destabilization of lipid-based DNA formulations with anionic liposomes is shown in Fig. 5. Rapid (< 1 min) and significant decreases in dye exclusion were observed for all DNA formulations after the addition of NaCl (final concentration of 150 mM). The percentage of dye exclusion decreased from >95% to values of 70%, 30%, 60%, and 40% for DODAC:DOPE LDPs, DODAC:DOPC LDPs, DODAC:DOPE LDAs, and DODAC:DOPC LDAs, respectively. Increased labeling of DNA by TO-PRO-1 after the addition of NaCl was greatest for those formulations prepared with DODAC:DOPC. Comparable

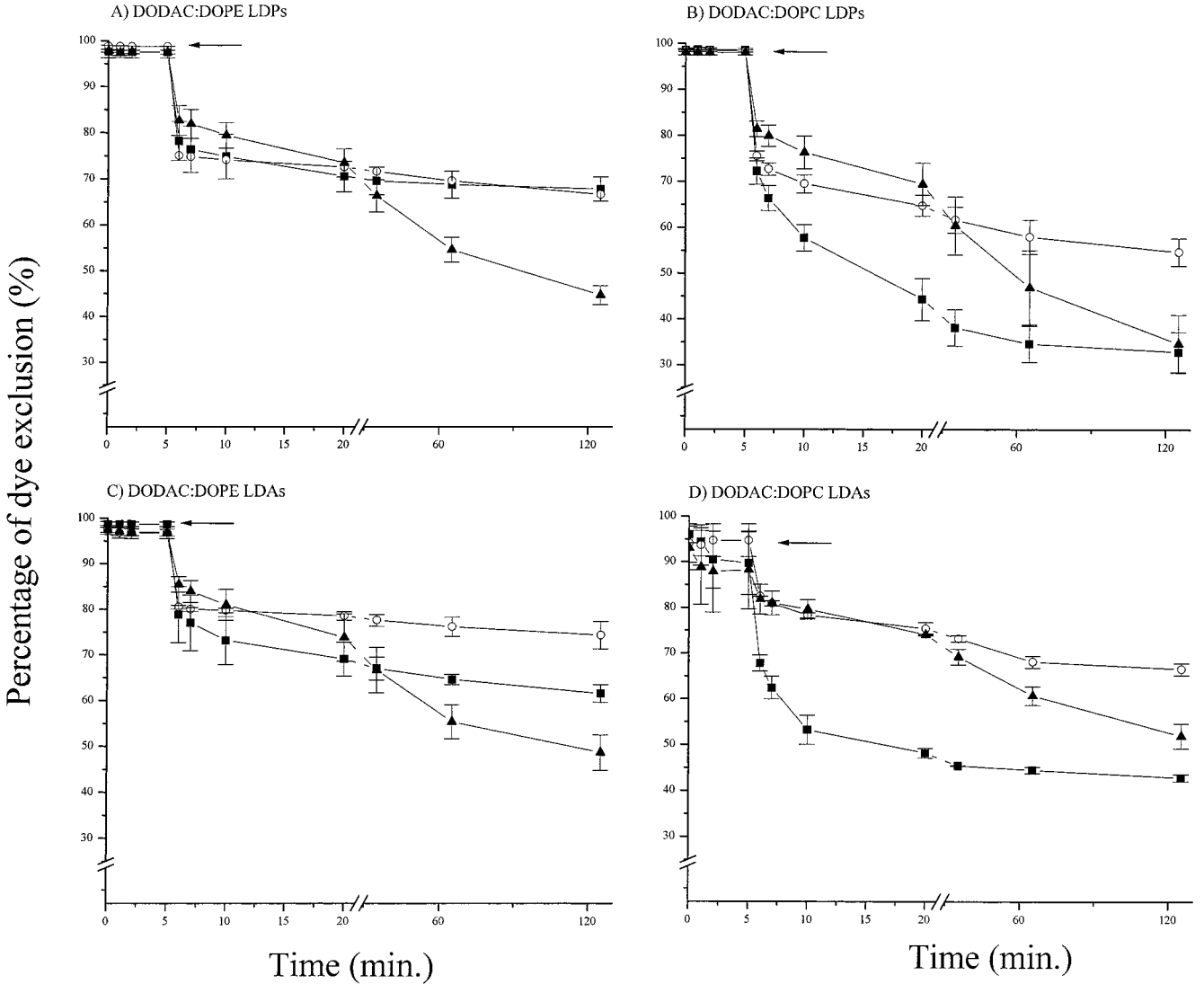

FIGURE 4 TO-PRO-1 accessibility after lipid/DNA carrier (LDPs or LDAs) destabilization with different anionic liposomes. (*A*) DODAC:DOPE (1:1) lipid-DNA particles; (*B*) DODAC:DOPC (1:1) lipid/DNA particles; (*C*) DODAC:DOPE (1:1) liposome/DNA aggregates; (*D*) DODAC:DOPC (1:1) liposome/DNA aggregates. Anionic MLVs: ■, DOPS:DOPE:DOPC (1:2:1); ○, DOPS:DOPC (1:1); ▲, DMPG:DOPC (1:1). Percentage of dye exclusion reflected by the TO-PRO-1 accessibility has been calculated by $(I_{OGP} - I(I_{OGP} - I)/I_{OGP}) \times 100$. Values represent the mean of three different experiments ± SE. The arrow indicates anionic liposome addition.

decreases were observed for all of the lipid-based DNA formulations when 150 mM NaCl with 10% serum was added (Fig. 5, *dashed lines*). After the addition of DOPS: DOPC liposomes, the dye exclusion indices decreased to 0 for all formulations in the presence of 150 mM NaCl, indicating that TO-PRO-1 binding was comparable to that observed when the lipid-based formulations were completely solubilized in detergent. Interestingly, in the presence of fetal bovine serum (final concentration of 10%), the anionic liposome-mediated increase in TO-PRO-1 binding was less than that observed in the absence of added serum. After 50 min, dye exclusion of 20% or more was observed for all formulations with added serum. The addition of detergent to these samples did result in efficient DNA labeling, suggesting that serum was not affecting TO-PRO-1 binding attributes (results not shown).

Because TO-PRO-1 dye binding may be influenced by the presence of bound lipids in the lipid-based DNA formulations evaluated, we also choose to assess DNA protection by measuring its degradation in the presence of DNase I. DNA analysis was completed as described in Materials and Methods, using agarose gel electrophoresis of plasmid DNA as a qualitative measure of DNA stability. As shown in the agarose gel in Fig. 6, free plasmid DNA was completely hydrolyzed under the incubation conditions employed. Using identical conditions, the DNA formulated in LDPs and LDAs was well protected, with similar banding patterns observed in the presence and absence of DNase I. It should be noted that there were shifts in the banding patterns observed for the different formulations. There was consistently more DNA retained in the wells when it was derived from the lipid-based formulations compared to free

