Tunable pH-Sensitive Liposomes Composed of Mixtures of Cationic and Anionic Lipids

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ABSTRACT The pH-dependent fusion properties of large unilamellar vesicles (LUVs) composed of binary mixtures of anionic and cationic lipids have been investigated. It is shown that stable LUVs can be prepared from the ionizable anionic lipid cholesteryl hemisuccinate (CHEMS) and the permanently charged cationic lipid *N,N*-dioleoyl-*N,N*-dimethylammonium chloride (DODAC) at neutral pH values and that these LUVs undergo fusion as the pH is reduced. The critical pH at which fusion was observed (pH_f) was dependent on the cationic lipid-to-anionic lipid ratio. LUVs prepared from DODAC/CHEMS mixtures at molar ratios of 0 to 0.85 resulted in vesicles with pH_f values that ranged from pH 4.0 to 6.7, respectively. This behavior is consistent with a model in which fusion occurs at pH values such that the DODAC/CHEMS LUV surface charge is zero. Related behavior was observed for LUVs composed of the ionizable cationic lipid 3α-[*N*-(*N′,N′*-dimethylaminoethane)-carbamoyl] cholesterol hydrochloride (DC-Chol) and the acidic lipid dioleoylphosphatidic acid (DOPA). Freeze-fracture and ^{31}P NMR evidence is presented which indicates that pH-dependent fusion results from a preference of mixtures of cationic and anionic lipid for "inverted" nonbilayer lipid phases under conditions where the surface charge is zero. It is concluded that tunable pH-sensitive LUVs composed of cationic and anionic lipids may be of utility for drug delivery applications. It is also suggested that the ability of cationic lipids to adopt inverted nonbilayer structures in combination with anionic lipids may be related to the ability of cationic lipids to facilitate the intracellular delivery of macromolecules.

INTRODUCTION

Liposomes that exhibit triggered release properties have potentially important applications in drug delivery. It has been shown that liposomes can be constructed that are sensitive to a variety of physical and chemical stimuli, including temperature, light, or pH (Gerasimov et al., 1996). Liposomes that can be triggered to release their contents or fuse in response to pH stimuli are of particular interest, as they can potentially respond to acidic environments in vivo (Yatvin et al., 1980; Straubinger, 1993). Such environments include those encountered in tumor tissue (Tannock and Rotin, 1989) and primary endocytic vesicles (Tycko and Maxfield, 1982).

pH-sensitive liposomes are typically prepared from lipid mixtures containing dioleoylphosphatidylethanolamine (DOPE), a lipid that adopts the nonbilayer inverted hexagonal (H_{II}) phase in isolation (Cullis and de Kruijff, 1978), and an ionizable acidic lipid such as cholesteryl hemisuccinate (Lai et al., 1985b). At pH values above the p*K* of the acidic lipid, the negatively charged form of the acidic lipid can stabilize the DOPE in the bilayer organization, allowing the formation of bilayer vesicles. These vesicles then fuse as the pH is reduced toward the p*K* of the acidic lipid. Protonation of the anionic lipid diminishes the ability of this component to stabilize DOPE in the bilayer organization (Lai et al., 1985b). Lipids such as phosphatidylserine (Hope et al., 1983), palmitoylhomocysteine (Connor et al., 1984), and α-tocopherol hemisuccinate (Jizomoto et al., 1994) have also been used in concert with DOPE to prepare pH-sensitive liposomes.

This approach gives limited control of the pH at which fusion will occur unless one resorts to using a number of anionic lipids with different p*K* values (Collins et al., 1989). We describe here the properties of LUVs containing cholesteryl hemisuccinate (CHEMS) and increasing amounts of the permanently charged cationic lipid *N,N*-dioleoyl-*N,N*-dimethylammonium chloride (DODAC). It is shown that these LUVs exhibit pH-sensitive fusion properties and that the pH at which fusion is observed increases as the content of cationic lipid is increased. It is also demonstrated that pH-induced fusion correlates with a preference for nonbilayer inverted lipid structures. Related effects were observed for LUVs composed of dioleoylphosphatidic acid (DOPA) and the ionizable cationic lipid 3α-[*N*-(*N′,N′*-dimethylaminoethane)-carbamoyl] cholesterol hydrochloride (DC-Chol). It is concluded that the pH at which fusion of LUVs containing anionic lipids occurs can be readily modulated by the inclusion of cationic lipid. It is also demonstrated that mixtures of cationic and anionic lipids exhibit interesting polymorphism that may be related to the ability of cationic lipids to promote the intracellular delivery of macromolecules.

MATERIALS AND METHODS

DC-Chol, DOPA, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2–1,3-benzoxadiazol-4-yl) (NBD-PE), and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-lissamine rhodamine b sulfonyl (Rh-PE) were

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obtained from Avanti Polar Lipids (Alabaster, AL). Cholesteryl hemisuccinate (morpholine salt), dimethylamine, oleoyl bromide, HEPES, 2-[*N*-morpholino]ethanesulfonic acid (MES), and *t*-octylphenoxypolyethoxyethanol (Triton X-100) were obtained from Sigma Chemical Company (St. Louis, MO).

