

Acknowledgments

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[7] Tunable pH-Sensitive Liposomes

By ISMAIL M. HAFEZ and PIETER R. CULLIS

Overview

Liposomes are membrane-enclosed vesicles composed of a lipid bilayer shell surrounding an aqueous core. Liposomes have historically been studied and developed as biophysical models of cellular membranes and as drug delivery systems—an application for which an appropriate response to a physiological stimulus such as bilayer lysis or lipid chemical breakdown is highly desirable. Smart liposome systems that are able to alter their permeability or change their chemical composition in response to external signals have utility in drug delivery applications¹ and as components of biosensor elements² in the emerging fields of nano and biotechnology.

One of the most attractive stimuli to exploit *in vivo* for drug delivery applications is that of local pH change. During the cellular process of endocytosis, early endosomal vesicles become acidified by the vacuolar-ATPase proton pump, producing a large vesicular proton gradient versus cytosolic pH.³ The pH inside endosomal compartments can become as low as pH 5.5–4.5, sufficiently acidic to be exploited by microbial and viral agents to trigger mechanisms that allow the escape of pathogens and toxins from endosomal membranes and access to the cell interior. Liposome systems that contain pH-sensitive components can be designed to respond appropriately to low pH environments encountered during endocytosis.

pH-sensitive liposome systems are traditionally composed of a mixture of a titratable anionic lipid and a neutral or zwitterionic lipid.⁴ The neutral lipids used, such as dioleoylphosphatidylethanolamine (DOPE), adopt nonbilayer lipid phases in isolation, but mixtures with titratable anionic lipids, such as cholesteryl hemisuccinate (CHEMS), can be stabilized into

¹ O. V. Gerasimov, J. A. Boomer, M. M. Qualls, and D. H. Thompson, *Adv. Drug. Deliv. Rev.* **38**, 317 (1999).

² M. B. Esch, A. J. Baeumner, and R. A. Durst, *Anal. Chem.* **73**, 3162 (2001).

³ B. Tycko and F. R. Maxfield, *Cell* **28**, 643 (1982).

⁴ R. M. Straubinger, *Methods Enzymol.* **221**, 361 (1993).

a bilayer phase. The titratable anionic lipid can stabilize the nonbilayer lipid only in the ionized state and thus does so in a pH-dependent manner. The range of pH sensitivity of systems composed of mixtures of anionic and neutral lipids is, in general, dependent on the pK_a of the anionic stabilizing lipid⁵ and may also be altered slightly based on the molar ratio of the anionic and neutral lipid in the bilayers.⁶ An alternative method for the preparation of tunable pH-sensitive liposomes has been described by our group.⁷

This article describes a novel type of pH-sensitive liposome system composed of a mixture of cationic and anionic lipids. In this system the pH of membrane instability can be predictably altered, simply by changing the binary ratio of the cationic and anionic components of the liposome membrane. This article discusses the unique polymorphic and fusogenic properties of tunable pH-sensitive liposome systems that derive from the presence of both cationic and anionic lipids in the same lipid membrane. Details of the design, preparation, and methods for characterization of several formulations of tunable pH-sensitive liposomes are highlighted.

Introduction

Equimolar Mixtures of Cationic and Anionic Lipids Form Nonbilayer Phases

Mixtures of cationic and anionic amphipaths dispersed in aqueous media assemble into aggregates with the charged head groups near the membrane/water interface forming charge neutralized ion pairs.⁸ This property has been exploited to prepare bilayer vesicles from mixtures of cationic and anionic detergents such as cetyl trimethylammonium tosylate (CTAT) and sodium dodecyl benzene sulfonate (SDBS), each of which adopt micellar phases in isolation.⁹ Bilayer vesicles can be formed from mixtures of single chain cationic and anionic surfactants in a range of charge ratios, making vesicles that are neutral, anionic, or cationic in net charge.⁹

Surprisingly, only until very recently have the phase properties of mixtures of bilayer-forming cationic lipids in mixtures with bilayer-forming anionic lipids been described. Physicochemical studies have revealed that

⁵ D. F. Collins, F. Maxfield, and L. Huang, *Biochim. Biophys. Acta* **987**, 47 (1989).

⁶ H. Ellens, J. Bentz, and F. C. Szoka, *Biochemistry* **24**, 3099 (1985).

⁷ I. M. Hafez, S. Ansell, and P. R. Cullis, *Biophys. J.* **79**, 1438 (2000).

⁸ S. Bhattacharya and S. S. Mandal, *Biochemistry* **37**, 7764 (1998).

⁹ E. W. Kaler, A. K. Murthy, B. E. Rodriguez, and J. A. Zasadzinski, *Science* **245**, 1371 (1989).

mixtures of bilayer-forming cationic and anionic lipids exhibit unique polymorphic phase behavior.^{7,10–12} Specifically, mixtures of bilayer-forming cationic and anionic lipids adopt nonbilayer phases such as the inverted hexagonal (H_{II}) phase when mixed under conditions of zero surface charge. **Figure 1** shows that this phenomenon holds true for mixtures of different cationic and anionic lipids.¹² Alternatively, mixtures of cationic and anionic lipid can exist as bilayer vesicles if stabilized sufficiently by strong bilayer forming lipids such as phosphatidylcholine¹² or in the presence of an excess of either the anionic or the cationic lipid component.⁷ In the case of ionizable lipid species comprising either vesicular cationic or anionic lipid components, the net charge of the vesicle systems and the resultant phase properties are directly a function of pH and the cationic-to-anionic lipid molar ratio.

Tunable pH-Sensitive Lipid Phases and Liposomes from Mixtures of Cationic and Anionic Lipids

Ionizable Anionic Lipid Present in Excess over a Permanently Charged Cationic Lipid. Consider the properties of a binary mixture of an ionizable anionic lipid in molar excess over a monovalent cationic lipid containing a quaternary amine. In this case, the pH at which the liposome surface charge will be zero (and thus favors the formation of nonbilayer phases) is determined by the amount of ionized anionic lipid and the molar ratio of the cationic and anionic lipids. The pH at which the lipid mixture is neutralized (pH_n) occurs when the molar fraction of the anionic lipid (X_{anionic} with an ionization constant $\text{p}K_a$) that is negatively charged equals the cationic lipid (X_{cationic}) fraction of the membrane. Numerically, this is given by the modified Henderson–Hasselbach equation,⁷

$$\text{pH}_n = \text{p}K_a + \log_{10}[X_{\text{cationic}}/(X_{\text{anionic}} - X_{\text{cationic}})] \quad (1)$$

Equation (1) may be used to predict the pH when a mixture of an ionizable anionic lipid and a permanently charged cationic lipid become neutralized and thus prefer to adopt nonbilayer lipid phases over bilayer phases. **Equation (1)** shows that pH_n is theoretically in the range of $\text{p}K_a \pm 1.99$ for liposome formulations prepared from a mixture of a permanently charged cationic lipid and an ionizable anionic lipid with cationic/anionic lipid molar ratios ranging from 0.01 to 0.99. This suggests that tunable bilayer systems prepared in this manner can be designed to respond to a

¹⁰ R. N. Lewis and R. N. McElhaney, *Biophys. J.* **79**, 1455 (2000).

