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# Phospholipid-cationic lipid interactions: influences on membrane and vesicle properties

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**Abstract**

Liposomes composed of synthetic dialkyl cationic lipids and zwitterionic phospholipids such as dioleoylphosphatidylethanolamine have been studied extensively as vehicles for gene delivery, but the broader potentials of these cationic liposomes for drug delivery have not. An understanding of phospholipid-cationic lipid interactions is essential for rational development of this potential. We evaluated the effect of the cationic lipid DOTAP (*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium) on liposome physical properties such as size and membrane domain structure. DSC (differential scanning calorimetry) showed progressive decrease and broadening of the phase transition temperature of dipalmitoylphosphatidylcholine (DPPC) with increasing fraction of DOTAP, in the range of 0.4–20 mol%. Laurdan (6-dodecanolyldimethylamino-naphthalene), a fluorescent probe of membrane domain structure, showed that DOTAP and DPPC remained miscible at all ratios tested. DOTAP reduced the size of spontaneously-forming PC-containing liposomes, regardless of the acyl chain length and degree of saturation. The anionic lipid DOPG (dioleoylphosphatidylglycerol) had similar effects on DPPC membrane fluidity and size. However, DOTAP/DOPC (50/50) vesicles were taken up avidly by OVCAR-3 human ovarian tumor cells, in contrast to DOPG/DOPC (50/50) liposomes. Overall, DOTAP exerts potent effects on bilayer physical properties, and may provide advantages for drug delivery. 

*Keywords:* Membrane domain; Drug delivery; Cationic liposome; Fluorescence spectroscopy; Vesicle size

## 1. Introduction

Liposomes composed of cationic lipids and helper phospholipids have been shown to be effective vehicles for cellular delivery of RNA and DNA [1–4]. For example, liposomes containing the cationic lipids DOTMA (*N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium) or DOTAP (*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium) (Fig. 1), form strong complexes with DNA, and the addition of positive charge to the liposome surface enhances the interaction with cellular membrane components [1,5–7].

There exists a wide range of situations in which the ability to provide overall cationic electrostatic charge

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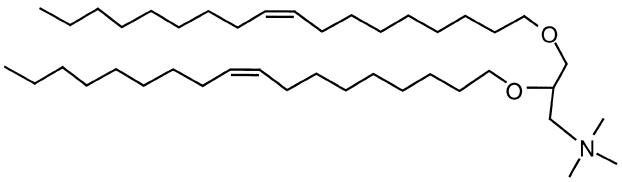
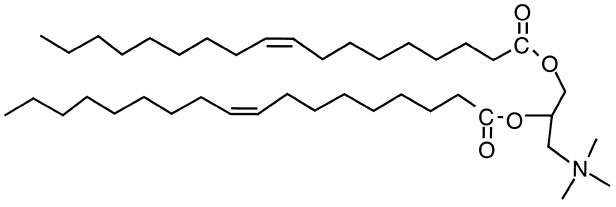


DOTMA



DOTAP

Fig. 1. Chemical structure of cationic lipids.

to liposomes could be advantageous [8]. For example, the addition of charge can inhibit aggregation of liposomes, but in some cases, such as the encapsulation of polycationic drugs or peptides, anionic lipids would be unsuitable. Stearylamine (octadecylamine), a monoalkyl cationic lipid, has been used widely to provide cationic charge to liposomes [9–12]. However, rapid extraction or exchange of stearylamine from the bilayer in the presence of cells or plasma proteins, as well as potential toxic effects, represent limitations [8,13–15]. Dialkyl or diacyl lipids such as DOTAP and DOTMA should be more stable in the membrane bilayer, based on the enthalpic and entropic contributions resulting from the incorporation of additional hydrocarbon chains in the membrane bilayer. These or similar lipids have been used both in vitro and in vivo, and have shown minimal toxicity in a number of applications [16–19].

Cationic liposomes designed for nucleic acid delivery generally are intrinsically unstable and undergo a structural transition upon interacting with the polyanionic nucleic acids or serum components [3,5,20–22]. Membrane conformational instability, which appears essential to the delivery function, is enhanced by inclusion of a helper lipid; the most common is phosphatidylethanolamine (PE). PE is a zwitterionic phospholipid that is unable to form stable bilayer vesicles at neutral pH in physiological saline, and it readily undergoes a bilayer to hexagonal phase transition [23,24]. In contrast, liposomes used for drug or peptide delivery should be intrinsically stable in order to retain their contents. Such stability might be achieved nominally by the substitution of phosphatidylcholine (PC), a zwitterionic phospholipid that forms stable bilayers, for PE, and by the inclusion of high phase transition lipids that inhibit interaction with serum proteins and promote the retention of liposome contents [25].

In the present work, we have studied the interactions of cationic lipids with phospholipids that form stable bilayer vesicles, and have examined their effects on liposome physical properties. DSC (differential scanning calorimetry) was used to evaluate the physical effect of DOTAP on thermotropic phase behavior. Fluorescence spectroscopy using Laurdan (6-dodecanolyldimethylamino-naphthalene) and DPH (1,6-diphenyl-1,3,5-hexatriene) was employed to probe the membrane interfacial region and domain structure, as well as the acyl chain region of membrane vesicles. QLS (quasi-elastic light scattering) was used to evaluate the effect of cationic lipids on the size of spontaneously-forming vesicles. Finally, fluorescence microscopy was used to provide an overview of cellular interactions of PC-containing cationic liposomes.

## 2. Materials and methods

### 2.1. Materials

Dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylglycerol (DOPG), phosphatidylglycerol (PG) from egg yolk and the cationic lipid DOTAP were obtained from Avanti Polar Lipids (Alabaster, AL, USA). All lipids were stored at −80°C in chloroform under an inert atmosphere. HPLC grade solvents (water and ethanol) were obtained from Fisher Scientific. Laurdan (6-dodecanolyldimethylamino-naphthalene), DPH (diphenylhexatriene), and rhodamine-dipalmitoylphosphatidylethanolamine were obtained from Molecular Probes (Eugene, OR, USA). Cell culture media and serum were obtained from Gibco-BRL (Gaithersburg, MD, USA).