Synthesis of DODAC

Oleoyl bromide (5 g) was stirred in a saturated solution of dimethylamine in methanol (200 ml) overnight. The solvent and most of the dimethylamine was removed on a rotovap. The residue was treated with 10 ml of 5 N NaOH solution, and all of the remaining dimethylamine was removed under vacuum. Chloroform (40 ml) was added to the mixture, followed by oleoyl bromide (10 g). The mixture was refluxed overnight, diluted with water (50 ml), and extracted three times with chloroform. The organic phase was washed six times with 3% hydrochloric acid, decolorized with hydrochloric acid-washed charcoal, and washed twice with saturated aqueous NaCl. The solvent was removed on a rotovap, and the residue was dried by azeotropic removal of water with ethanol. The residue was passed down a silica gel column (200 g) with a 2–20% MeOH/CH_2Cl_2 gradient. Fractions containing the hydroxyl analog of DODAC were discarded. Fractions containing DODAC were combined and the solvent was removed under vacuum. Fractions contaminated with the hydroxyl analog of DODAC were stored for rechromatography. The residue from the pure DODAC fractions was hydrated in water (50 mg/ml) at 60°C, passed through 0.1-μm-pore polycarbonate filters with an Extruder (Lipex Biomembranes, Vancouver, BC), and lyophilized, yielding DODAC as a colorless powder (5 g). Purity was determined to be greater than 99% by ^{1}H NMR and thin-layer chromatography.

Preparation of LUVs

LUVs were prepared from binary mixtures of DODAC and CHEMS or DC-Chol and DOPA. Lipids were dissolved in chloroform at the desired molar ratios and dried to a thin film under a stream of nitrogen gas. Typically 5–10 μmol of total lipid was dried per 13 $\times$ 100 mm glass test tube. The resulting thin films were placed under high vacuum for at least 1 h to remove residual organic solvent. DODAC/CHEMS lipid films were hydrated by vortex mixing in 1.0 ml of aqueous buffer containing 10 mM HEPES and 150 mM NaCl (pH 8.1). DC-Chol/DOPA lipid films were hydrated by vortex mixing in 1.0 ml of either 10 mM HEPES, 150 mM NaCl (pH 8.1) or 10 mM HEPES, 150 mM NaCl (acidified to pH 3.9 with acetic acid). After hydration the multilamellar vesicles (MLVs) were subjected to five freeze-thaw cycles (liquid nitrogen/room temperature). The MLV suspensions were then extruded 10 times through two stacked 0.2-μm-pore polycarbonate filters with an extruder (Lipex Biomembranes) to produce LUVs (Mayer et al., 1986). The mean diameter of LUV systems was determined with a Nicomp C270 submicron particle sizer operating in the particle mode.

Lipid mixing fusion assays

A membrane fusion assay based on fluorescence resonance energy transfer between two fluorescent lipid probes was used to assess the fusion of LUVs in response to pH (Struck et al., 1981). The fluorescent lipids NBD-PE and Rh-PE were included at 1 mol% each to produce labeled LUVs. Labeled and unlabeled vesicles were mixed at a 1:5 molar ratio and injected into 2 ml of buffer containing 10 mM HEPES, 10 mM 2-[*N*-morpholino]ethanesulfonic acid, 10 mM acetate, and 140 mM NaCl adjusted to pH values between 3.0 and 8.5. Ammonium chloride (10 mM) was included in the medium to eliminate transmembrane pH gradients for the DC-Chol/DOPA systems. The final lipid concentration was 150 μM. NBD-PE fluorescence dequenching was monitored in a stirred cuvette in a Perkin-Elmer LS-50B at 25°C, using excitation and emission wavelengths of 467 nm and 540 nm, respectively. Lipid mixing was monitored for ~400 s. Complete dilution of the fluorescent probes was determined by the addition of Triton X-100 to a final concentration of 0.1% (v/v). The extent of lipid mixing was calculated with the equation Lipid mixing (%) = $(F - F_o)/(F_{max} - F_o) \times 100$, where F is the NBD-PE fluorescence during the lipid mixing assay, F_o is the initial fluorescence, and F_{max} is the NBD-PE fluorescence upon infinite probe dilution in the presence of 0.1% (v/v) Triton X-100. The value of F_{max} was corrected for the quenching of NBD-PE fluorescence due to Triton X-100 by measuring the NBD-PE fluorescence of vesicles that had a concentration of NBD-PE and Rh-PE of 0.16 mol%, the molar fraction expected if the labeled and unlabeled LUVs had undergone complete lipid mixing. To allow a comparison of pH sensitivity between different LUV compositions, the normalized lipid mixing (%) for each lipid composition was expressed as a percentage of the maximum lipid mixing observed for a given LUV composition.

Freeze-fracture electron microscopy

The structures formed by hydrated DODAC/CHEMS mixtures were analyzed by freeze-fracture electron microscopy. DODAC/CHEMS lipid films were hydrated with 50 mM HEPES, 150 mM NaCl (pH 8.1), followed by five freeze-thaw cycles. DODAC/CHEMS LUVs that were subsequently acidified were formed from lipid films hydrated with 10 mM HEPES, 150 mM NaCl (pH 8.1) to reduce the buffering capacity. DODAC/CHEMS LUVs were treated with a volume of acidic buffer containing 50 mM acetate, 150 mM NaCl (pH 4.6) to obtain the final desired pH values. Glycerol was added to all samples to a final concentration of 30% (v/v). Samples were dispensed onto gold cups and rapidly frozen in Freon 22 cooled in liquid nitrogen. Platinum/carbon replicas were prepared using a Balzers freeze-etching unit (BAF 400D) and observed by transmission electron microscopy as described elsewhere (Hope et al., 1989).

^{31}P nuclear magnetic resonance spectroscopy

Lipid films composed of DC-Chol/DOPA (1.6 molar ratio) were hydrated in 20 mM HEPES (pH 7.6) or 20 mM HEPES, 20 mM acetate (pH 3.8) to achieve a final concentration of 15 mM phospholipid. The DC-Chol/DOPA dispersion hydrated at pH 3.8 was alkalized with ~60 μl of 500 mM NaOH to achieve a final pH of 6.1. ^{31}P NMR spectra were obtained with a Bruker MSL-200 spectrometer operating at 81.3 MHz. Acquisition parameters included a 60° pulse, a 10-kHz sweep width, and a 1-s interpulse time, and spectra were accumulated in the presence of broad-band proton decoupling. The temperature was 300 K.