¹¹ Y. S. Tarahovsky, A. L. Arsenault, R. C. MacDonald, T. J. McIntosh, and R. M. Epand, *Biophys. J.* **79**, 3193 (2000).

¹² I. M. Hafez, N. Maurer, and P. R. Cullis, *Gene Ther.* **8**, 1188 (2001).

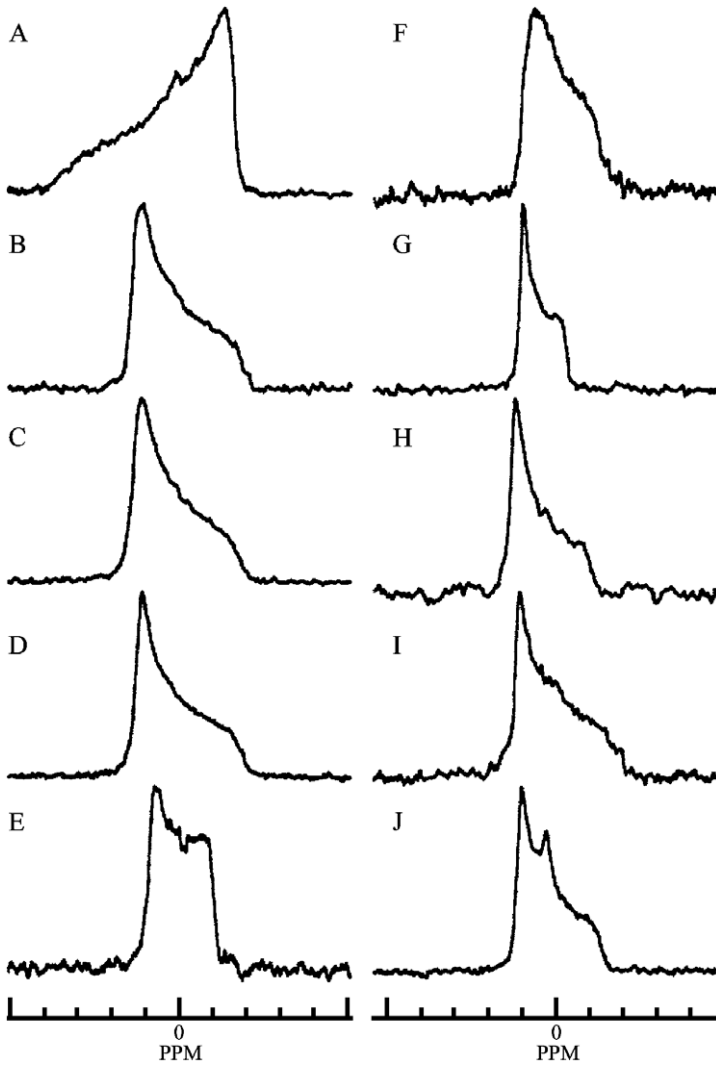


FIG. 1. Equimolar mixtures of cationic lipids and anionic phospholipids adopt the inverted hexagonal (H_{II}) phase. ^{31}P NMR spectra obtained from aqueous dispersions of (A) DOPS, (B) DODAC/DOPS, (C) DOTMA/DOPS, (D) DOTAP/DOPS, (E) DC-Chol/DOPS, (F) DODAC/DOPA, (G) DODAC/LBPA, (H) DODAC/CL (2:1; molar ratio), (I) DODAC/PI, and (J) DODAC/POPG. Cationic lipid abbreviations: *N,N*-dioleoyl-*N,N*-dimethylammonium chloride (DODAC), *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-tri-methylammonium chloride (DOTMA), 1,2-dioleoyloxy-3-(trimethylammonio) propane (DOTAP), 3 α -[*N,N'*-dimethyl-aminoethane]-carbamoyl] (DC-Chol). Anionic lipid abbreviations: dioleoylphosphatidylserine (DOPS), dioleoylphosphatidic acid (DOPA), *sn*-(3-oleoyl-2-hydroxy)-glycerol-1-phospho-*sn*-

range of proton concentrations that differs up to 10,000 \times . It will be demonstrated that lipid vesicles composed of the anionic lipid CHEMS and the cationic lipid *N, N*-dioleoyl-*N, N*-dimethylammonium chloride (DODAC) adhere very closely to the behavior predicted by Eq. (1).

Ionizable Cationic Lipid in Excess over an Anionic Lipid. Ionizable cationic lipids such as 3 α -[*N*-(*N, N*'-dimethylaminoethane)carbonyl] cholesterol hydrochloride (DC-Chol) may be included into bilayer phases with mixtures of an anionic lipid such as dioleoylphosphatidic acid (DOPA). In this case, DOPA may be considered as the permanently charged lipid having an ionizable phosphate group with at least one negative charge at pH values above ~ 4.0 . With the DC-Chol in excess over the anionic lipid, bilayer phases are favored at acidic pH values when the net charge of the system is positively charged. In this system, bilayer stability is maintained at acidic pH and nonbilayer phases are favored under neutral and basic conditions. Interestingly with this system, some mixtures of DC-Chol/DOPA (e.g., 1.6 molar ratio) will adopt bilayer phases at both acidic and basic pH values when the system is either positively or negatively charged, and nonbilayer phases when neutralized.⁷ Vesicle and bilayer phases that are prepared from these lipid mixtures may have application not only in the area of smart drug delivery systems, but also in the development of biosensor elements based on supported bilayer membranes.¹³ However, this article restricts the content to the preparation of tunable pH-sensitive liposomes that are acid sensitive, as these present the most potential for intracellular drug delivery applications.

Materials

Lipids and Chemicals

DODAC is from Steven Ansell of Inex Pharmaceuticals Corporation (Burnaby, BC). 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-*sn*-glycero-3-phosphoserine (DOPS), 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-PE), 1,2-dioleoyl-*sn*-glycerol-3-phospho-L-serine-

¹³ E. Sackmann, *Science* **271**, 43 (1996).

3'-(1'-oleoyl-2'-hydroxy)-glycerol (lysobisphosphatidic acid/LBPA), tetraoleoylcardiolipin (CL), phosphatidylinositol liver derived (PI), 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG). Reproduced from Hafez *et al.*¹² with permission.

N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-PS), and 1,2-dioleoyl-*sn*-glycerol-3-phosphoethanolamine-*N*-lissamine rhodamine b sulfonyl (Rh-PE) are from Avanti Polar Lipids (Alabaster, AL). Cholesterol, cholesteryl hemisuccinate (morpholine salt), 2-[*N*-morpholino]ethanesulfonic acid (MES), Triton X-100, HEPES, and sodium dithionite are from Sigma Chemical Co. (St. Louis, MO). Lipids are checked for purity by thin-layer chromatography and are used if samples produce only one spot by phospholipid detection or sulfuric acid char.