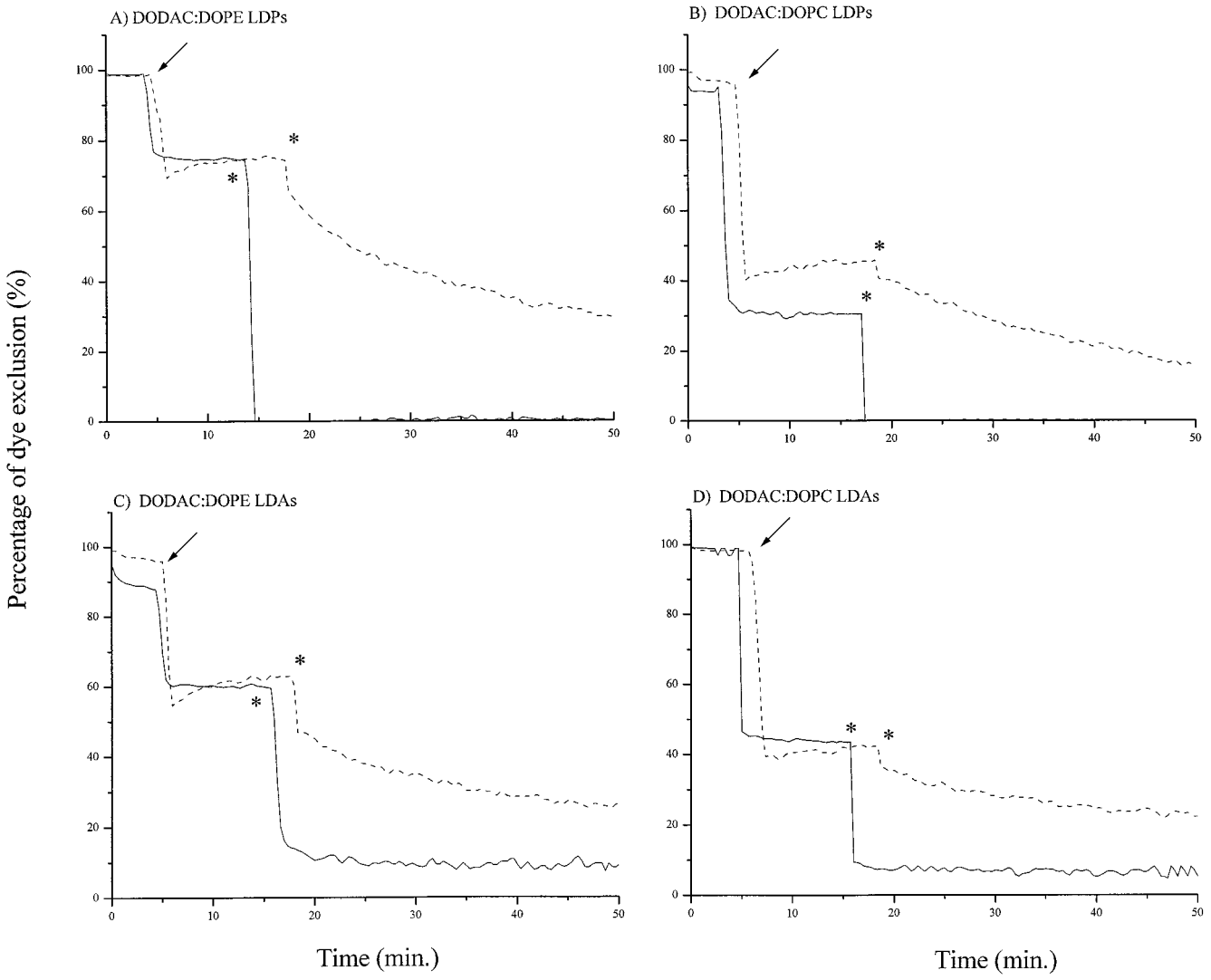

FIGURE 5 TO-PRO-1 accessibility after lipid/DNA carrier (LDPs or LDAs) destabilization with 150 mM NaCl (——) or with 150 mM NaCl containing 10% FBS (– – –) for 10 min, followed by a second destabilization with DOPS:DOPC anionic liposomes. (*A*) DODAC:DOPE (1:1) lipid-DNA particles; (*B*) DODAC:DOPC (1:1) lipid-DNA particles; (*C*) DODAC:DOPE (1:1) liposome/DNA aggregates; (D) DODAC:DOPC (1:1) liposome/DNA aggregates. The percentage of dye exclusion reflected by TO-PRO-1 accessibility has been calculated by $(I_{OGP} - I(I_{OGP} - I)/I_{OGP}) \times 100$. The curve is representative of three different experiments. Arrows represent the addition of 150 mM NaCl or 10% FBS in 150 mM NaCl, and * represent DOPS:DOPC (1:1) anionic liposome addition.

plasmid, and this made quantitative assessments difficult. Because a similar amount of DNA was loaded onto each well, the reduced labeling intensity observed for DNA samples derived from DODAC:DOPE lipid-based formulations suggests that these formulations were more sensitive to DNase I. In addition, the migration patterns were different, depending on which lipid formulation was used. DODAC:DOPE LDPs and LDAs consistently showed two bands, whereas the DODAC:DOPC LDPs and LDAs showed three bands. The two bands observed for the DOPE-containing formulations migrated as relaxed and linear plasmid DNA configurations would. The third band observed with the DOPC formulations migrated as supercoiled DNA, and the banding pattern observed for the DOPC formulations was more reflective of the banding pattern observed for free plasmid.

DNase I sensitivity assays were also conducted after the addition of DOPS:DOPC liposomes to the different lipid-based DNA formulations; these results are shown in Fig. 7. Agarose gel electrophoresis of DNA isolated after the addition of DOPS:DOPE liposomes or 150 mM NaCl (Fig. 7, *A* and *B*) clearly suggested that the addition of anionic liposomes mediated an increase in DNase I degradation. The hydrolysis was greatest for DNA samples derived from DOPE-containing formulations, suggesting that DODAC:DOPE LDPs and LDAs were more sensitive to destabilization. Interestingly, agarose gel electrophoresis indicated that the most significant effect of anionic liposome- or NaCl-mediated disassembly of the DODAC:DOPC LDPs and LDAs was loss of the supercoiled configuration of plasmid DNA. As shown in Fig. 7 *C*, the combination of DOPS:DOPE and the addition of 150 mM NaCl resulted in desta-