RESULTS

DODAC/CHEMS lipid mixtures can form bilayer vesicles on hydration

It is well established that CHEMS adopts a bilayer structure on hydration at neutral pH (Lai et al., 1985a; Janoff et al., 1988) and can stabilize nonbilayer lipids such as DOPE in the bilayer organization (Ellens et al., 1984). Similarly, cationic lipids such as DODAC can form bilayers and stabilize DOPE in a bilayer structure (Mok and Cullis, 1997). Less is known concerning the structural properties of mixtures of bilayer-forming cationic and anionic lipids, which may be expected to exhibit unusual behavior due to interactions between the positively and negatively charged headgroups. It was found that hydration of lipid films com-

posed of DODAC and CHEMS at cationic-to-anionic lipid molar ratios of less than 1 resulted in white milky dispersions visually similar to those observed for MLV phospholipid dispersions. The structure of these DODAC/CHEMS lipid dispersions was examined by freeze-fracture electron microscopy, revealing the formation of MLVs (Fig. 1 *A*). It was also found that MLVs consisting of DODAC/CHEMS at molar ratios less than 1 could be extruded through 0.2-μm-pore filters to give rise to uniformly sized LUV structures (Fig. 1 *B*). The mean diameter $\pm$ mean SD of these vesicles as determined by dynamic light scattering was 153 $\pm$ 35 nm for DODAC/CHEMS molar ratios of 0–0.72 and 274 $\pm$ 94 nm for LUVs composed of DODAC/CHEMS at a molar ratio of 0.85.

DODAC/CHEMS LUVs undergo pH-sensitive fusion that can be modulated by adjusting the DODAC/CHEMS ratio

Previous work has shown that LUVs composed of CHEMS alone undergo pH-dependent fusion as the pH is lowered below pH 4.2 (Hafez and Cullis, 2000). An objective of this investigation was to determine whether the addition of cationic lipid resulted in an increase in the pH at which fusion occurred in these pH-sensitive LUVs. The next experiments therefore characterized the influence of increasing amounts of DODAC on the pH-dependent fusion properties of DODAC/CHEMS LUVs. Lipid mixing assays were performed as a function of pH for DODAC/CHEMS LUVs at molar ratios ranging from 0 to 0.85. As shown in Fig. 2 *A*, increases in DODAC content resulted in an in-

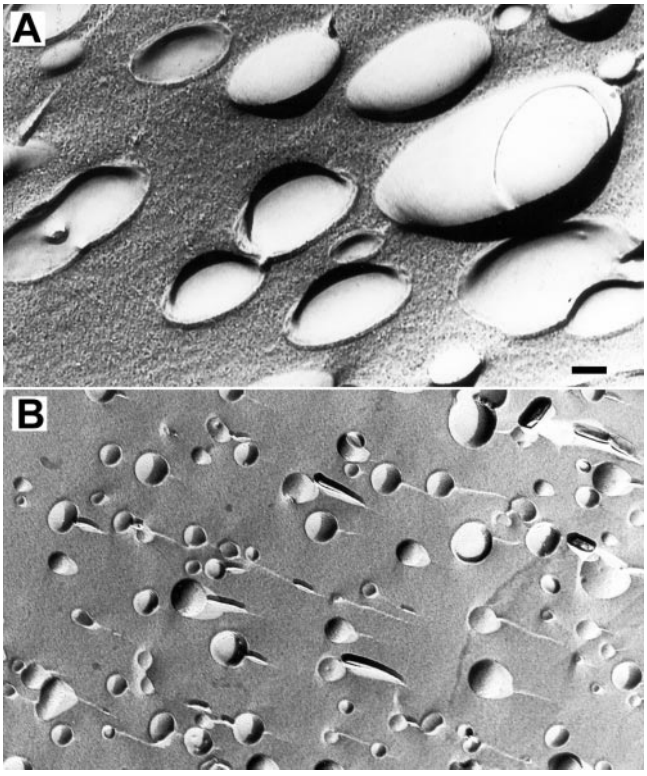

FIGURE 1 Structural characteristics of aqueous dispersions of DODAC/CHEMS (0.72 molar ratio). The lipid was hydrated in 50 mM HEPES, 150 mM NaCl (pH 8.1), and freeze-fracture electron micrographs were prepared as described in Materials and Methods. (*A*) DODAC/CHEMS MLVs formed on hydration of the lipid film. (*B*) DODAC/CHEMS LUVs formed after extrusion through two stacked filters with 0.2-μm pore size. Scale bar: 200 nm.