Methods

General Preparation of Large Unilamellar Vesicles

Lipids are obtained as dry powders, dissolved in chloroform, and stored at -20° until use. Large unilamellar vesicles (LUVs) of uniform size distribution are prepared by a freeze-thaw and extrusion technique.¹⁴ Positive displacement pipettes that utilize glass capillary-like tips (Digital Microdispenser, VWR Scientific, West Chester, PA) are used to dispense accurately chloroform solutions of lipids in the 1- to 100- μ l range. Lipids are codissolved in chloroform at desired molar ratios and dried to a thin film under a stream of nitrogen gas. Fluorescent lipids used for the preparation of labeled LUVs for the lipid-mixing assays are included at 1 mol% each; combinations are either NBD-PE/Rh-PE or NBD-PS/Rh-PE. Typically, between 5 and 10 μ mol of lipid is dried in 13×100 -mm borosilicate glass test tubes. The resulting thin films are placed in high vacuum for 1 to 2 h to remove residual solvent. Lipid films are hydrated in 1 to 2 ml of buffer with vigorous vortex mixing to form multilamellar vesicles (MLVs). Occasionally, some lipid mixtures do not hydrate well using this method. In these cases, the test tubes containing the dried lipid and buffer are frozen either by careful swirling in liquid nitrogen or placement in a -80° freezer until the buffer and suspended lipid are frozen. Vortex mixing during the thawing of frozen samples allows the lipids to be removed from the walls of the test tubes. This preliminary freeze-thaw and vortex procedure is repeated several times if required. This method is very effective for hydrating lipid films that adhere strongly to the walls of the glass test tubes. Lipid suspensions are extruded through two stacked 0.2- μ m pore-size polycarbonate filters using the extruder (Lipex Biomembranes, Vancouver, BC). The size of extruded samples is determined by quasi-elastic light scattering with a Nicomp C270 submicrometer particle sizer operating in particle size mode. LUVs are stored at 4° and used within 2 weeks. Standard freeze-fracture

¹⁴ L. D. Mayer, M. J. Hope, and P. R. Cullis, *Biochim. Biophys. Acta* **858**, 161 (1986).

transmission electron microscopy methods are also used to characterize the liposome systems.

Preparation of Tunable pH-Sensitive Liposomes

Tunable pH-sensitive liposomes are prepared from binary mixtures of cationic and anionic lipids. The majority of our studies have used the mixture of cholesteryl hemisuccinate (CHEMS) and DODAC as the anionic and cationic amphipaths, respectively, although it is possible to prepare vesicles with similar properties from CHEMS and the commercially available DOTAP. Binary lipid mixtures contain only one species of anionic and cationic lipid with the exception of fluorescently labeled vesicles used in lipid mixing experiments. To prepare labeled LUVs, NBD-PE and LUVs, NBD-PE, and Rh-PE are included at 1 mol% each. Dried lipid films composed of the various formulations of DODAC/CHEMS are, hydrated with an aqueous buffer containing 150 mM NaCl, 10 mM HEPES, pH 8.1 and processed into LUVs using the freeze-thaw and extrusion methods described earlier.

Preparation of "Target" Liposomes

LUVs are prepared from mixtures of zwitterionic, neutral, and anionic lipids for use as target membranes in lipid mixing fusion assays for tunable pH-sensitive LUVs. Target LUVs are used to determine the extent of lipid mixing of DODAC/CHEMS vesicles with stable membranes that share no compositional similarity. Target membranes are composed of POPC, DOPS/POPC (30/70 mol%), and DOPS/cholesterol/POPC (30/30/40 mol%). LUVs are prepared as described earlier by freeze-thaw and extrusion in an aqueous buffer containing 150 mM NaCl, 10 mM HEPES, pH 7.8. For the preparation of labeled vesicles for lipid mixing assays, fluorescent lipids are included at 1 mol% each; the combinations are either NBD-PE/Rh-PE or NBD-PS/Rh-PE. Caution should be observed when using the fluorescent lipids NBD-PE, NBD-PS, and Rh-PE in target vesicles. These fluorescent lipids have negatively charged head groups and thus impart a negative charge to vesicles harboring them.

Preparation of Cationic and Conventional pH-Sensitive Liposomes

For comparison with tunable pH-sensitive liposomes, cationic liposomes composed of DODAC/cholesterol, DODAC/DOPE, and conventional pH-sensitive liposomes composed of DOPE/CHEMS are prepared. DODAC/DOPE and DODAC/cholesterol lipid films are hydrated in 10 mM Na-HEPES, pH 7.5, whereas DODAC/CHEMS and DOPE/CHEMS systems

are prepared with in 150 mM NaCl, 10 mM HEPES, pH 8.1. These systems are processed into LUVs by freeze-thaw and extrusion. Cationic formulations of DODAC/DOPE and DODAC/cholesterol are both made at a molar ratio of 85:15. DOPE/CHEMS is formulated at 70/30 mol% to produce pH-sensitive liposomes, which destabilize at \sim pH 5.⁶

Lipid Mixing Fusion Assays

A lipid mixing assay based on fluorescence resonance energy transfer (FRET) is used to assess lipid mixing properties of tunable pH-sensitive liposomes at various pH values.^{15–17} To assay “self-fusion,” lipid-mixing experiments are preformed with labeled and unlabeled DODAC/CHEMS vesicles of identical composition, with the exception that labeled vesicles contain 1 mol% NBD-PE and Rh-PE. The lipid mixing assay is used to measure the differential acid sensitivity of each of the DODAC/CHEMS formulations. To examine the interaction of DODAC/CHEMS vesicles with target membranes of different compositions, labeled DODAC/CHEMS vesicles are mixed with unlabeled target vesicles or vice versa.

Acid-Induced Lipid Mixing of DODAC/CHEMS Vesicles

NBD-PE/Rh-PE-labeled and unlabeled DODAC/CHEMS vesicles are mixed at a 1:5 ratio (labeled to unlabeled) and are injected into 2 ml of aqueous buffer (150 μ M final lipid concentration) containing 140 mM NaCl, 10 mM HEPES, 10 mM MES, and 10 mM acetate set to desired pH values. The injected lipid is less than 2% of the total buffer volume. To prepare easily many buffers with different pH values, two solutions are made, a basic (pH \sim 8.0) buffer and an acidic (pH \sim 3.8) buffer. They are mixed appropriately to prepare buffers with desired pH values in a reproducible matter. NBD-PE fluorescence is monitored at 25° in a stirred cuvette on a Perkin-Elmer LS-50B fluorometer using excitation and emission wavelengths of 467 and 540 nm, respectively, with an emission filter of 530 nm. Lipid mixing is monitored for \sim 400 s. Complete dilution of the fluorescent probes is estimated by the addition of highly purified Triton X-100 (10%, v/v) to a final concentration of 0.1%. The pH of the buffer does not change with the addition of lipid or Triton X-100. The extent of lipid mixing is calculated using the equation: lipid mixing (%) = $(F_t - F_o)/(F_{max} - F_o) \times 100\%$, where F_t is the NBD-PE fluorescence during the time course, F_o is the initial NBD-PE fluorescence of the labeled sample,

¹⁵ D. K. Struck, D. Hoekstra, and R. E. Pagano, *Biochemistry* **20**, 4093 (1981).