### 2.2. Preparation of liposomes

The required amount of lipid was dissolved in chloroform at 20 mM, and the solvent was removed using a rotary evaporator to form a thin film on the walls of a screw-cap tube. The film was then hydrated with water in an inert atmosphere, incubated above the bilayer phase transition temperature of the phospholipid employed, and vortexed intermittently. The specific concentration of lipid used for each study was determined by the experimental requirements, and was typically 10–20 mM.

### 2.3. Differential scanning calorimetry

Thermograms were obtained using a Perkin Elmer DSC-2 calorimeter with samples sealed in aluminum pans. The instrument was calibrated with an indium standard. The thermograms were recorded using a heating rate of 2.5 K/min and a range of 1 mCal/s. For each scan, 13 μl of a liposome suspension of approximately 80 mM phospholipid was used. Samples previously equilibrated above their phase transition were held at an initial temperature of 25°C for at least 15 min before obtaining each thermogram. To generate a phase diagram, triplicate samples were prepared for each phospholipid/DOTAP ratio and immediately analyzed. For each sample, the peak height and width, and the transition temperature were measured.

### 2.4. Membrane lamellar domains

Excitation and emission spectra of Laurdan (6-dodecanolyldimethylamino-naphthalene) were obtained using an SLM Aminco 8000 series instrument. Laurdan, which is fully miscible in gel and liquid crystalline phases of membranes, was used to characterize the effects of DOTAP on the phase domain properties [26–28] of DPPC vesicles over the temperature range 30–70°C. Laurdan was incorporated in liposomes at a ratio of 1/3000 (Laurdan/lipid). The total concentration of lipid used for each measurement was 0.5 mM. The excitation spectra were acquired over the range 320–400 nm, and the emission wavelengths were set sequentially at 440 nm and 490 nm. The temperature was equilibrated for at least 15 min before acquiring spectra.

### 2.5. Generalized polarization

Laurdan excitation spectra were obtained as described above, using fixed emission wavelengths of 440 and 490 nm. Generalized polarization (GP) values at each point in the excitation spectrum were calculated according to the following formula:

$$\mathrm{GP} = (I_{440} - I_{490})/(I_{440} + I_{490})$$

[26–28], where '*I*' represents the fluorescence intensity for each discrete excitation wavelength as detected at 440 and 490 nm.

### 2.6. Fluorescence polarization and fluidity

Measurements were made using an excitation wavelength of 355 nm, an emission wavelength of 430 nm, 4 nm excitation and emission slits, and FP110 film polarizers. To incorporate DPH into small unilamellar vesicles, a stock solution of 2 mM DPH was prepared in tetrahydrofuran, and 2 μl was added to 2 ml of cationic liposomes (0.6 mM phospholipid). The sample was mixed vigorously above the phase transition of the phospholipid employed. The cuvette chamber was maintained at the required temperature using a circulating water bath, and was allowed to equilibrate for 15–20 min prior to fluorescence measurements. Fluorescence polarization (*P*) was calculated by the following formula:

$$P = I(0,0) - G \times I(0,90)/I(0,0) + G \times I(0,90),$$

$$[G = I(90,0)/I(90,90)]$$

[29], where '*I*' represents the fluorescence intensity using an excitation wavelength of 355 nm and an emission wavelength of 430 nm.

### 2.7. Quasi-electric light scattering

A particle sizer (Nicomp Particle Sizing Systems model 370, Santa Barbara, CA, USA), was used to investigate the effect of DOTAP on liposome size. All liposome formulations were made in triplicate and analyzed without sonication or extrusion. The measurements were carried out at 25°C, with an acquisition time of approximately 2 min and a lipid concentration of 2.9 mM. The volume-weighted

NICOMP analysis was applied to estimate size distribution.

### 2.8. Fluorescence microscopy

Liposomes labeled with rhodamine-DPPE (*N*-(Lissamine rhodamine B sulfonyl)-dipalmitoylphosphatidylethanolamine) were used to investigate the effect of charge on uptake of liposomes by human ovarian tumor cells. The cell line OVCAR-3, obtained as described previously [30], was plated on sterile cover slips in 6-well plates (Costar, Cambridge, MA, USA) at a density of $5\times10^5$ cells per ml of media. The cells were allowed to adhere overnight in RPMI 1640 media containing 10% fetal bovine serum, and were treated with 100 nmol of rhodamine labeled liposomes on the following day. The liposome formulations consisted of DOPC/DOTAP (50/50) or DOPG/DOTAP (50/50), and included 1 mol% rhodamine-DPPE. After 1 h at 37°C, the cells were washed with cold phosphate-buffered saline (to remove free liposomes and any cellular debris), and the cells were analyzed by fluorescence microscopy (40× objective lens).

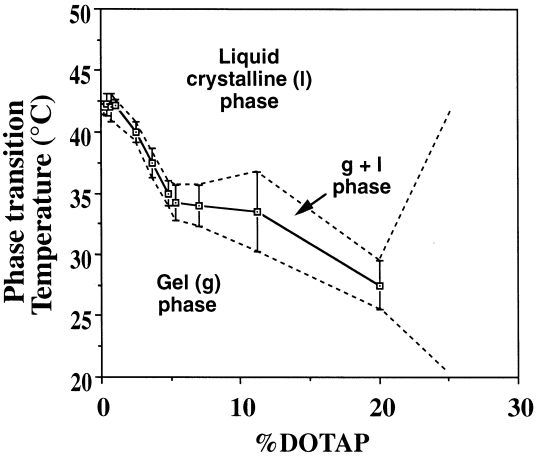



Fig. 2. Effect of DOTAP on thermal phase transition of DPPC vesicles. Multilamellar liposomes containing varying concentrations of DOTAP (0–20 mol%) were prepared as described in Section 2. The DPPC concentration was 80 mM for all samples. Samples were prepared in triplicate and analyzed by DSC immediately. The phase transition temperature was determined graphically from analog scans, and is plotted as a function of the mol% DOTAP in DPPC. Vertical bars at each point represent the peak width at half-height. Dotted lines connecting the maxima and minima of the vertical bars indicate approximate phase boundaries. Boundaries drawn beyond the data (>20 mol%) are estimated from Laurdan fluorescence data, as described in the text.