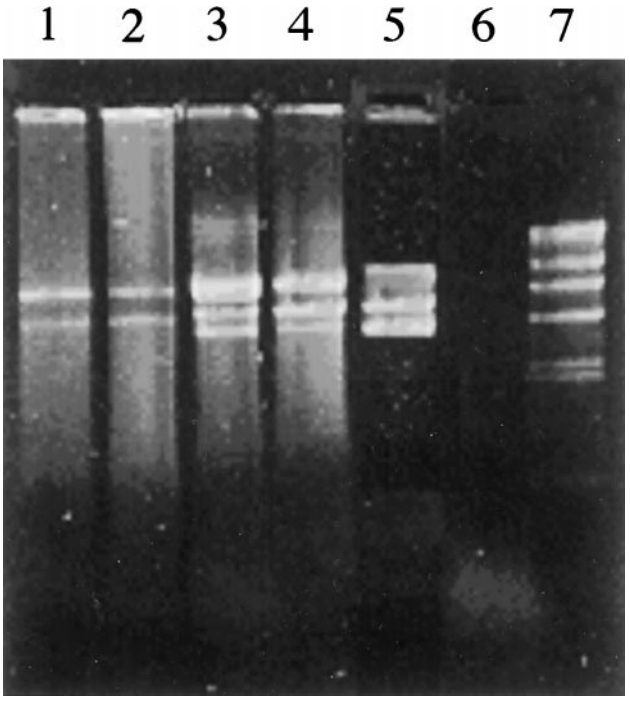

FIGURE 6 Agarose gel electrophoresis for assessment of DNA integrity after DNase I incubation with liposome/DNA aggregates or lipid-DNA particles at 37°C for 20 min. Lane 1, DODAC:DOPE (1:1) LDAs; lane 2, DODAC:DOPE (1:1) LDPs; lane 3, DODAC:DOPC (1:1) LDAs; lane 4, DODAC:DOPC (1:1) LDPs (lanes 1–4 have been treated with DNase I); lane 5, free DNA without DNase I; lane 6, free DNA with DNase I; lane 7, λ phage DNA digested with *Hin*dIII.

bilization of all of the lipid-based formulations studied, and 100% degradation was observed under the conditions used.

DISCUSSION

It is well established that lipid composition can affect the transfection activity of lipid-based DNA formulations, as illustrated by the data in Fig. 2. It is important to question why such differences are observed, because the answers will be essential to increasing our understanding of the transfection process, as well as establishing the role(s) of lipid constituents used. It has been argued previously that DOPE was required to promote membrane fusion. Results from other laboratories (Hui et al., 1996) and those reported here question whether membrane fusion plays any role in the activity of lipid-based formulations of plasmid expression vectors. It must be stressed, therefore, that anionic membrane-mediated destabilization of lipid-based DNA formulations described here was not studied in an effort to characterize bilayer fusion. Rather, the objective of these studies was to investigate the factors that are important in the regulation of lipid-based DNA carrier disassembly, a process that can be initiated by the addition of anionic membranes (Fig. 8). The results are considered by focusing the discussion on three areas: 1) the transfection-enhancing role of the ethanolamine headgroup; 2) how differences in formulation procedures may affect transfection activity; and 3) the role of DNA conformation in gene expression.

Two techniques were used to prepare lipid-based DNA formulations exhibiting comparable lipid compositions and DNA-to-lipid ratios (Fig. 1). The methods differ primarily in the manner in which lipids bind DNA. The more commonly employed procedure uses preformed cationic liposomes that organize into an aggregate structure when mixed with DNA (Sternberg et al., 1994). The alternative procedure relies on generating macromolecular structures from a hydrophobic cationic lipid/DNA complex intermediate (Zhang et al., 1997). As shown in Fig. 2, both formulations are effective in terms of in vitro transfection. Furthermore, the transfection efficiency of both formulation types is increased when they are prepared using phosphatidylethanolamine rather than phosphatidylcholine. This result is consistent with other data that rely on transgene expression as a primary end point in defining gene transfer (Fasbender et al., 1997; Hui et al., 1996; Liu et al., 1997).