¹⁶ D. Hoekstra and N. Düzgüneş, *Methods Enzymol.* **220**, 15 (1993).

¹⁷ N. Düzgüneş, *Methods Enzymol.* **372**, 260 (2003).

and F_{\max} is the NBD-PE fluorescence in the presence of 0.1% Triton X-100. Fluorescence of the NBD fluorophore is not sensitive to pH in the range used in our experiments.

Fusion with Target Membranes: Acid-Induced Lipid Mixing of DODAC/CHEMS Vesicles with Target Membranes

Unlabeled DODAC/CHEMS vesicles and NBD-PE/Rh-PE-labeled target vesicles are added to a final concentration of 75 μM each into 2 ml 140 mM NaCl, 10 mM HEPES, 10 mM MES, 10 mM acetate, with the pH set to desired values. Alternatively, NBD-PE/Rh-PE-labeled DODAC/CHEMS vesicles and unlabeled target vesicles (1:1 ratio, 150 μM total lipid) and mixed in 2 ml of 150 mM NaCl, 5 mM HEPES, pH 7.8, and the buffer is "acid shocked" with an aliquot of 1 M acetic acid. The extent of lipid mixing is assayed and calculated as described previously.

Inner Monolayer Membrane Fusion Assay

To assess whether lipid mixing of DODAC/CHEMS vesicles with target membranes encompasses a complete fusion reaction or only lipid mixing with the outer monolayer (hemifusion), a modification of the lipid mixing assay, which should only be sensitive to the dilution of the lipids of the inner monolayer, is used. The assay employs dithionite to selectively quench the fluorescence of the NBD-lipid in the outer monolayer of LUVs.¹⁸ Changes in NBD-lipid fluorescence should only be due to the dilution of probe in the inner monolayer, provided that the NBD-lipid asymmetry is maintained. For this purpose, NBD-PS, a lipid conjugate that does not undergo membrane flip-flop, is utilized.¹⁹ A method similar to this has been used to show inner monolayer lipid mixing in reconstituted liposomes harboring recombinant exocytotic membrane fusion proteins.²⁰

Outer monolayer NBD-PS fluorescence is eliminated by chemical reduction using dithionite. Asymmetrically labeled LUVs are prepared by freeze-thaw and extrusion of a hydrated dispersion of DOPS/POPC/NBD-PS/Rh-PE (30/70/1/1 molar ratios) at a 20 mM lipid concentration in 150 mM NaCl, 10 mM HEPES pH 7.8. Sodium dithionite is dissolved freshly to a concentration of 1 M dithionite in 1 M Tris and added to the LUV suspension within 1 min of preparation. The dithionite solution is added to the 20 mM LUV suspension at a 1:10 volume ratio, resulting in

¹⁸ J. C. McIntyre, D. Watson, and R. G. Sleight, *Chem. Phys. Lipids* **66**, 171 (1993).

¹⁹ B. R. Lentz, W. Talbot, J. Lee, and L. X. Zheng, *Biochemistry* **36**, 2076 (1997).

²⁰ T. Weber, B. V. Zemelman, J. A. McNew, B. Westermann, M. Gmachl, F. Parlati, T. H. Sollner, and J. E. Rothman, *Cell* **92**, 759 (1998).

a dithionite to NBD-PS ratio of $\sim 500:1$. Upon addition of dithionite to the fluorescent lipid suspension, the color of the suspension changes instantly from a bright pink to a dull cherry color. The mixture is mixed by vortex mixing and is allowed to stand in the dark at room temperature for 10 min. Elution of the dithionite/liposome reaction mixture through a Sephadex CL-4B column (1×20 cm) with 150 mM NaCl, 5 mM HEPES, pH 7.8, exchanges the external buffer and removes the dithionite from the vesicle suspension. Vesicles elute in the void volume of the column, and the lipid concentration is estimated from the final dilution.

Quantitation of NBD of the outer monolayer NBD-PS reduction by dithionite is assessed using Rh-PE as an internal standard. The ratio of Rh-PE fluorescence emission (excitation 560 nm and emission 590 nm) to NBD-PS fluorescence emission (excitation 467 nm and emission 540 nm), before and after dithionite reduction, in the presence of 0.1% Triton X-100 is measured. For one reduction experiment the relative fluorescence ratio of Rh-PE/NBD-PS was 1.59 for untreated LUVs and 3.08 for dithionite-reduced LUVs. The total NBD-PS reduced in this LUV sample was $\sim 51\%$. NBD-PS reduction by dithionite typically yielded between 50 and 51% of the original NBD-PS fluorescence. This result correlates well with the nearly equal distribution of lipid in the inner and outer monolayer of LUVs and is in agreement with a previous report using this method to assay bilayer membrane asymmetry.²¹

Inner monolayer-labeled target LUVs are studied with the lipid-mixing assay using mixtures of unlabeled DODAC/CHEMS, DOPE/CHEMS, DODAC/DOPE, and DODAC/cholesterol LUVs vesicles in a 1:1 lipid ratio with inner monolayer-labeled NBD-PS/symmetrically labeled Rh-PE target vesicles. DODAC/CHEMS vesicles and inner monolayer-labeled DOPS/POPC/Rh-PE/NBD-PS vesicles are diluted in 150 mM NaCl, 5 mM HEPES pH 7.5, to a final lipid concentration of 150 μM and the buffer is acidified by the addition of an aliquot of 1 M acetic acid. In the case of non-pH-sensitive cationic liposomes, lipid mixing between DODAC/DOPE or DODAC/cholesterol is initiated by injection of the cationic liposomes into a stirred solution of inner monolayer-labeled target vesicles (1:1 lipid ratio) to a final lipid concentration 150 μM . NBD-PS fluorescence is monitored at excitation and emission wavelengths of 467 and 540 nm, respectively. Inner monolayer lipid mixing is calculated as described earlier using F_{max} values obtained from the addition of Triton X-100, which is 0.5 the fraction of the total NBD fluorescence of the symmetrically labeled target vesicles. Lipid mixing (%) in inner monolayer-labeled

²¹ C. Balch, R. Morris, E. Brooks, and R. G. Sleight, *Chem. Phys. Lipids* **70**, 205 (1994).

vesicles represents the relative extent of inner monolayer NBD-PS dilution. Lipid mixing (%) is calculated as described previously.