## 3. Results

### 3.1. The effect of DOTAP on the phase transition of DPPC

Differential scanning calorimetry (DSC) was carried out to investigate the effect of DOTAP on membrane physical properties. Fig. 2 shows the effect of DOTAP mol fraction on the thermal phase transition of large DPPC vesicles. Liposomes composed of DPPC alone showed a highly cooperative acyl chain melting transition at 41.3°C (314.3 K; data not shown) [31]. At lower DOTAP concentrations (≤0.2%), the effect of DOTAP on the thermal phase transition of DPPC was minimal. As the concentration of DOTAP increased (0.4 to 2.5 mol%), a shift in the thermal phase transition toward lower temperatures was observed (Fig. 2). Such a shift may suggest packing defects at the membrane interface. As the mol fraction of DOTAP increased, broadening of the DPPC peak was observed, and the DPPC phase transition was abolished ≥25 mol% DOTAP (Fig. 2).

The DSC thermograms were used to construct a phase diagram in order to characterize the effects of DOTAP on the thermal phase behavior of DPPC (Fig. 2). For pure DPPC, a single-component system, two phases (gel and liquid crystalline) coexist over a narrow range of temperatures. As the concentration of DOTAP increased (≤10 mol%), there was an observable increase in the temperature range over which the gel and liquid crystalline phases coexist. Above 20 mol% of DOTAP, the coexisting phase was completely abolished, and the DOTAP/DPPC mixtures formed an apparently homogeneous liquid crystalline phase. Because of extensive peak broadening, it was not possible to use DSC to investigate bilayer state at DOTAP ratios above 20 mol%.

### 3.2. Laurdan fluorescence: properties of lamellar domains

The state of the bilayer above 20 mol% DOTAP is of potential interest for drug delivery [1,2]; high charge densities may be required for optimal cell

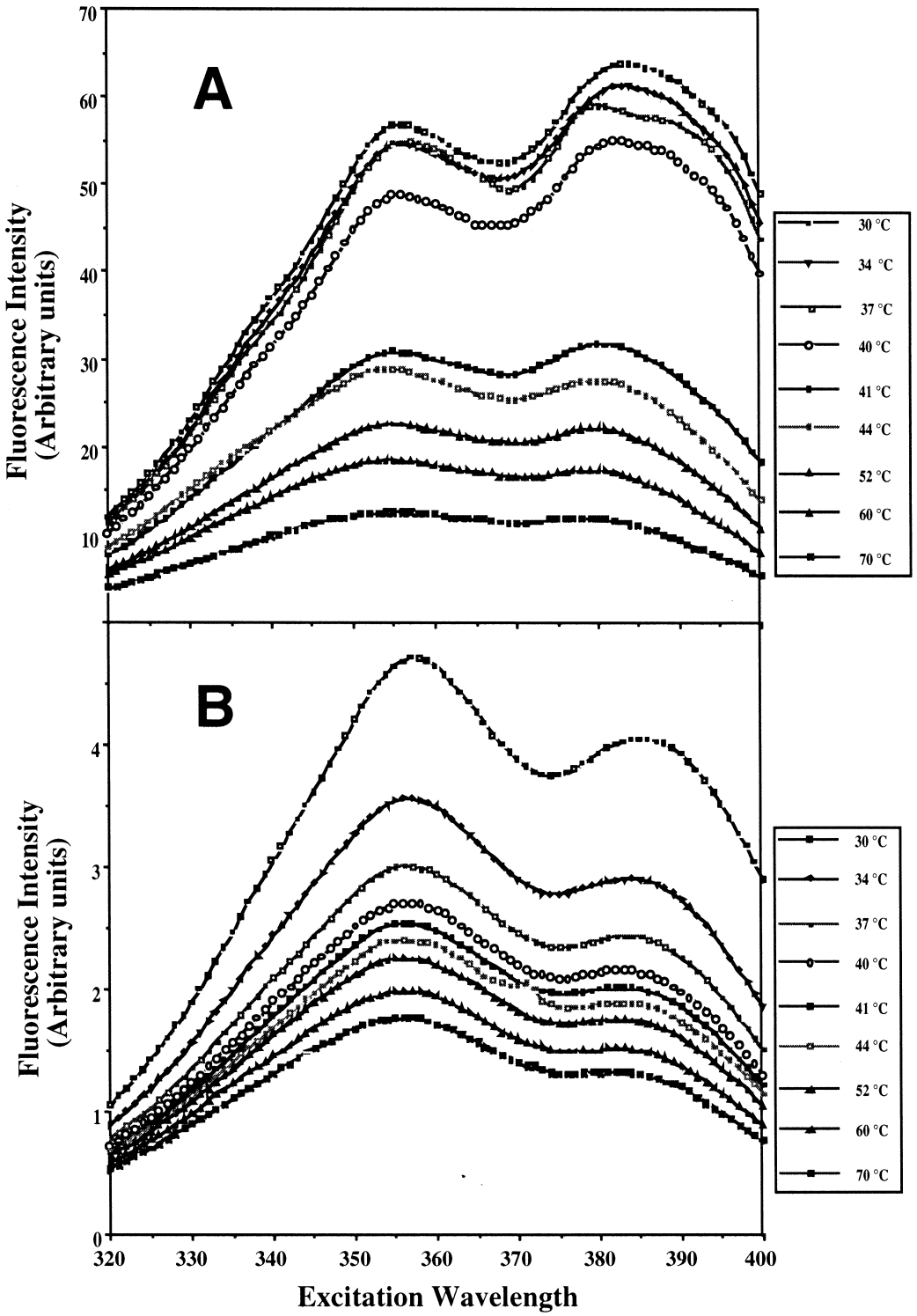



Fig. 3. Temperature-dependent excitation spectra of Laurdan in DPPC and DPPC/DOTAP liposomes. Laurdan was incorporated into multilamellar liposomes of (A) DPPC (100%), and (B) DPPC/DOTAP (50/50 mol%). Excitation spectra were acquired at various temperatures spanning the phase transition temperature of DPPC, using an emission wavelength of 440 nm. The symbols and temperatures to which they are assigned are shown in the inset for each panel.