Because the role of membrane fusion in governing gene transfer activity has been questioned (Stegmann and Legendre, 1997), we have suggested that the ethanolamine headgroup may play a role in facilitating the dissociation of cationic lipids that are bound to DNA (Wong et al., 1996, and manuscript submitted for publication). When rationalizing the beneficial properties of PE in comparison to PC, it is important to note that differences in the behavior of these lipids have often been attributed to their hydration properties (Marra and Israelachvili, 1985). Diacylphosphatidylcholines adopt a lamellar phase as a consequence of attractive (van der Waals) and repulsive (hydration) forces (McIntosh, 1996), and similar forces play a role in the macromolecular structure adopted by PE. However, an additional interaction between the amine group and the nonesterified oxygen of phosphate groups within and between lipid bilayers has been proposed for PE (Damodaran and Merz, 1997). Intra- and intermolecular interactions and hydration-repulsion forces help stabilize lipids into a bilayer (lamellar) phase; however, the added interaction between amine groups and phosphate groups in facing bilayers can induce local dehydration and facilitate close contact between membranes (McIntosh, 1996). These effects, when combined with factors that influence the acyl chain packing properties, are thought to be responsible for the polymorphic phase behavior of hydrated lipid systems (Slater et al., 1993). Although the potential for the amine group of PEs to interact with phosphate groups of phospholipids has been documented, we suggest that a similar interaction can occur between the amine group of PE and DNA phosphate groups. Such an interaction would serve to weaken the binding reaction between cationic lipids and DNA. It can be predicted that PE containing lipid-based formulations of DNA should be more susceptible to factors that promote disassembly due to the weaken binding reactions, a prediction that is confirmed in this report.

It is clear from results shown in Tables 1 and 2 that disassembly of the lipid-based DNA carriers is enhanced as

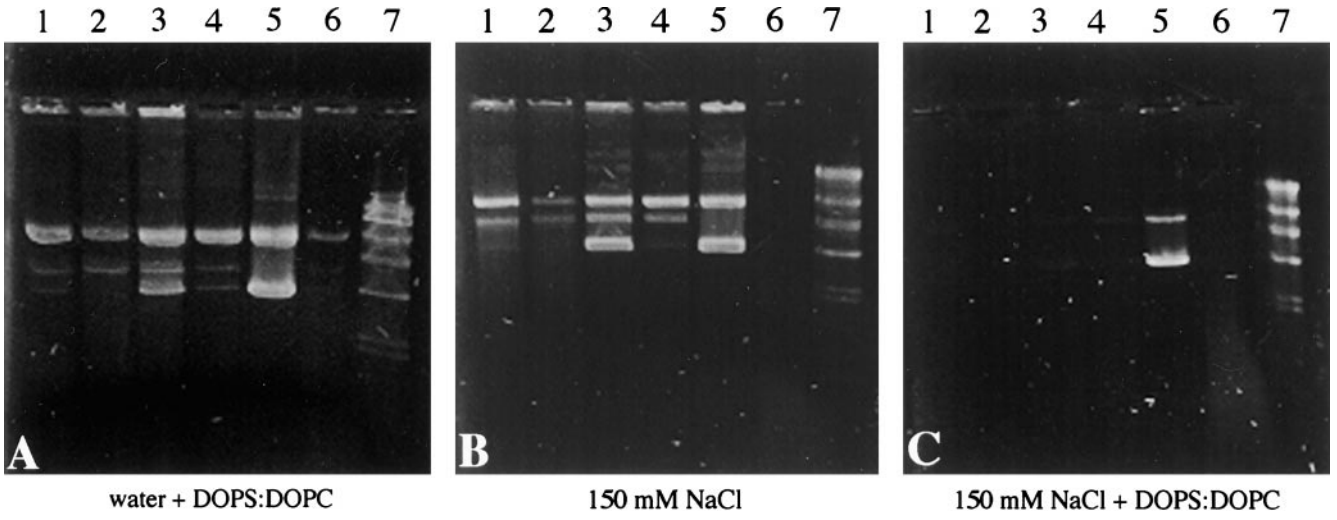

FIGURE 7 Agarose gel electrophoresis for assessment DNA integrity after 30 min of incubation with DNase I. (*A*) Incubated in water and destabilized with DOPS:DOPC 10-fold charge excess; (*B*) destabilization with 150 mM NaCl only; (*C*) after destabilization with 150 mM NaCl containing DOPS:DOPC anionic liposomes. After 30 min of destabilization, the mixture was incubated with 160 units of DNase I for 30 min at room temperature. Lane 1, DODAC:DOPE LDAs; lane 2, DODAC:DOPE LDPs; lane 3, DODAC:DOPC LDAs; lane 4, DODAC:DOPC LDPs; lane 5, free DNA without DNase I; lane 6, free DNA with DNase I; lane 7, λ DNA digested with *Hin*dIII.