Formation of Liposomes from Mixtures of Cationic and Anionic Lipids

Mixtures of DODAC and CHEMS could be formulated into LUVs in mildly alkaline buffer at cationic-to-anionic lipid molar ratios less than one. The structure of DODAC/CHEMS dispersions was examined by freeze-fracture electron microscopy. Figure 2A shows that MLVs are formed upon hydration of DODAC/CHEMS (0.72 molar ratio), which could be extruded through 0.2- μm pore-size filters to give rise to uniformly sized LUV structures (Fig. 2B).

Table I gives the vesicle sizes of various DODAC/CHEMS formulations following extrusion through 0.2- μm pore-size filters. Lipid films composed of an equimolar mixture of DODAC and CHEMS cannot be extruded, yielding precipitates upon hydration. Vesicles composed of DODAC/CHEMS at a molar ratio of 0.85 have a larger mean diameter and larger size distribution than vesicles composed of lower DODAC/CHEMS molar ratios. The vesicle size is stable for at least 1 month when stored at 4°.

Tunable pH-Sensitive Fusion of DODAC/CHEMS Vesicles

The pH-dependent fusion properties of DODAC/CHEMS vesicles were examined using the lipid-mixing assay. The lipid-mixing properties of DODAC/CHEMS vesicles with molar ratios from 0 to 0.85 were measured as a function of pH. As shown in Fig. 3A, the lipid-mixing properties of DODAC/CHEMS vesicles prepared at a molar ratio of 0.11 show an onset of lipid mixing at pH 4.7 with maximum lipid-mixing occurring at approximately pH 3.8. In contrast, the lipid-mixing properties of vesicles with a DODAC/CHEMS molar ratio of 0.85 show an onset of lipid mixing at pH 6.8 with a maximum occurring at approximately pH 6.4 (Fig. 3B). Figure 3C summarizes lipid mixing data for each formulation; in Fig. 3D the data are normalized to the maximum lipid mixing. From normalized lipid-mixing data from Fig. 3D it is possible to calculate the pH at which half-maximum membrane fusion (pH_f) was observed and to compare this to the predicted pH of charged neutralization (pH_n) determined from Eq. (1). As shown in Fig. 4, the predicted pH_n values correlate well with the experimentally obtained values for pH_f when using value for the apparent pK_a of CHEMS of 5.8.⁷ This supports the hypothesis that the fusion of DODAC/CHEMS vesicles proceeds upon surface charge neutralization and that the pH of fusion can be tuned by adjusting the molar ratio of the cationic and anionic lipids in the vesicles.

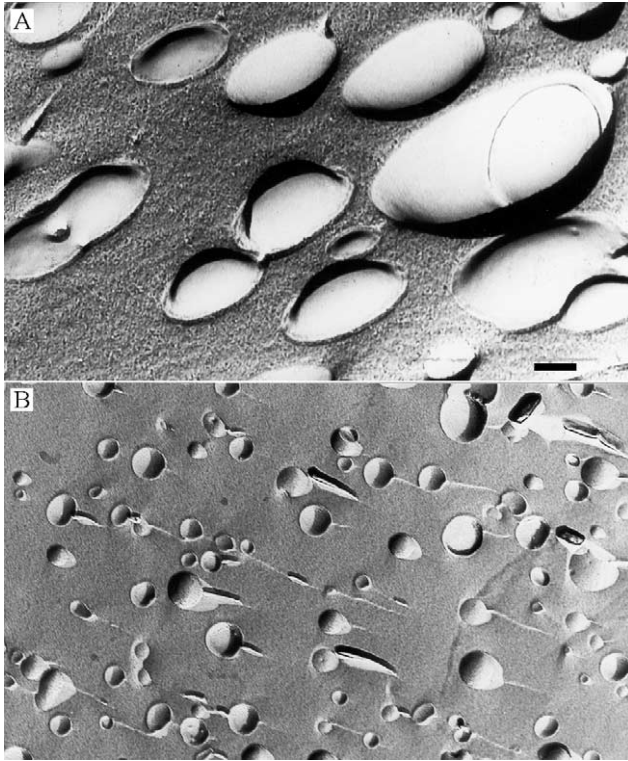


FIG. 2. Structural characteristics of aqueous dispersions of DODAC/CHEMS (0.72 molar ratio). The lipid was hydrated in 50 mM HEPES, 150 mM NaCl (pH 8.1), and freeze-fracture electron micrographs were prepared as in Hafez *et al.*⁷ (A) DODAC/CHEMS MLVs formed on hydration of the lipid film. (B) DODAC/CHEMS LUVs formed after extrusion through two stacked filters with 0.2 μm pore size. Scale bar: 200 nm. Figure reproduced from Hafez *et al.*⁷ with permission.

Fusion of DODAC/CHEMS Liposomes with “Target” Vesicles

Preparation of vesicles that only exhibit pH-sensitive lipid mixing or fusion with vesicles of identical composition are not particularly useful in terms of designing an efficient delivery vehicle that must destabilize or fuse with biological membranes in order to deliver its cargo to the cell interior. We hypothesized that the presence of the cationic lipid present in mixed DODAC/CHEMS liposomes would enhance the affinity of these vesicles for “target” membranes that have a negative surface charge. The cationic nature of the liposomes would only become apparent once a sufficient amount of anionic lipid was neutralized, thus exposing the positive charge

TABLE I
SIZE OF VARIOUS FORMULATIONS OF DODAC/CHEMS VESICLES
AFTER EXTRUSION

DODAC/CHEMS (molar ratio)	Size following extrusion (nm)
0.11	142 ± 28 ^a
0.43	144 ± 30
0.52	152 ± 31
0.61	146 ± 32
0.72	153 ± 35
0.85	274 ± 94
1.0	N/A ^b

^aThe size of DODAC/CHEMS LUV formulations following hydration in 10 mM HEPES, 150 mM NaCl, pH 8.1, and extrusion through two-stacked filters with 0.2 μm pore size. The mean diameter of the LUV systems was determined with a NICOMP C270 submicrometer particle sizer operating in the particle mode.

^bHydration of lipid films of DODAC/CHEMS mixtures at a molar ratio of 1.0 produced precipitates that could not be extruded.

of the cationic lipid in the membrane. Lipid-mixing assays were therefore performed to evaluate the pH-dependent fusion of DODAC/CHEMS vesicles with pH-stable target vesicles.

Figure 5 illustrates that upon acidification to pH 4.4, fluorescently labeled DODAC/CHEMS/NBD-PE/Rh-PE (DODAC/CHEMS molar ratio of 0.61) liposomes undergo substantial lipid mixing with target vesicles composed mainly of POPC, only if the target vesicle contain the anionic lipid phosphatidylserine (PS). Enhanced lipid mixing is observed with target membranes containing both PS and cholesterol. This result is not unexpected, as cholesterol is known to be able to induce negative membrane curvature and to promote the formation of inverted lipid phases and thus fusion in some lipid mixtures.²² As a validation of the assay, no fluorescence changes are observed in the absence of target vesicles, indicating that the observed changes in fluorescence are due to dilution of the NBD-PE/Rh-PE probes from the DODAC/CHEMS liposomes into the target vesicle membranes. The pH change to 4.4 for this experiment is lower than that required for the self-fusion of DODAC/CHEMS vesicles (0.61 molar ratio; pH_f 6.11) and was purposely chosen so that a large proportion of the anionic lipid should be neutralized, thus exposing the positive charge of the cationic lipid DODAC. The following set of experiments characterized the

²² P. R. Cullis and B. de Kruijff, *Biochim. Biophys. Acta* **507**, 207 (1978).