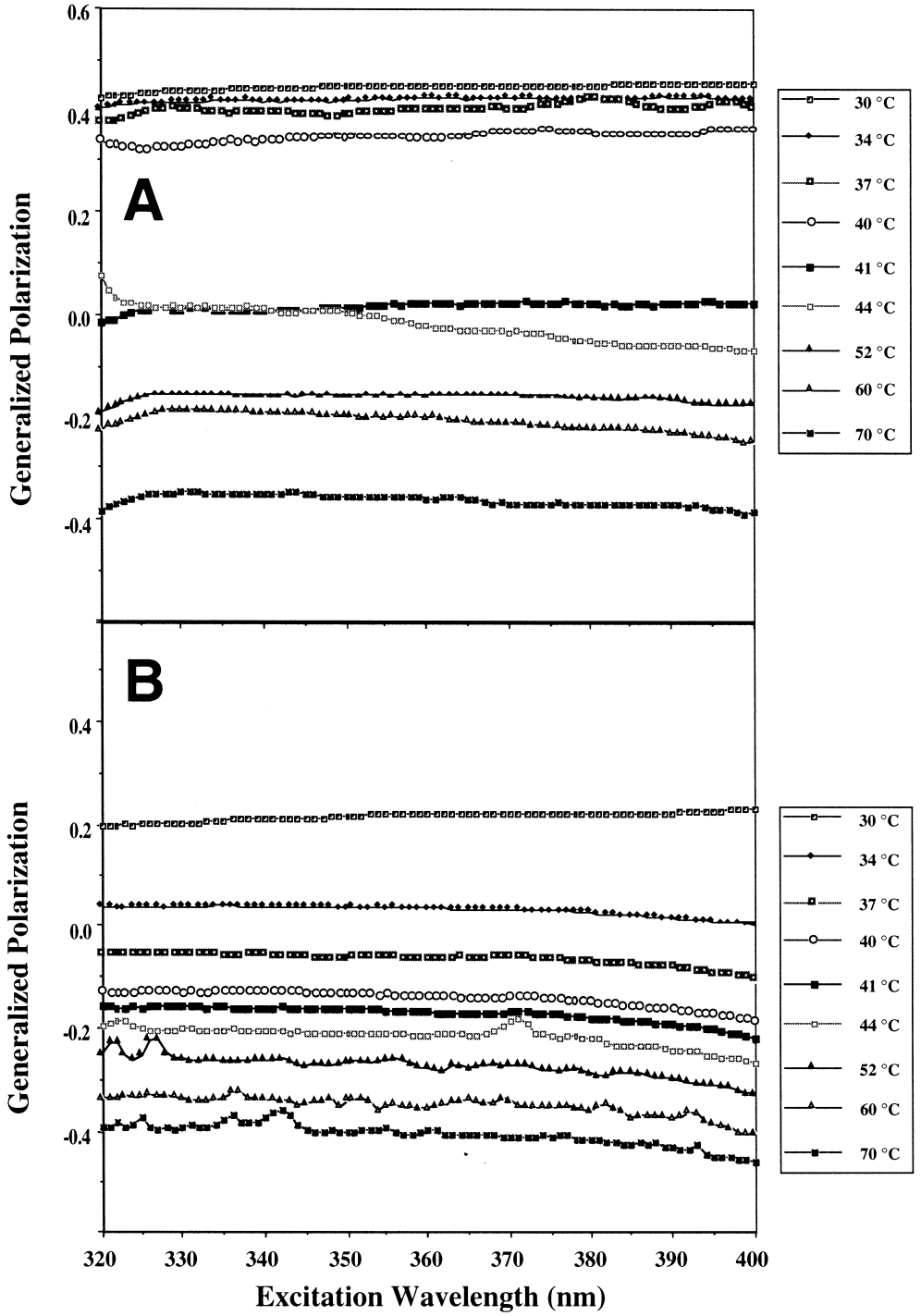



Fig. 4. General polarization as a function of wavelength for DPPC and DPPC/DOTAP liposomes. Excitation spectra of Laurdan-containing multilamellar liposomes composed of (A) DPPC (100%), and (B) DPPC/DOTAP (50/50) were acquired at various temperatures spanning the phase transition temperature of DPPC, using emission wavelengths of 440 nm and 490 nm. General polarization was calculated at each excitation wavelength as described in Section 2. The symbols and temperatures to which they are assigned are shown in the inset for each panel.

interaction or incorporation of substances for encapsulation. Fluorescence spectroscopy was used to probe the compositions of DPPC/DOTAP not amenable to study by DSC, and to test the specific hypotheses that DOTAP perturbs the membrane water/hydrocarbon interfacial region or alters lipid miscibility.

The excitation spectrum of Laurdan is sensitive to lamellar phase properties, and does not partition selectively in different lipid domains [26]. The excitation spectrum has two peaks: one at 350 nm and the other at 390 nm. The relative intensity of these peaks is sensitive to the phase state of the membrane; in the gel state ($T \leq 41.3$°C), the intensity at 350 nm is greater than that at 390 nm, whereas in the liquid crystalline phase ($T \geq 41.3$°C), the 390 nm band is slightly more intense than the band at 350 nm.