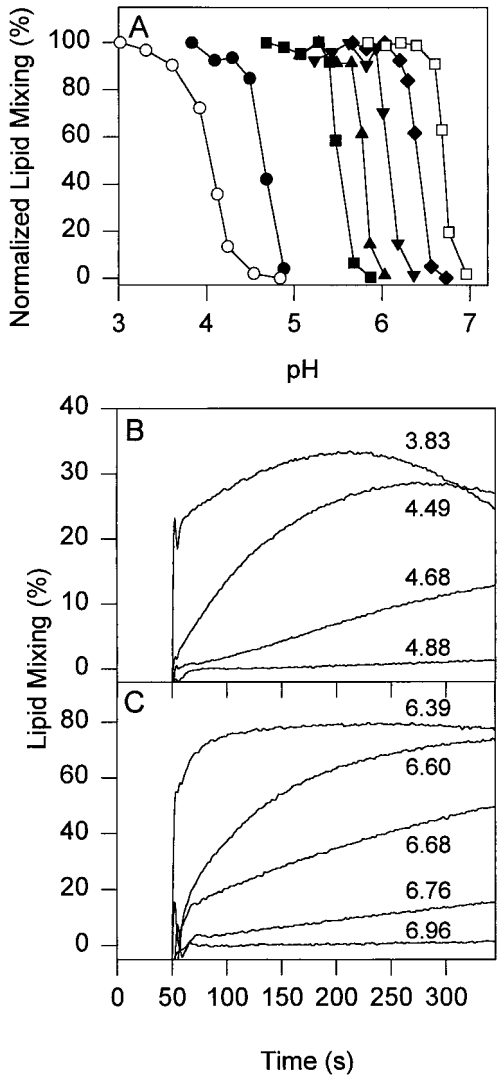

FIGURE 2 pH-dependent fusion properties of DODAC/CHEMS LUV containing increasing amounts of DODAC. (*A*) DODAC/CHEMS LUV prepared at pH 8.1 were then introduced into buffer with the pH values indicated. The normalized lipid mixing (%) was determined as described in Materials and Methods. Data are presented for LUVs with DODAC/CHEMS molar ratios of 0 (○), 0.11 (●), 0.43 (■), 0.52 (▲), 0.61 (▼), 0.72 (♦), and 0.85 (□). (*B*) Membrane fusion kinetics observed for LUVs composed of DODAC/CHEMS (0.11 molar ratio) and (*C*) LUVs composed of DODAC/CHEMS (0.85 molar ratio) after acidification. LUVs were added to buffer with the indicated pH at 50 s. The lipid mixing (%) was determined as described in Materials and Methods.

crease in the pH at which fusion occurred. For example, the pH for half-maximum fusion (pH_f) for pure CHEMS LUVs is 4.0, whereas for LUVs composed of DODAC/CHEMS at a molar ratio 0.11, a pH_f of 4.7 was observed. Further increases in the DODAC/CHEMS molar ratio to 0.85 resulted in LUVs with pH_f values as high as 6.7. The fusion kinetics observed with the lipid mixing assay for LUVs composed of DODAC/CHEMS at molar ratios of 0.11 and 0.85 are shown in Fig. 2, *B* and *C*, respectively.

It is logical to suggest that fusion between DODAC/CHEMS LUVs will not proceed unless the surface charge is neutralized, allowing close contact between LUVs. For the surface charge to be zero the proportion of CHEMS that is negatively charged must equal the DODAC content of the membrane. Under these conditions it is straightforward to show that the pH at which the surface charge is neutral (pH_n) can be written as

$$pH_n = pK_{CHEMS} + \log_{10}[(X_{DODAC}/X_{CHEMS} - X_{DODAC})] \quad (1)$$

where pK_{CHEMS} is the apparent p*K* of CHEMS and X_{DODAC} and X_{CHEMS} are the molar fractions of CHEMS and DODAC, respectively. Previous work has shown that the apparent p*K* of CHEMS in a lipid bilayer is 5.8 (Hafez and Cullis, 2000), allowing a comparison between pH_f and pH_n. The assumption that the apparent p*K* of CHEMS remains constant is only an approximation. As shown in Fig. 3, pH_f correlates well with pH_n, supporting the conclusion that fusion proceeds between DODAC/CHEMS LUVs when the surface charge is zero.

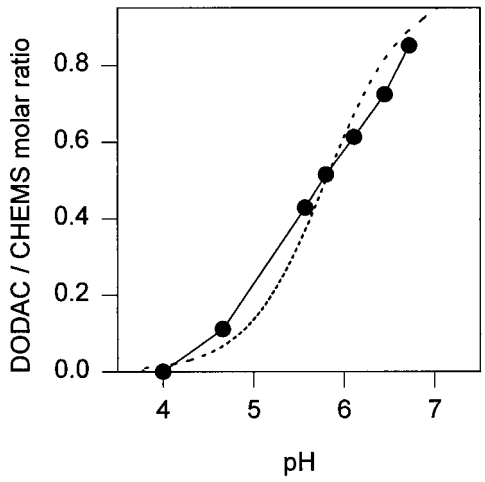

FIGURE 3 Correlation between membrane fusion and surface charge neutralization of DODAC/CHEMS LUVs. Half-maximum fusion (pH_f) values were determined from Fig. 2 for each DODAC/CHEMS LUV formulation as the pH at which lipid mixing was 50% of the maximum observed lipid mixing and plotted as a function of the DODAC/CHEMS molar ratio (●). The pH at which the surface charge was predicted to be zero (*dashed line*) was determined from Eq. 1, using $pK_{CHEMS} = 5.8$.

pH-sensitive fusion that is modulated by the cationic-to-anionic lipid ratio can also be observed for LUVs containing an ionizable cationic lipid

pH-sensitive fusion of DODAC/CHEMS LUVs is modulated by the neutralization of CHEMS, the anionic lipid component, which is in excess compared to the cationic lipid. These systems exhibit progressively lower levels of negative surface charge as the pH is lowered, fusing when the surface charge is zero. It is of interest to examine the properties of systems in which the cationic lipid species is the ionizable component and is present in excess over the anionic lipid species. By analogy with the behavior of DODAC/CHEMS LUVs, such systems should form stable bilayers at low pH, where the cationic lipid species is fully charged, and should fuse as the pH is raised toward the p*K* of the cationic lipid. DC-Chol, which was chosen as the ionizable cationic lipid, has a p*K* of 8.0 (Zuidam and Barenholz, 1997). The anionic lipid chosen was DOPA, which exhibits p*K* values of ~3.0 and ~8.0 (Tocanne and Teissié, 1990). It was found that DC-Chol/DOPA LUVs at molar ratios ranging from 1.6 to 4 could be prepared at pH 3.9, and as predicted, fusion between these LUVs was observed as the pH was raised (Fig. 4 *A*). The fusion kinetics observed with the lipid mixing assay for LUVs composed of DC-Chol/DOPA at molar ratios of 1.6 and 4.0 are shown in Fig. 4, *B* and *C*, respectively. Variations in the DC-Chol/DOPA molar ratio over the range 1.6–4.0 resulted in changes in the pH_f from 4.9 to 7.7. It was not possible to correlate pH_f with pH_n, the pH valve giving rise to zero surface charge, because of the indeterminate nature of the p*K* values of DC-Chol and DOPA in these mixed systems.