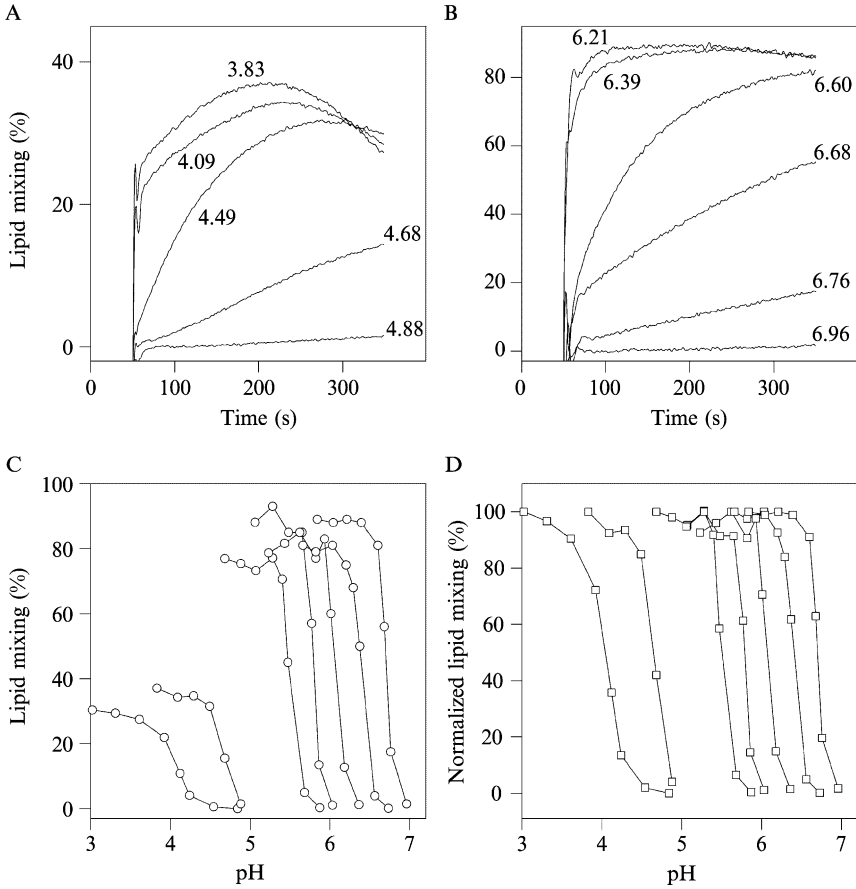


FIG. 3. pH-dependent fusion properties of DODAC/CHEMS LUVs containing increasing amounts of the cationic lipid DODAC determined by lipid mixing. Labeled and unlabeled (5:1 lipid ratio) DODAC/CHEMS LUVs were mixed and introduced into buffer with pH values indicated at 50 s. Lipid mixing traces are presented for DODAC/CHEMS molar ratios of (A) 0.11 and (B) 0.85. Summarized lipid-mixing data presented for DODAC/CHEMS molar ratios of 0, 0.11, 0.43, 0.52, 0.61, 0.72, and 0.85 (from left to right) in (C) actual lipid mixing (%) and (D) normalized lipid mixing (%). Lipid mixing (%) was determined as described in the text. Figure reproduced from Hafez *et al.*⁷ with permission.

lipid-mixing properties of DODAC/CHEMS vesicles with anionic target vesicles as a function of pH and found that the pH required for fusion with a target membrane is lower than that required for self-fusion of DODAC/CHEMS liposomes.

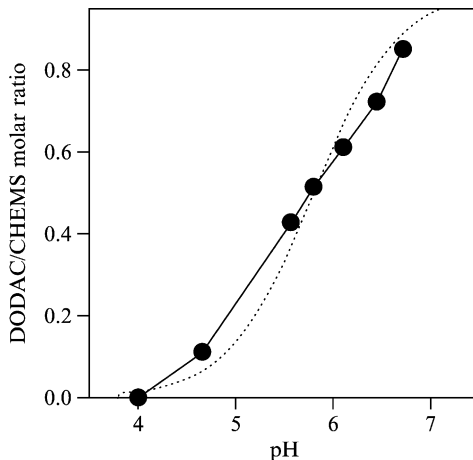


FIG. 4. Correlation between membrane fusion and surface charge neutralization of DODAC/CHEMS LUVs. The pH of half-maximum fusion (pH_f) values plotted as a function of the DODAC/CHEMS molar ratio (solid circles). The pH at which the surface charge was predicted to be zero (dashed line) was determined from Eq. (1) using $\text{p}K_{\text{CHEMS}} = 5.8$. pH_f was 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 value. Figure reproduced from Hafez *et al.*⁷ with permission.

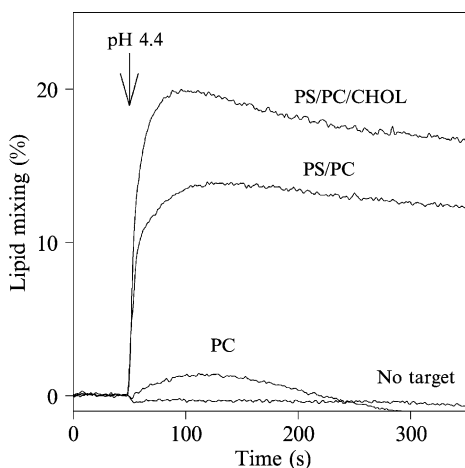


FIG. 5. Fusion properties of DODAC/CHEMS LUVs with target LUVs assayed by lipid mixing. NBD-PE/Rh-PE-labeled DODAC/CHEMS LUVs (0.61 molar ratio) were mixed with unlabeled POPC, DOPS/POPC (30/70 mol%), or DOPS/POPC/cholesterol vesicles (30/30/40 mol%) at a lipid ratio of 1:1 labeled to unlabeled vesicles as indicated on the graph. The pH was adjusted from 7.4 to 4.4 with an aliquot of 1 M acetic acid. Acidification of labeled DODAC/CHEMS LUVs in the absence of unlabeled target was also performed (no target).