a consequence of membrane interactions that bring about lipid mixing. As judged by the lipid mixing assay, disassembly of the lipid-based DNA formulations that relied on formation of a hydrophobic cationic lipid-DNA complex intermediate (LDPs) was more complete in comparison to formulations prepared with preformed liposomes (LDAs). We believe that such a result is likely a consequence of changes (aggregation and fusion) in cationic liposomes induced by the addition of DNA (Mok and Cullis, 1997). The fusion events observed when polyvalent anions are mixed with cationic liposomes must be distinguished, however, from events that lead to the disassembly of the lipid-based DNA formulation (Fig. 8). It has been demonstrated, for example, that addition of POPS:DOPC to DODAC:DOPE liposomes in the absence of DNA induces a lipid mixing reaction (Bailey and Cullis, 1997). Similarly, DNA addition to DODAC:DOPE liposomes also engenders a lipid mixing reaction, membrane fusion, and formation of nonbilayer structures (Bailey and Cullis, 1997). In the studies with LDAs, significant changes in the cationic liposomes have already occurred. Importantly, the data presented here do not suggest that transfection efficiencies would correlate with the potential for a lipid-based formulation to support a lipid mixing reaction. The tendency for these formulations to exhibit membrane reactions, such as lipid mixing, decreases significantly as the formulations are mixed with salts and serum proteins (see Table 3). It is for this reason that we believe that the destabilization reactions that lead to DNA release from the lipid-based carrier are most significant in terms of defining transfection activity.

In general, the addition of anionic liposomes caused increases in TO-PRO-1 (a DNA intercalating dye) binding and an associated increase in DNase I sensitivity. It should be noted that before the addition of anionic liposomes

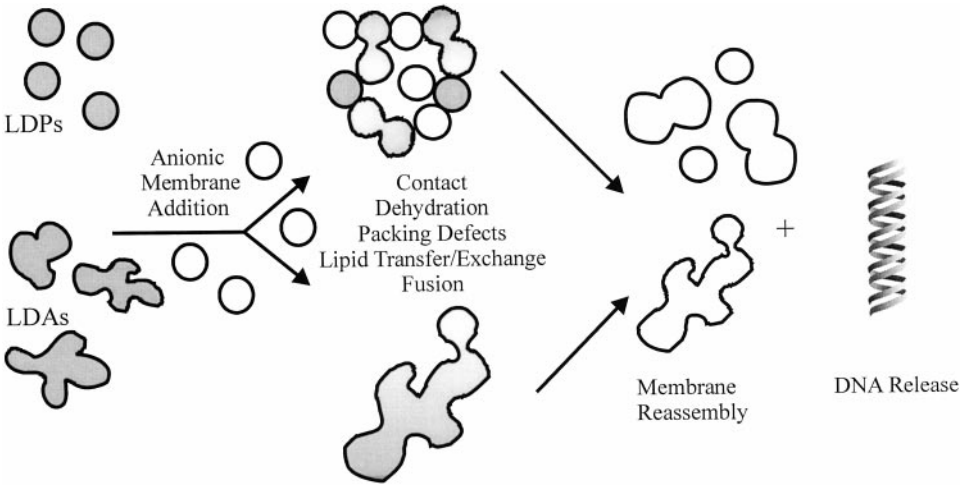

FIGURE 8 Schematic description of disassembly process mediated by anionic membrane on lipid-DNA particles (LDPs) or liposome/DNA aggregate (LDA).

and/or salts, all lipid-based DNA formulations maintained DNA in a protected form. The percentage dye exclusion, as measured by TO-PRO-1, was always greater than 90% (see Fig. 3 before the addition of anionic liposomes), and DNA in the formulations was protected in the presence of DNase I. The addition of anionic liposomes, regardless of lipid composition, resulted in rapid destabilization of the lipid-based DNA formulations. If comparisons are made between DNA formulations prepared using PE and PC, it can be suggested that increases in TO-PRO-1 labeling are greatest for the PC-containing systems. From these data it was anticipated that anionic membrane-mediated increases in DNase I sensitivity would be greatest in the PC formulations. The results shown in Fig. 7 did not support this. Considering that TO-PRO-1 is a hydrophobic molecule, it is perhaps not surprising that there may be lipid-dependent differences in dye labeling of DNA in these formulations. In this regard, DNA intercalating dye binding may provide more information about the lipid structure protecting the DNA than about whether the DNA is protected. Previous results from our laboratory, for example, have shown complete accessibility of DNA to TO-PRO-1 binding under conditions in which the DNA is protected from DNase I degradation (Zhang et al., 1997). Crook et al., (1996) also observed that DNA fixed to cationic liposomes is resistant to DNase I digestion, but is accessible for ethidium bromide staining.