It was also found that DC-Chol/DOPA LUVs could be produced at basic pH values above the p*K* of the DC-Chol component. In particular, LUVs were generated at pH 8.1 from DC-Chol/DOPA at a molar ratios of 1.1–1.6. These systems underwent fusion as the pH was lowered toward neutrality (results not shown). This behavior was attributed to the ability of the negatively charged DOPA to stabilize the bilayer organization in the presence of neutral DC-Chol. Fusion as the pH was reduced can then be attributed to the increasing protonation of DC-Chol and DOPA, leading to a reduced surface charge.

Fusion of LUVs composed of cationic and anionic lipid is accompanied by the appearance of "inverted" nonbilayer lipid structures

The structural features of LUVs composed of cationic and anionic lipids when the pH is adjusted to levels that promote fusion are of interest. Although surface charge neutralization may be necessary to allow close apposition of vesicle bilayers, it is unlikely that this condition alone is sufficient to induce fusion. It was observed during the lipid mixing

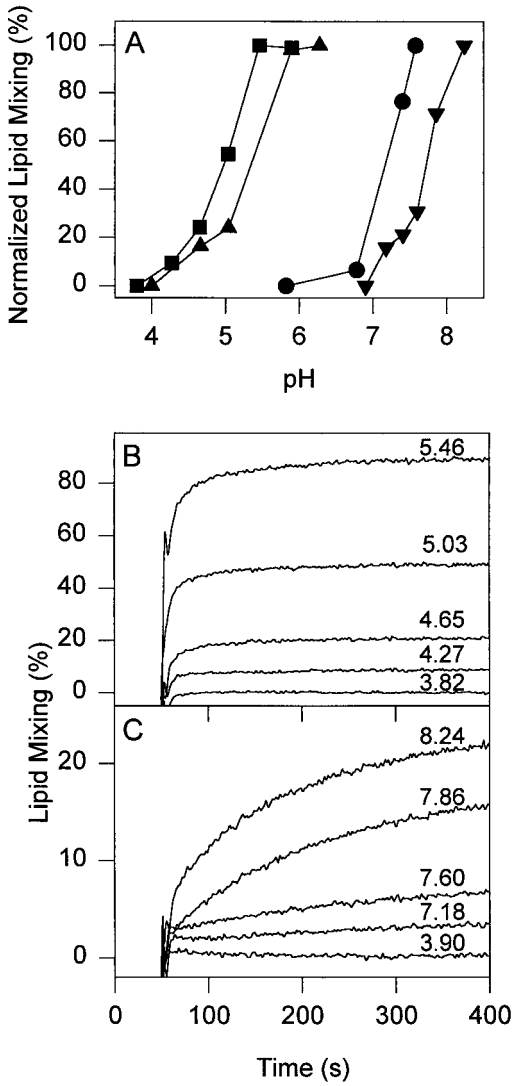

FIGURE 4 pH-dependent fusion properties of DC-Chol/DOPA LUVs containing increasing amounts of DC-Chol. (*A*) DC-Chol/DOPA LUVs were prepared at pH 3.9 and were then introduced into buffer with the pH values indicated. The normalized lipid mixing (%) was determined as described in Materials and Methods. Data are presented for LUVs with DC-Chol/DOPA LUV molar ratios of 1.6 (■), 2.0 (▲), 3.0 (●), and 4.0 (▼). (*B*) Membrane fusion kinetics observed for LUVs composed of DC-Chol/DOPA (1.6 molar ratio) and (*C*) LUVs composed of DC-Chol/DOPA (4.0 molar ratio) after the pH increase. LUVs were added to buffer with the indicated pH at 50 s. The lipid mixing (%) was determined as described in Materials and Methods.

assays that fusion of DODAC/CHEMS LUVs was accompanied by an increase in the turbidity of the LUV dispersion, followed by precipitation of the lipid. The structures formed by DODAC/CHEMS (0.85 molar ratio) LUVs after incubation at pH 6.9 (the approximate pH_f for this system) were examined by freeze-fracture electron microscopy. As shown in Fig. 5, *A–C*, large structures containing characteristic "lipidic particle" (Verkleij et al., 1980) or "interlamellar attachment site" (Siegel, 1999) structures are observed. Such structures are commonly observed in fusion between vesicles induced by the tendency of component lipids to adopt nonbilayer structures (Verkleij et al., 1980). The propensity of DODAC/CHEMS (0.85 molar ratio) LUVs to adopt a nonbilayer structure was further demonstrated by examining the structures formed after incubation at pH 5.4, a value below the pH_f for this system. As shown in Fig. 5 *D*, the freeze-fracture micrographs obtained reveal the characteristic striated pattern of lipid organized in the hexagonal H_{II} phase.