Laurdan spectra were acquired for DPPC/DOTAP vesicles at temperatures spanning the bilayer phase transition of DPPC (Fig. 3A). From the relative heights of the 350 and 390 nm excitation peaks, the data suggest that the bilayer exists in the gel phase over the temperature range 30–40°C. As the temperature increased above 40°C, an abrupt decrease was observed in fluorescence intensity at 390 nm, indicating transition from the gel to the liquid crystalline phase.

Fig. 3B shows Laurdan excitation spectra for DPPC/DOTAP (50/50) liposomes. At the lowest temperatures (30°C), the membrane existed almost entirely in the liquid crystalline phase, based on the intensity of the 350 and 390 nm peaks. Therefore, DOTAP induced an earlier onset of the liquid crystalline phase in DPPC membranes. This observation is consistent with the DSC studies that showed DOTAP to lower the phase transition temperature of DPPC membranes containing $\leq 20$ mol% DOTAP.

### 3.3. General polarization of the bilayer phases

Laurdan can provide additional information on the state of the bilayer phase, such as the relative fractions of gel and liquid crystalline domains when those phases coexist. Therefore, the generalized polarization (GP) of Laurdan was calculated from fluorescence spectral scans. GP values of approximately 0.6 and −0.2 correspond to the gel and liquid crystalline phases, respectively [26–28]. In addition, the wavelength dependence of GP is smaller for gel state lipid than for lipid in the liquid crystalline phase state; this phenomenon arises from the high rate of solvent relaxation in the liquid crystalline membrane at higher temperatures, which leads to excited state stabilization and a red shift in the fluorescence emission [26–28].

The steady-state GP value was calculated for each excitation wavelength [26], as described in Section 2, and data were acquired to determine GP as a function of temperature and liposome composition. From the excitation spectra of 100% DPPC (Fig. 4A), the gel phase at 30°C has a GP value of 0.4, and the GP was independent of excitation wavelength. The excitation spectra at 41°C, a temperature at which gel and liquid phase coexist, gave a GP value of 0.0, and the GP was slightly more dependent on wavelength than it was in the gel phase. A stronger dependence of GP on wavelength was observed for the liquid crystalline phase at 70°C (GP = −0.4).

At most temperatures, the GP values of DPPC liposomes containing 50% DOTAP suggest a decrease in the relative amount of gel phase compared to compositions lacking DOTAP (Fig. 4B). At 30°C, the GP values of DPPC/DOTAP resembled those of DPPC at 41°C. The GP values of DPPC/DOTAP at $\geq 34$°C, having a magnitude of $\leq 0.0$, suggest the existence of liquid crystalline phase. Overall, the data are consistent with broadening and early onset of the liquid crystalline phase transition in the presence of 50 mol% DOTAP.

Fig. 5 shows the effect of DOTAP on the lamellar domain structure of DPPC as a function of temperature. In general, the excitation $GP_{380nm}$ of DPPC and DPPC/DOTAP liposomes decreased as a function of increasing temperature, with the lowest GP values reported at 70°C and a sharp decrease in $GP_{380nm}$ observed at approximately 41°C, the temperature at which DPPC undergoes the gel to liquid crystalline transition.

### 3.4. DOTAP effects on the hydrocarbon chain region of DPPC membranes

Laurdan suggested significant perturbation of the interfacial region of DPPC membranes by DOTAP. The fluorescent probe DPH (diphenylhexatriene) is located deeper within the bilayer core, and is sensi-

tive to membrane fluidity because the probe has greater rotational freedom in the liquid crystalline phase [32,33]. In liposomes of DPPC alone, DPH had a polarization value of approximately 0.45 at 25°C (Fig. 6). 10 mol% DOTAP in DPPC, which caused significant lowering and broadening of the DPPC phase transition as detected by DSC (data not shown), had no significant effect on DPH mobility (data not shown). In contrast, 50 mol% DOTAP exerted a significant effect on acyl chain mobility of DPPC-containing liposomes (Fig. 6).

To investigate whether the observed fluidizing effect arises from a unique property of the dialkyl cationic lipid, or whether the effect is conferred by the incorporation of unsaturated hydrocarbon chains into the bilayer, a variety of other charged and unsaturated phospholipids were substituted for DOTAP in DPPC membranes. Anionic and zwitterionic phospholipids of the same hydrocarbon chain length and saturation exerted an effect similar to that of DOTAP; 50 mol% DOPG (dioleoylphosphatidylglycerol; Fig. 6) or DOPC (dioleoylphosphatidylcholine; data not shown) in DPPC altered acyl chain mobility to the same extent as did 50% DOTAP. Naturally-occurring phospholipids having a blend of different acyl chain lengths and saturation had an intermediate effect compared to dioleoyl lipids (Fig. 6).

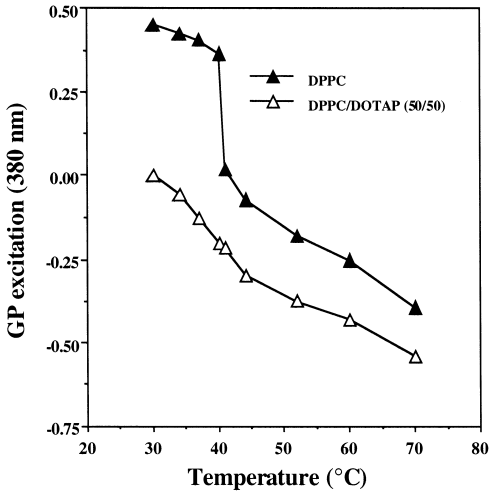



Fig. 5. GP as a function of temperature for DPPC and DPPC/DOTAP liposomes. GP values for Laurdan plotted in the presence and absence of DOTAP (see Section 2), and the GP values for 380 nm are plotted as a function of temperature for each liposome formulation.