Tunable pH-Sensitive Fusion of DODAC/CHEMS Liposomes with Anionic Target Membranes

The pH-dependence of lipid mixing of DODAC/CHEMS liposomes with anionic target vesicles composed of labeled PS/PC (30/70 mol%) was examined. DODAC/CHEMS (0.43 molar ratio) undergo lipid mixing with PS/PC target vesicles at \sim pH 5 and below (Fig. 6A), whereas DODAC/CHEMS (0.72 molar ratio) does so below pH 6.2 (Fig. 6B). A summary of the extent of lipid mixing with anionic target vesicles as a function of pH for each of the DODAC/CHEMS formulations tested is shown in Fig. 6C. DODAC/CHEMS liposomes undergo lipid mixing with anionic target vesicles, and the pH threshold for this event depends on the ratio of cationic to anionic lipid in the membrane. The level of lipid mixing for DODAC/CHEMS vesicles of molar ratio 0.11 is low and is due to the small amount of cationic lipid in this formulation. This contention is supported by the result that purely anionic CHEMS vesicles do not undergo lipid mixing with anionic target vesicles. Above a DODAC/CHEMS molar ratio of 0.43 the extent of lipid mixing appears to decrease with an increasing DODAC/CHEMS molar ratio (Fig. 6C). This effect is likely an artifact of the slow mixing of the two populations of DODAC/CHEMS and target vesicles. We have found that in the absence of anionic target vesicles, DODAC/CHEMS vesicles fuse and undergo a phase transition to the inverted H_{II} phase,⁷ thus losing the ability to undergo a lipid mixing reaction with anionic PS/PC vesicles. DODAC/CHEMS vesicles likely fuse with membranes of like composition before adequate sample mixing occurs between the DODAC/CHEMS vesicles and the anionic target vesicle population. Normalization of lipid-mixing values given in Fig. 6C allows for the determination of half-maximum lipid-mixing values, pH_{50} , of the DODAC/CHEMS liposomes with anionic target membranes (Fig. 6D).

A summary of the half-maximum values from lipid-mixing assays for self-fusion (pH_f) and fusion with anionic target membranes (pH_{50}) is given in Table II. Lipid mixing of DODAC/CHEMS with anionic target membranes occurs with pH_{50} values between 0.4 and 0.53 pH units lower than that required for self-fusion (pH_f). Fusion of all DODAC/CHEMS vesicle formulations with anionic target liposomes occurs in a pH range where DODAC/CHEMS vesicles are predicted to carry a net positive charge. During the time course of lipid-mixing assays between DODAC/CHEMS and anionic target vesicles, an increase in solution turbidity signaling fusion of DODAC/CHEMS vesicles occurs at values near pH_f without lipid mixing of DODAC/CHEMS vesicles with anionic target vesicles. These

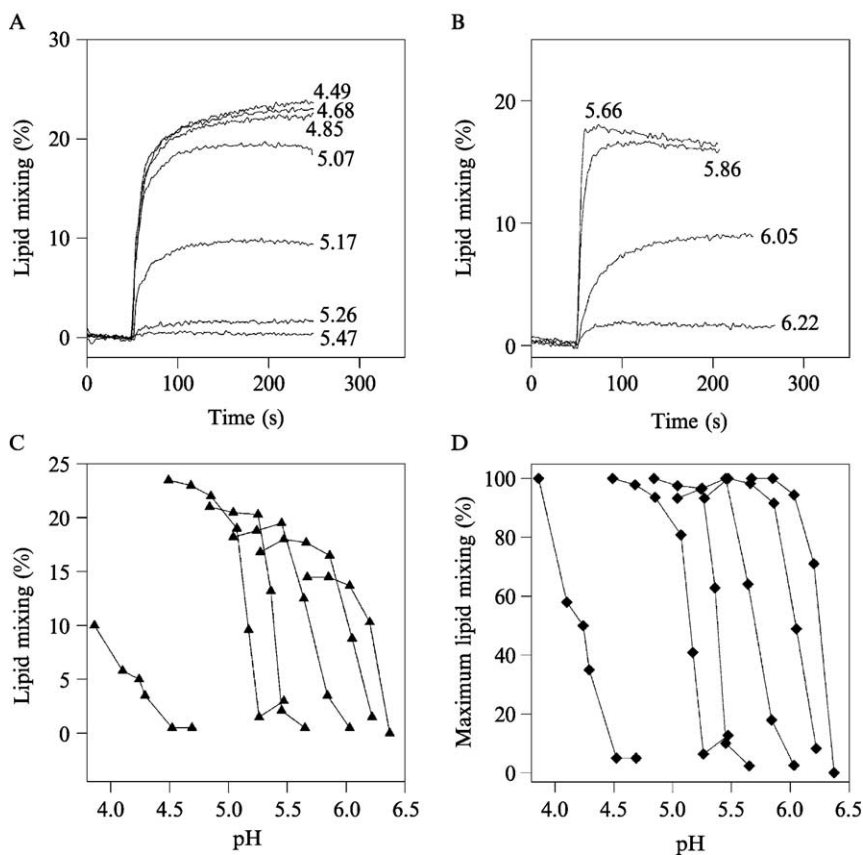


FIG. 6. Fusion properties of DODAC/CHEMS LUVs containing increasing amounts of DODAC with target DOPS/POPC (30/70 mol%) LUVs. Unlabeled DODAC/CHEMS LUVs were mixed with labeled target DOPS/POPC LUVs and introduced into buffer with the pH values indicated at 50 s. Lipid-mixing traces are presented for DODAC/CHEMS molar ratios of (A) 0.43 and (B) 0.72. Summarized lipid-mixing data presented for DODAC/CHEMS molar ratios of 0.11, 0.43, 0.52, 0.61, 0.72, and 0.85 (from left to right) with DOPS/POPC LUVs in (C) actual lipid mixing (%) and (D) normalized lipid mixing (%). Lipid mixing (%) was determined as described in the text.

results suggest strongly that fusion of DODAC/CHEMS vesicles with anionic target vesicles requires the exposure of a cationic lipid, which is masked by the anionic lipid until a sufficiently low pH is reached, allowing the presentation of the positive character of the DODAC/CHEMS vesicles.

TABLE II
COMPARISON OF PREDICTED AND EXPERIMENTAL FUSOGENIC PROPERTIES OF
DODAC/CHEMS LUVs

DODAC/CHEMS (molar ratio)	Predicted pH of neutralization (pH _n)	Experimental 50% maximum "self" lipid mixing (pH _f)	Experimental 50% maximum lipid mixing with anionic target ^a
0	3.80	4.00	No lipid mixing
0.11	4.90	4.65	4.22
0.43	5.68	5.57	5.10
0.52	5.83	5.80	5.41
0.61	6.00	6.11	5.71
0.72	6.22	6.45	6.00
0.85	6.56	6.72	6.19

^a Lipid mixing with an anionic target refers to fusion with anionic LUVs composed of DOPS/POPC (30/70 mol%).