Although the agarose gel electrophoresis results suggest that the plasmid DNA can be protected by the use of either formulation approach, considerable changes in DNA conformation are observed, and these changes appear to be dependent on lipid composition. In particular, after isolation of DNA from PC-containing formulations three bands were resolved. These correspond to those bands observed with the free plasmid that exists in the supercoiled (migrates furthest), relaxed (migrates the least), and linear (migrates in between) conformation. The free plasmid was primarily in the supercoiled configuration; however, after DNA isolation from PC-containing formulations, there was an apparent predominance of the relaxed configuration. After anionic membrane-mediated destabilization of the DOPC: DODAC LDPs, DNase I exposure resulted in selective loss of the supercoiled plasmid. This was not observed for the PC-containing LDA formulations. Only two bands were observed after agarose gel electrophoresis of DNA isolated from LDAs and LDPs prepared with PE (see Fig. 5), with no apparent supercoiled configuration present. These observations are interesting in view of data that suggest that optimal transfection is observed using supercoiled DNA (Meyer et al., 1995). The importance of these lipid- and formulation-dependent changes in DNA conformation is not understood, and future studies will attempt to address whether such changes in conformation can explain the improved transfection activity of PE-containing formulations. We have concluded from these studies that lipid-based DNA transfer systems prepared with PE disassemble more readily than those prepared with PC, and that release of DNA after internalization is likely a key factor controlling the transfection activity of these formulations.

This work was supported by a grant from the Medical Research Council of Canada and Inex Pharmaceuticals Corp.

REFERENCES

Bailey, A. L., and P. R. Cullis. 1997. Membrane fusion with cationic liposomes: effects of target membrane lipid composition. *Biochemistry.* 36:1628–1634.

Bally, M. B., Y. Zhang, F. M. P. Wong, E. K. Wasan, and D. L. Reimer. 1997. Lipid/DNA complexes as an intermediate in the preparation of particles for gene transfer: an alternative to cationic liposome/DNA aggregates. *Adv. Drug Del. Rev.* 24:275–290.

Boulikas, T. 1996. Liposome DNA delivery and uptake by cells. *Oncol. Rep.* 3:989–995.

Chowdhury, N. R., C. H. Wu, G. Y. Wu, P. C. Yerneni, V. R. Bommineni, and J. R. Chowdhury. 1993. Fate of DNA targeted to the liver by asialoglycoprotein receptor-mediated endocytosis in vivo. Prolonged persistence in cytoplasmic vesicles after partial hepatectomy. *J. Biol. Chem.* 268:11265–11271.

Cristiano, R. J., and D. T. Curiel. 1996. Strategies to accomplish gene delivery via the receptor-mediated endocytosis pathway. *Cancer Gene Ther.* 3:49–57.

Crook, K., G. McLachlan, B. J. Stevenson, and D. J. Porteous. 1996. Plasmid DNA molecules complexed with cationic liposomes are protected from degradation by nucleases and shearing by aerosolisation. *Gene Ther.* 3:834–839.

Damodaran, K. V., and K. M. Merz. 1997. A comparison of DMPC- and DLPE-based lipid bilayers. *Biophys. J.* 66:1076–1087.

Farhood, H., N. Serbina, and L. Huang. 1995. The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer. *Biochim. Biophys. Acta.* 1235:289–295.

Fasbender, A., J. Marshall, T. O. Moninger, T. Grunst, S. Cheng, and M. J. Welsh. 1997. Effect of co-lipids in enhancing cationic lipid-mediated gene transfer in vitro and in vivo. *Gene Ther.* 4:716–725.

Felgner, P. L., T. R. Gadek, M. Holm, R. Roman, H. W. Chan, M. Wenz, J. P. Northrop, G. M. Ringold, and M. Danielsen. 1987. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. USA.* 84:7413–7417.

Felgner, P. L., and G. M. Ringold. 1989. Cationic liposome-mediated transfection. *Nature.* 337:387–388.

Hirons, G. T., J. J. Fawcett, and H. A. Crissman. 1994. TOTO and YOYO: new very bright fluorochromes for DNA content analyses by flow cytometry. *Cytometry.* 15:129–140.

Holland, J. W., C. Hui, P. R. Cullis, and T. D. Madden. 1996. Poly(ethylene glycol)-lipid conjugates regulate the calcium-induced fusion of liposomes composed of phosphatidylethanolamine and phosphatidylserine. *Biochemistry.* 35:2618–2624.

Hope, M. J., M. B. Bally, L. D. Mayer, A. S. Janoff, and P. R. Cullis. 1986. Generation of multilamellar and unilamellar phospholipid vesicles. *Chem. Phys. Lipids.* 40:89–107.