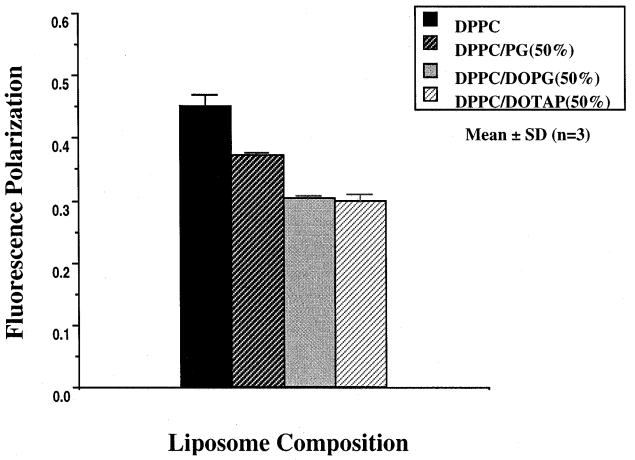



Fig. 6. Acyl chain mobility of DPPC liposome containing DOTAP. Liposomes of DPPC were prepared containing 50 mol% DOTAP, DOPG, or PG from egg yolk. DPH was added in trace quantity and fluorescence polarization was measured as described in Section 2. The temperature was 25°C for each experiment. The liposomes and symbols to which they are assigned are in the inset for each panel. Each point represents the mean of three separate determinations, and the vertical bars on each point indicate the standard deviation.

The effect of 50 mol% DOTAP on acyl chain mobility was not restricted to DPPC; similar effects were observed for DOTAP in dimyristoylphosphatidylcholine (DMPC) and distearoylphosphatidylcholine (DSPC) liposomes (data not shown).

### 3.5. Effect of DOTAP on liposome size

Liposome diameter and size dispersity are important parameters in drug delivery applications. QLS (quasi-elastic light scattering) was used to determine the effect of DOTAP on the size distribution of spontaneously-forming liposomes as a function of phospholipid acyl chain length (14:0, 16:0, 18:0), degree of unsaturation (18:1), and concentration of DOTAP (0–100 mol%). DOTAP reduced the mean diameter of PC-containing multilamellar liposomes, regardless of the phospholipid chain length and degree of unsaturation (Fig. 7). The greatest effect was observed in the range 0–20 mol% cationic lipid; for some compositions the effect was quite large. For example, DSPC liposomes had a mean diameter of 3 μm (Fig. 7); inclusion of 20 mol% DOTAP reduced liposome diameter nearly 5-fold, to 600 nm.

With increasing mol% DOTAP, both the diameter

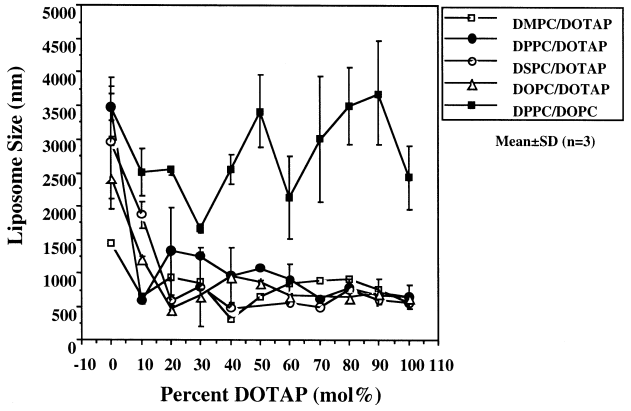



Fig. 7. Effect of DOTAP on the size distribution of multilamellar vesicles. Liposomes were prepared with varying ratios of DOTAP in DMPC, DPPC, DOPC, and DSPC, or with DOPC in DPPC. QLS was used to determine liposome diameter; samples were prepared in triplicate and analyzed at 25°C using a 2 min acquisition time. The volume-weighted NICOMP analysis was applied to estimate the size distribution. Liposome diameter is shown as a function of phospholipid acyl chain length (14:0, 16:0, and 18:0), degree of unsaturation (18:1), and percent DOTAP (0–100 mol%). The liposomes and symbols to which they are assigned are in the inset of the panel.

and the dispersity of the vesicle population were reduced (data not shown); regardless of the PC used, binary mixtures containing 50 mol% DOTAP had a mean diameter of 0.5–1 μm (Fig. 7). Liposomes of 100% DOTAP had a mean diameter of approximately 0.6 μm.

To investigate whether the reduction in vesicle size arises primarily from the inclusion of a lipid with unsaturated alkyl chains, DOPC was substituted for DOTAP in DPPC liposomes. Over a range of 10–100% DOPC, liposome diameter was highly variable, and tended to larger mean diameters than the equivalent DOTAP-containing DPPC formulation (Fig. 7). Anionic unsaturated lipids also were investigated; DOPG or egg PG at 50 mol% reduced the diameter of DPPC vesicles to sizes equivalent to DOTAP-containing liposomes (data not shown).

### 3.6. Cellular interaction

DOTAP-containing liposomes, like anionic liposomes, have been shown to interact with many different cell types. However, most compositions tested previously contain large amounts of phosphatidylethanolamine, are highly unstable in the presence of cells, and carry complexed polyanions such as DNA which may modify their electrostatic charge. Furthermore, cells vary in their ability to bind or internalize liposomes [6,7,34–36]. We investigated the extent to which liposomes of PC and high concentrations of DOTAP can interact with cells, and