The ability of mixtures of cationic lipid and anionic lipid to adopt nonbilayer structures under conditions of zero surface charge is intriguing, and suggests that equimolar mixtures of charged cationic and anionic lipids may prefer nonbilayer structure on hydration, whereas either species in isolation adopts a lamellar organization. This possibility was investigated for aqueous dispersions of equimolar amounts of DODAC and CHEMS prepared at pH 8.1. Freeze-fracture electron microscopy studies revealed systems containing lipidic particle structures (Fig. 6 *A*). Extensive regions of linear arrays of lipidic particles were also observed (Fig. 6, *B* and *C*); such structure has been associated with lipids in the cubic phase (Ellens et al., 1989).

The polymorphic phase preferences of DC-Chol/DOPA lipid mixtures could be investigated by using ^{31}P NMR, because of the presence of the phosphate group of the phosphatidic acid. As shown in Fig. 7 *A*, DC-Chol/DOPA (1.6 molar ratio) dispersions prepared at pH 3.8 reveal the characteristic asymmetric lineshape with a low field shoulder and a high field peak associated with bilayer structure (Cullis and de Kruijff, 1979). Adjustment of the pH to 6.1 (Fig. 7 *B*) results in the appearance of a ^{31}P NMR signal with reversed asymmetry that is a factor of 2 narrower and is characteristic of phospholipid in the hexagonal H_{II} phase (Cullis and de Kruijff, 1979). Integration of the spectra shown in Fig. 7, *A* and *B*, indicated a constant ^{31}P NMR signal intensity after correction for the number of scans accumulated for each spectrum. At pH 7.6, the DC-Chol/DOPA mixture adopts a bilayer organization, as indicated by ^{31}P NMR (Fig. 7 *C*). These results illustrate the ability of the charged form of either DC-Chol or DOPA to stabilize the ensemble into a bilayer organization, whereas when the amounts of charged DC-Chol and DOPA are equal, the hexagonal H_{II} phase is adopted. Changes in the width of the chemical shift anisotropy of the spectra, indicating a bilayer organization at acidic and alkaline pH values (Fig. 7, *A* and *C*), may be ascribed to changes in the ionization of phosphatidic acid (Pott et al., 1995).

DISCUSSION

The results presented here provide information on a new class of liposomal systems composed of mixtures of cationic and anionic lipids. There are three major points of interest. First, stable liposomes can be generated from mix-

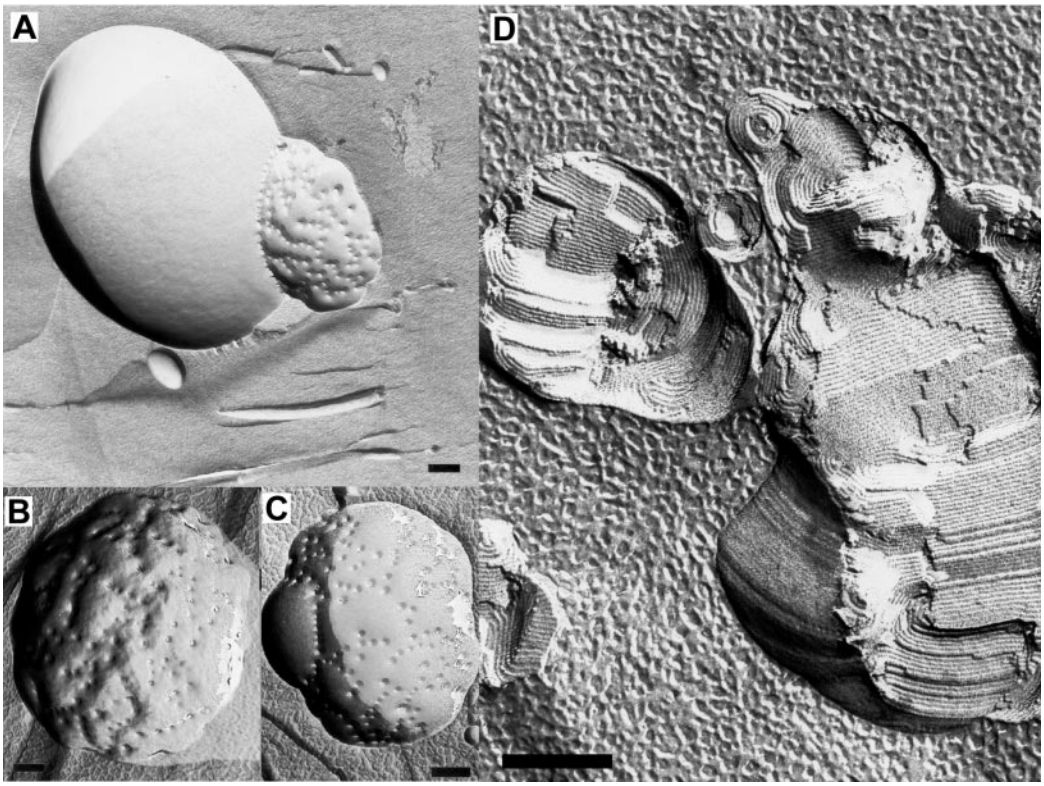

FIGURE 5 Freeze-fracture electron micrographs of DODAC/CHEMS LUVs (0.85 molar ratio) prepared at pH 8.1 and then incubated at lower pH values. DODAC/CHEMS LUVs adopt nonbilayer phases at low pH values. (*A–C*) Incubation at pH 6.9 induces fusion and lipidic particle structures. (*D*) Incubation at pH 5.4 results in fusion and formation of the inverted hexagonal phase. Scale bars: 200 nm.

tures of cationic and anionic lipids when either the cationic or anionic lipid species is in excess. Second, fusion between these liposomes is stimulated by a preference for nonbilayer "inverted" lipid phase structures when the surface charge is zero. Finally, if an ionizable cationic or anionic lipid is used, pH-dependent fusion between them is observed, and varying the proportions of cationic and anionic lipids can modulate the pH at which this fusion occurs. We discuss these features in turn.