Hui, S. W., M. Langner, Y. Zhao, P. Ross, E. Hurley, and K. Chan. 1996. The role of helper lipids in cationic liposome-mediated gene transfer. *Biophys. J.* 71:590–599.

Lasic, D. D., and N. S. Templeton. 1996. Liposomes in gene therapy. *Adv. Drug Delivery Rev.* 20:221–266.

Liu, Y., L. C. Mounkes, H. D. Liggitt, and R. J. Debs. 1997. Factors influencing the efficacy of cationic liposome-mediated intravenous gene delivery. *Nature Biotechnol.* 15:167–173.

Marra, J., and J. N. Israelachvili. 1985. Direct measurements of forces between phosphatidylcholine and phosphatidylethanolamine bilayers in aqueous electrolyte solutions. *Biochemistry.* 24:4608–4618.

McIntosh, T. J. 1996. Hydration properties of lamellar and non-lamellar phases of phosphatidylcholine and phosphatidylethanolamine. *Chem. Phys. Lipids.* 81:117–131.

Meyer, K. B., M. M. Thompson, M. Y. Levy, L. G. Barron, and F. C. J. Szoka. 1995. Intratracheal gene delivery to the mouse airway: characterization of plasmid DNA expression and pharmacokinetics. *Gene Ther.* 2:450–460.

Mok, K. W. C., and P. R. Cullis. 1997. Structural and fusogenic properties of cationic liposomes in the presence of plasmid DNA. *Biophys. J.* 73:2534–2545.

Reimer, D. L., S. Kong, and M. B. Bally. 1997. Analysis of cationic liposome-mediated interactions of plasmid DNA with murine and human melanoma cells in vitro. *J. Biol. Chem.* 272:19480–19487.

Reimer, D. L., Y. Zhang, S. Kong, J. J. Wheeler, R. W. Graham, and M. B. Bally. 1995. Formation of novel hydrophobic complexes between cationic lipids and plasmid DNA. *Biochemistry.* 34:12877–12883.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *In* Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Seed, B., and J. Sheen. 1988. A simple phase-extraction assay for chloramphenicol acetyltransferase activity. *Gene.* 67:271–277.

Slater, S. J., C. Ho, F. J. Taddeo, M. B. Kelly, and C. D. Stubbs. 1993. Contribution of hydrogen bonding to lipid-lipid interactions in membranes and the role of lipid order: effects of cholesterol, increased phospholipid unsaturation, and ethanol. *Biochemistry.* 32:3714–3721.

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

Sternberg, B., F. L. Sorgi, and L. Huang. 1994. New structures in complex formation between DNA and cationic liposomes visualized by freeze-fracture electron microscopy. *FEBS Lett.* 356:361–366.

Struck, D. K., D. Hoekstra, and R. E. Pagano. 1981. Use of resonance energy transfer to monitor membrane fusion. *Biochemistry.* 20: 4093–4099.

Tanford, C. 1991. The Hydrophobic Effect: Formation of Micelles and Biological Membranes. Krieger Publishing Company, Malabar, FL.

Templeton, N. S., D. D. Lasic, P. M. Frederik, H. H. Strey, D. D. Roberts, and G. N. Pavlakis. 1997. Improved DNA: liposome complexes for increased systemic delivery and gene expression. *Nature Biotechnol.* 15:647–52.

Wagner, E., C. Plank, K. Zatloukal, M. Cotten, and M. L. Birnstiel. 1992. Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: toward a synthetic virus-like gene-transfer vehicle. *Proc. Natl. Acad. Sci. USA.* 89:7934–7938.

Wasan, E. K., P. Harvie, and M. B. Bally. 1997. Lipid mixing and aggregation properties of cationic liposomes used for gene transfer. *Proc. Am. Assoc. Pharm. Sci.* 14:S-64.

Wong, F. M. P., D. L. Reimer, and M. B. Bally. 1996. Cationic lipid binding to DNA: characterization of complex formation. *Biochemistry.* 35:5776–5763.

Xu, Y., and F. C. J. Szoka. 1996. Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. *Biochemistry.* 35: 5616–5623.

Zelphati, O., and F. C. Szoka. 1996. Mechanism of oligonucleotide release from cationic liposomes. *Proc. Natl. Acad. Sci. USA.* 93:11493–11498.

Zhang, Y., D. L. Reimer, G. Zhang, P. H. Lee, and M. B. Bally. 1997. Self-assembling DNA-lipid particles for gene transfer. *Pharm. Res.* 14:190–196.