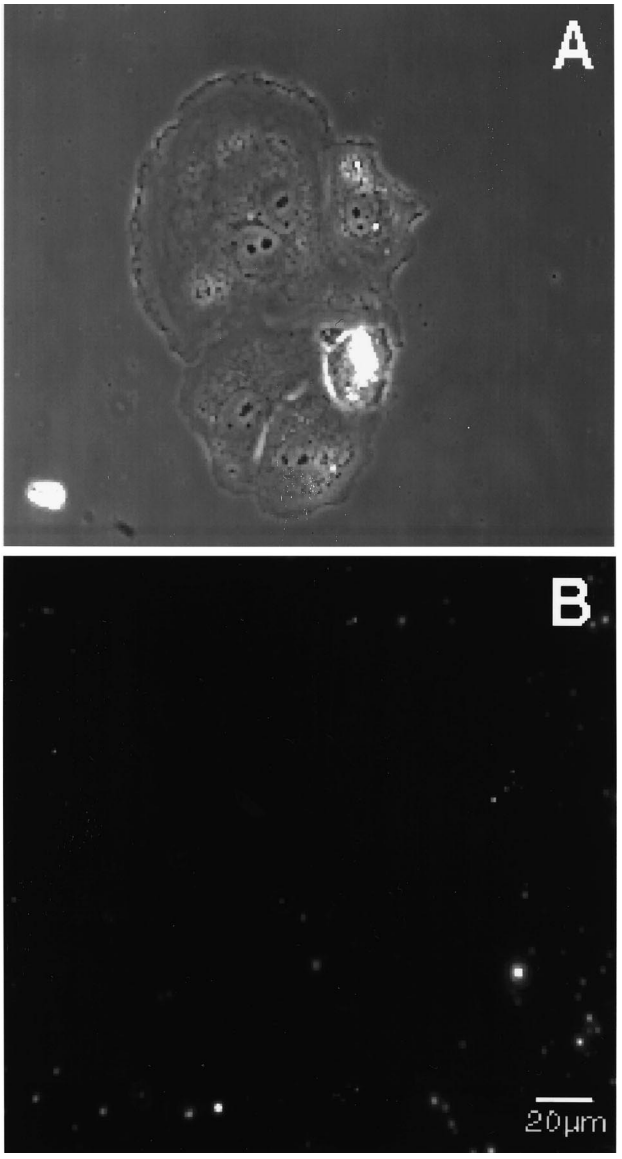



Fig. 8. Uptake of rhodamine-DPPE labeled anionic liposomes by OVCAR-3 cells in vitro. Cells in culture were incubated for 1 h at 37°C with 100 nmol of liposomes containing 1 mol% of the fluorescent phospholipid rhodamine-DPPE. Cells were incubated with 100 nmol of DOPC/DOPG (50/50) liposomes, and then washed free of unbound liposomes. Phase contrast (A) and fluorescence images (B) were acquired by microscopy (bar = 20 μm).

focused on OVCAR-3, an epithelioid human ovarian cancer cell line that has been shown to bind anionic liposomes so poorly that it interferes with drug delivery [30,34]. As a control, anionic liposomes were prepared in which DOTAP was substituted with an equivalent mol fraction of DOPG.

When OVCAR-3 cells were exposed to 100 nmol/ml DOPC/DOPG (50/50) for 1 h at 37°C, few rhodamine-DPPE labeled liposomes were associated with cells (Fig. 8). In contrast, DOPC/DOTAP (50/50) liposomes showed greater cell association (Fig. 9), and the perinuclear localization of fluorescence in many cells suggested internalization (Fig. 10).

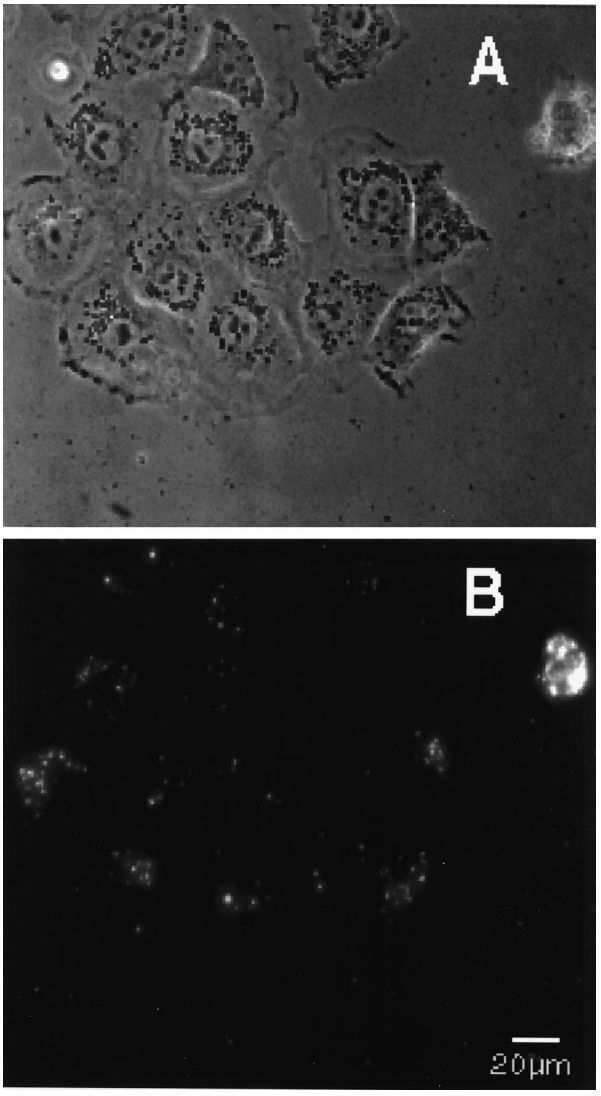



Fig. 9. Uptake of rhodamine-DPPE labeled cationic liposomes by OVCAR-3 cells in vitro. Cells in culture were incubated for 1 h at 37°C with 100 nmol of liposomes containing 1 mol% of the fluorescent phospholipid rhodamine-DPPE. Cells were incubated with 100 nmol of DOPC/DOTAP (50/50) liposomes, and then washed free of unbound liposomes. Phase contrast (A) and fluorescence images (B) were acquired by microscopy.

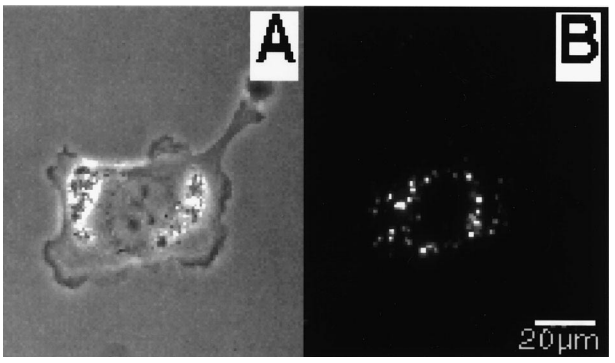



Fig. 10. Uptake of rhodamine-DPPE labeled cationic liposomes by OVCAR-3 cells in vitro. Phase contrast (A) and fluorescence image (B) demonstrating fluorescence in the perinuclear region of OVCAR-3 tumor cell 1 h after incubation with 100 nmol of DOPC/DOTAP (50/50) liposomes, and then washed free of unbound liposomes before analysis (bar = 20 μm).