The observation that stable liposomal systems can be generated from mixtures of cationic and anionic lipids is perhaps surprising, given the possibility of forming phase-separated crystalline domains of neutral cationic-anionic lipid pairs in these mixed lipid systems. The evidence pre-

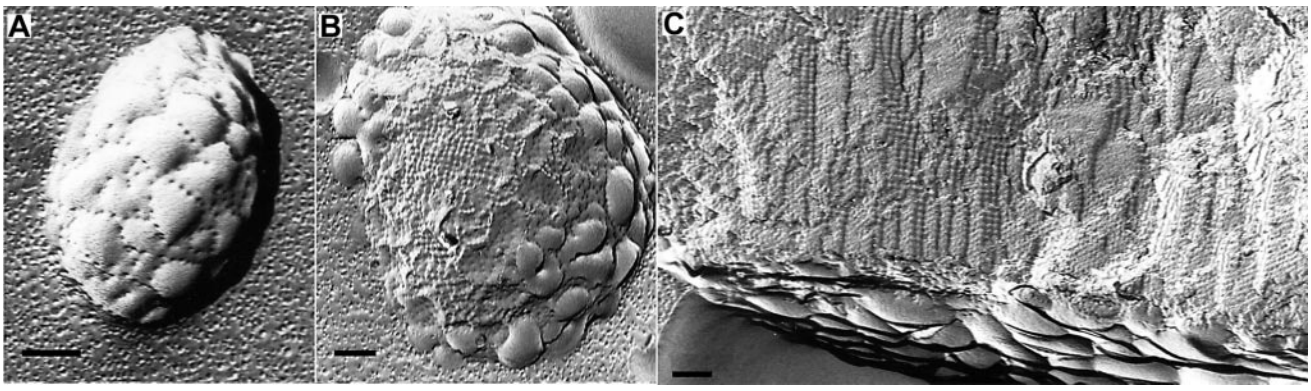

FIGURE 6 Equimolar mixtures of DODAC/CHEMS adopt nonbilayer structures after hydration at neutral pH values. Freeze-fracture electron micrographs of equimolar mixtures of DODAC/CHEMS hydrated in 50 mM HEPES, 150 mM NaCl (pH 8.1) reveal (*A* and *B*) lipidic particle structures and (*C*) regular arrays of lipidic particles. Freeze-fracture replicas were prepared and observed as described in Methods and Materials. Scale bars: 200 nm.

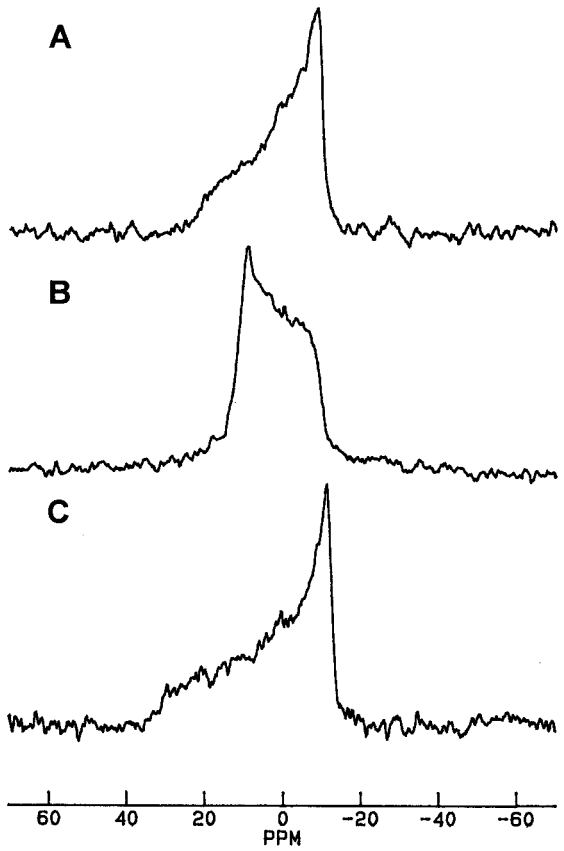

FIGURE 7 Influence of pH on the polymorphic phase properties of aqueous dispersions of DC-Chol/DOPA as detected by ^{31}P NMR. (*A*) ^{31}P NMR spectrum obtained from an aqueous dispersion of DC-Chol/DOPA (1.6 molar ratio) hydrated at pH 3.8. (*B*) ^{31}P NMR spectrum of the sample employed in *A* alkalized to pH 6.1 with NaOH. (*C*) ^{31}P NMR spectrum of an aqueous dispersion of DC-Chol/DOPA (1.6 molar ratio) hydrated at pH 7.6. A 50-Hz line broadening was applied to each spectrum. For other acquisition parameters see Materials and Methods.

sented here is consistent with the formation of liquid crystalline lipid bilayers from charged mixtures of cationic and anionic lipids, with no evidence for crystalline domains of cationic-anionic lipid pairs. Perhaps the most compelling evidence in this regard is provided by the ^{31}P NMR lineshapes observed for DOPA in mixtures with DC-Chol (Fig. 7) as well as the constant ^{31}P NMR signal intensity arising from the DOPA. The bilayer and hexagonal H_{II} ^{31}P NMR lineshapes are signatures of liquid crystalline phospholipids that are free to rotate rapidly around their long axes, with additional motional averaging in the hexagonal H_{II} phase due to lateral diffusion around the aqueous cores of the H_{II} phase cylinders (Cullis and de Kruijff, 1979). The constant ^{31}P NMR signal intensities that are observed independently of phase structure suggest that all of the DOPA, even if it is part of a DC-Chol:DOPA lipid pair, is contributing to these liquid crystalline lineshapes in both the bilayer and hexagonal phases. Previous work has shown that phospholipids in crystalline domains give rise to much broader ^{31}P NMR lineshapes with increased T_1 values (Tilcock et al., 1984).

The observations that LUVs containing cationic and anionic lipids fuse when the net surface charge is zero and that this fusion is due to a preference for nonbilayer structure are of particular interest for three reasons. First, fusion of LUVs when component lipids can adopt inverted lipid structures is consistent with the extensive literature indicating that membrane fusion proceeds via nonbilayer structures such as inverted micelles or stalks (see Chernomordik and Zimmerberg, 1995; Siegel, 1999 for reviews). These structures are favored by the presence of lipids that are able to adopt inverted lipid phases. Second, the ability of mixtures of cationic and anionic lipids to adopt cubic and hexagonal H_{II} phases under conditions of zero surface charge is also fully consistent with the phase behavior of mixtures of cationic and anionic surfactants (Kaler et al., 1989; Zemb et al., 1999) and intrinsic curvature or lipid shape arguments used to rationalize the phase preferences of lipids (Gruner et al., 1985). In particular, it has been shown that equimolar mixtures of single-chain cationic and anionic detergents can spontaneously form closed bilayer vesicles in aqueous solution (Kaler et al., 1989). In these systems, the cationic and anionic surfactants are suggested to form ion pairs that result in a diacyl zwitterion with a cylindrical molecular shape that is compatible with lamellar structure. Using the language of Gruner et al. (1985), because of the reduction in the headgroup area the intrinsic curvature of the lipid monolayers decreases substantially when the ion pairs are formed, leading to transitions from micellar to lamellar structures. Analogous behavior would be expected for ion pairs formed from mixtures of cationic and anionic lipids that adopt bilayer structure in isolation. In this case the reduction in intrinsic curvature on formation of zwitterions composed of cationic lipid and anionic lipid ion pairs would be expected to lead to transitions from bilayer structure to inverted lipid phases such as the hexagonal H_{II} phase or the cubic phase, as observed experimentally. Using shape arguments, this corresponds to a transition from charged lipids with a cylindrical shape to neutral ion pairs with a "cone" shape that is compatible with inverted lipid phase structures. Related behavior is observed, for example, when the negative headgroup charge of cardiolipin, a bilayer-forming tetra-acyl phospholipid, is neutralized by the addition of Ca^{2+}, triggering a bilayer-to-H_{II} phase transition (Rand and Sengupta, 1972; Cullis et al., 1978).

The third reason why fusion between LUVs containing cationic and anionic lipids, which reflects a preference for nonbilayer structure is of interest is that it represents the first demonstration that mixtures of two species of lipids that both adopt the bilayer configuration in isolation can form nonbilayer phases in combination. It is likely that this ability is basic to the mechanism whereby cationic lipids increase the intracellular delivery and transfection potency of nucleic acid polymers after incubation of cells with

cationic lipid-nucleic acid polymer complexes (Felgner et al., 1987). Previous work has shown that negatively charged lipids found in cell membranes can displace cationic lipid from nucleic acids in these complexes (Xu and Szoka, 1996; Zelphati and Szoka, 1996). The work presented here gives rise to the possibility that the displaced cationic lipid combines with the negatively charged lipid in cell membranes to actively promote formation of nonbilayer structures such as the hexagonal H_{II} phase. This would be expected to assist in the membrane disruption process that allows anionic polymers to penetrate the plasma or endosomal membrane to gain access to the cell interior.

The observation that LUVs composed of mixtures of DODAC/CHEMS and DC-Chol/DOPA undergo pH-dependent fusion at pH values that are regulated by the cationic-to-anionic lipid ratios has obvious potential for the design of pH-sensitive liposomes for tumor delivery or intracellular delivery applications. Tumor pH values measured by microelectrode techniques reveal that these tissues are acidic compared to normal tissue with a mean pH of 7.0 (range 5.8–7.6) (Tannock and Rotin, 1989) indicating the potential usefulness of liposomes that become unstable and release their contents, such as anticancer drugs, at these pH values. Alternatively, a major impediment to the utility of macromolecular drugs such as antisense oligonucleotides for the down-regulation of pathogenic genes (Loose-Mitchell, 1988) or plasmid expression vectors for gene therapy applications (Ledley, 1995) is the inability of these charged macromolecules to penetrate target cell membranes. Previous work has demonstrated the encapsulation of plasmid DNA within anionic CHEMS/DOPE pH-sensitive liposomes (Legendre and Szoka, 1992). This suggests that the encapsulation of nucleic acids within tunable pH-sensitive DODAC/CHEMS vesicle systems containing an excess of anionic lipid is possible. Delivery using liposomes that are tuned to become unstable in mildly acidic endocytic compartments could be of considerable utility. The particular advantage of the systems described here is that the pH value at which the liposome becomes unstable can be adjusted in a straightforward and systematic manner. Future work will determine the potential of these systems for intracellular delivery and other applications.

In summary, the studies presented show that stable liposomes can be constructed from mixtures of cationic and anionic lipids and that these liposomes fuse at pH values that can be readily adjusted by varying the ratio of the cationic and anionic lipid components. Fusion is accompanied by formation of nonbilayer structures formed under conditions of zero surface charge. Furthermore, the ability of mixtures of cationic and anionic lipids to adopt nonbilayer structures gives potentially important insight into the mechanism of action of cationic lipids used for the intracellular delivery of nucleic acids.

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