## 4. Discussion

DOTAP-containing liposomes have been studied most extensively for DNA delivery. Such liposomes are designed to deliver well hydrated macromolecules across cell membranes, and the role of the 'helper' phospholipid, in most cases a phosphatidylethanolamine, has been shown to be critical for delivery [1,2,37]. The intrinsic instability of PE-rich liposomes, as well as the specific non-bilayer structural transitions which PE undergoes [5,38], appear to be important factors in cellular delivery of nucleic acids.

In contrast, most drug delivery applications for liposomes require properties other than intrinsic instability; these include incorporation (encapsulation) efficiency, structural stability in biological media, and, for optimal circulation time in the blood, high phase transition lipids and small diameter. As DNA transfection helper lipids, the phosphatidylcholines are less efficient than phosphatidylethanolamines because of their lower propensity to undergo the structural transitions important for DNA delivery. However, by reason of their greater stability, PC-containing liposomes are more suitable for entrapping and delivering therapeutic agents. Formation

and stability of such drug/lipid formulations may be impacted greatly by the fundamental physical characteristics of the membrane, including phase transition temperature and miscibility of the cationic lipids in the presence of zwitterionic phospholipids. Therefore, we investigated the effects of DOTAP on the fundamental physical properties of liposomes.

DSC and Laurdan fluorescence spectroscopy were used to investigate the effect of cationic lipids on phase behavior and membrane fluidity; both reported a shift in the DPPC phase transition temperature toward lower temperatures, with DSC showing peak broadening at higher concentrations ($>10$ mol%) of DOTAP. DSC was not able to detect the physical events that occur above 20% DOTAP, owing to extensive peak broadening by DOTAP. However, fluorescence spectroscopy employing the probe Laurdan provided information on bilayer state at higher DOTAP concentrations. At 50% DOTAP, the DPPC bilayer existed predominately in a homogeneous liquid crystalline phase, and retained its structural integrity at elevated temperatures.

DPH polarization studies suggest DOTAP to have charge-, chain length-, and saturation-dependent effects on membrane fluidity. Phospholipid vesicles composed of unsaturated acyl chains with charged head groups (e.g. DOTAP and DOPG) fluidized membranes to a greater extent than did uncharged unsaturated (e.g. DOPC) or charged unsaturated lipids obtained from a more heterogeneous source (e.g. egg PG).

Liposome diameter or hydrodynamic radius bears a direct relationship to liposome clearance rates in blood, and can exert marked effects on the antitumor activity of therapeutic agents [39,40]. For some clinical applications, it may be desirable to prepare liposomes immediately prior to administration by the hydration of dry lipid powders [41–44]. Both the diameter and population dispersity of such spontaneously-forming liposomes are critical in this application.

The inclusion of DOTAP at $\geq$10 mol% both decreased the diameter of spontaneously-forming liposomes and decreased particle heterogeneity. Substitution of lipids matching either charge or acyl chain configuration indicated that the effect on size distribution did not appear to be chain length- or concentration-dependent at higher mole fractions of DOTAP. At lower mol fractions, DOTAP exerted the most marked effect on vesicle diameter.

Because 10% DOTAP exerted potent effect on vesicle size and peak broadening (observed by DSC), we investigated the role of membrane fluidity in reduction of vesicle size. The unsaturated zwitterionic lipid DOPC, which had a significant effect on membrane fluidity, did not reduce the size of liposomes composed predominantly of DPPC; rather DOPC inclusion resulted in a broadly variable size distribution of liposomes. In contrast, anionic charged unsaturated lipids not only conferred a significant increase in membrane fluidity, but also reduced DPPC vesicle size to a greater extent than uncharged unsaturated lipids (data not shown).

The molecular or physical interactions working in conjunction with membrane fluidity to reduce vesicle size is an important question. Alterations at the membrane interface induced by DOTAP could function in part to facilitate head group electrostatic interactions. These physical interactions could promote size reduction by decreasing the bilayer radius of curvature as a result of phospholipid-cationic head group contact. Molecular modeling studies conducted elsewhere [45], have reported DOTAP and DOPC head groups to be located at a similar plane of the bilayer. It was also suggested that the quaternary amine of DOTAP and the phosphate group of the phospholipid DOPC may form a salt bridge [45]. Such molecular interactions may explain, in part, the potent effect of DOTAP on vesicle size and bilayer phase state. These physical interactions may be exploited to improve the stability and encapsulation efficiency of liposomes containing drugs [46].

Stable cationic liposomes offer the potential for remarkable advancement of drug delivery applications. For example, although many cell lines bind anionic liposomes avidly, certain human ovarian cancer lines appear quite resistant to liposome-mediated delivery [30,34]. Inclusion of DOTAP results in a drastic increase in the binding of liposomes by OVCAR-3 human ovarian tumor cells; uptake of anionic liposomes of equivalent but opposite charge, was negligible. At relatively high concentrations of cationic liposomes (e.g. 400 nmol/$10^6$ cells) and percent charge ($\geq$50%), we observed no cytotoxicity (data not shown). Avid uptake of cationic liposomes by HeLa cells in culture [6], and angiogenic endothe-

lial cells in tumors [7], have been reported, and suggest applications in cell-specific targeting. Based on the effect of cationic lipids on the physical stability of liposomes [47], as well their apparent organ- and vasculature-specific uptake [4], stable cationic liposomes may have a wide range of applications in drug delivery, and represent an advance over previously described liposome formulations.

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