Complete Fusion of DODAC/CHEMS Liposomes with Anionic Target Membranes

It was important to assess whether DODAC/CHEMS vesicles undergo hemifusion (outer monolayer lipid mixing only) with anionic target vesicles or a complete fusion event involving both inner and outer monolayers of target liposomes. To address this issue, a lipid-mixing assay that is sensitive only to probe dilution of the inner monolayer of target vesicles was utilized. The measurement of inner monolayer lipid mixing relies on using target vesicles that are labeled fluorescently with NBD-PS only on the inner monolayer. Inner monolayer lipid-mixing (%) values will reach those of symmetrically labeled vesicles if equal lipid dilution of the inner and outer monolayers occurs. DOPS/POPC/NBD-PS/Rh-PE vesicles are treated with dithionite to eliminate outer monolayer NBD-PS. The fluorescent lipid analogue NBD-PS is not prone to transmembrane redistribution (unlike NBD-PE) and can, therefore, be used to prepare stable asymmetrically labeled vesicles.¹⁹ Changes in the fluorescence dequenching of NBD-PS in anionic vesicles should be due to lipid dilution of the inner monolayer of the target vesicles.

Figure 7 shows that DODAC/CHEMS liposomes are able to cause probe dilution in the inner membrane of target vesicles, but do so to various degrees depending on the DODAC/CHEMS vesicle composition. Vesicles with low cationic lipid content, e.g., DODAC/CHEMS (0.11, 0.42, and 0.52 molar ratios) induce inner monolayer lipid mixing that is not comparable to the total lipid mixing in symmetrically labeled targets, whereas

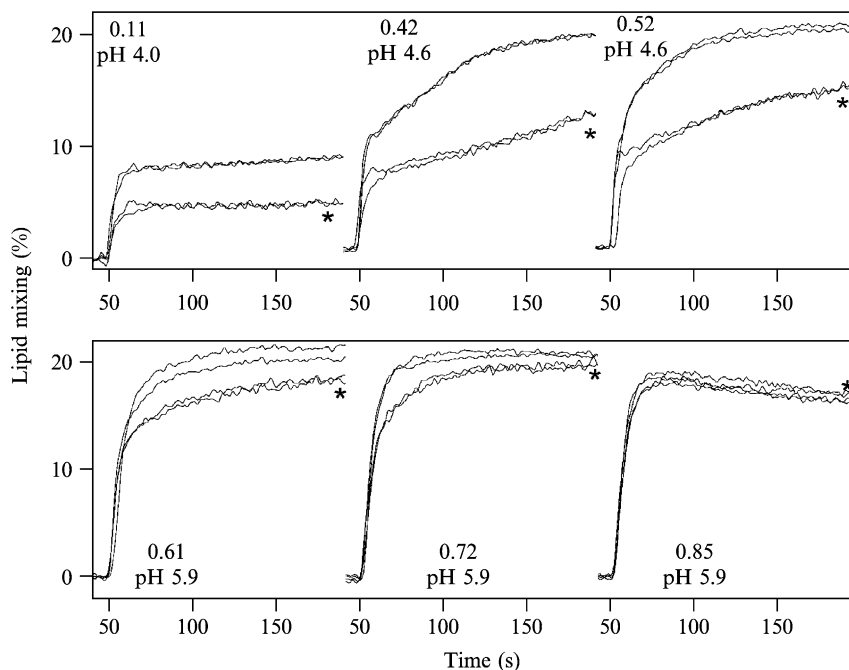


FIG. 7. Inner monolayer lipid mixing fusion assay. Lipid mixing of unlabeled DODAC/CHEMS vesicles was measured with symmetrically and inner monolayer-labeled NBD-PS/symmetrically labeled Rh-PE anionic DOPS/POPC (30/70 mol%) LUVs. The DODAC/CHEMS vesicle formulation is noted on each lipid-mixing trial as the DODAC/CHEMS molar ratio as well as the final pH after acidification. Inner monolayer lipid mixing traces are denoted by an asterisk. Two experimental trials are shown for each DODAC/CHEMS formulation for both inner monolayer labeled and symmetrically labeled target LUVs. Media were acidified at 50 s using an aliquot of 1 M acetic acid to the final pH value as indicated. Lipid mixing (%) was determined as described in the text.

DODAC/CHEMS vesicles with a high cationic lipid content (0.61, 0.72, and 0.85 molar ratios) produce nearly equivalent lipid mixing in both inner monolayer and symmetrically labeled PS/PC target vesicles (Fig. 7). This result indicates that vesicles composed of DODAC/CHEMS with high amounts of cationic lipid undergo a more complete membrane fusion reaction with anionic target vesicles than formulations with less cationic lipid.

The inner monolayer lipid mixing assay employed was able to detect variances among the fusogenicity of DODAC/CHEMS formulations with target membranes that were not apparent from traditional lipid-mixing assays utilizing the symmetric distribution of fluorescent probes in labeled

vesicles. We next turned our efforts toward comparing the fusogenic behavior of tunable pH-sensitive liposomes, which have intrinsic pH-sensitive and cationic characteristics, with commonly employed pH-sensitive liposomes and cationic liposomes.

Comparison of Tunable pH-Sensitive Liposomes with Traditional pH-Sensitive and Cationic Liposomes

We compared the properties of fusion between anionic target vesicles and tunable pH-sensitive DODAC/CHEMS liposomes, cationic liposomes composed of DODAC/cholesterol, DODAC/DOPE, and pH-sensitive liposomes composed of DOPE/CHEMS. The conventional and inner monolayer lipid-mixing assay was employed for this purpose. Data in Fig. 8 show that pH-sensitive DODAC/CHEMS and non-pH-sensitive DODAC/cholesterol and DODAC/DOPE undergo fusion with anionic target vesicles, with dilution of the inner monolayer lipids of target vesicles, indicating complete fusion (Fig. 8A–C). The amount of the cationic lipid present was the same for each of the formulations containing DODAC. As observed previously, DODAC/CHEMS LUVs undergo lipid mixing with anionic target vesicles only after an acidic stimulus is given (Fig. 8A). However, DODAC/cholesterol and DODAC/DOPE vesicles undergo lipid mixing immediately upon addition of the cationic vesicles to the PS/PC vesicle suspension, and this fusion reaction cannot be controlled (Fig. 8B and C). In sharp contrast, lipid mixing does not occur between pH-sensitive DOPE/CHEMS liposomes and anionic target vesicles even upon sample acidification (Fig. 8D). Clearly, DODAC/CHEMS vesicles have the ability to undergo lipid mixing with anionic target vesicles in a pH-sensitive manner, thus encompassing the best of the properties of both cationic liposomes and conventional pH-sensitive liposomes. It can be concluded that tunable pH-sensitive liposomes composed of mixtures of cationic and anionic lipid have properties that are highly desirable in a liposome delivery system. The vesicles exhibit pH-sensitive instability and exposure of a cationic surface charge that can promote fusion with anionic lipid membranes that approximate the composition of cellular membranes.

Conclusions

We have described the design and construction of a tunable pH-sensitive liposome system with the capacity to fuse with stable target anionic membranes. Tunable pH-sensitive liposomes represent a defined, non-phospholipid, synthetic membrane envelope that is able to respond to changes in surrounding pH. Subtle changes to the composition of the

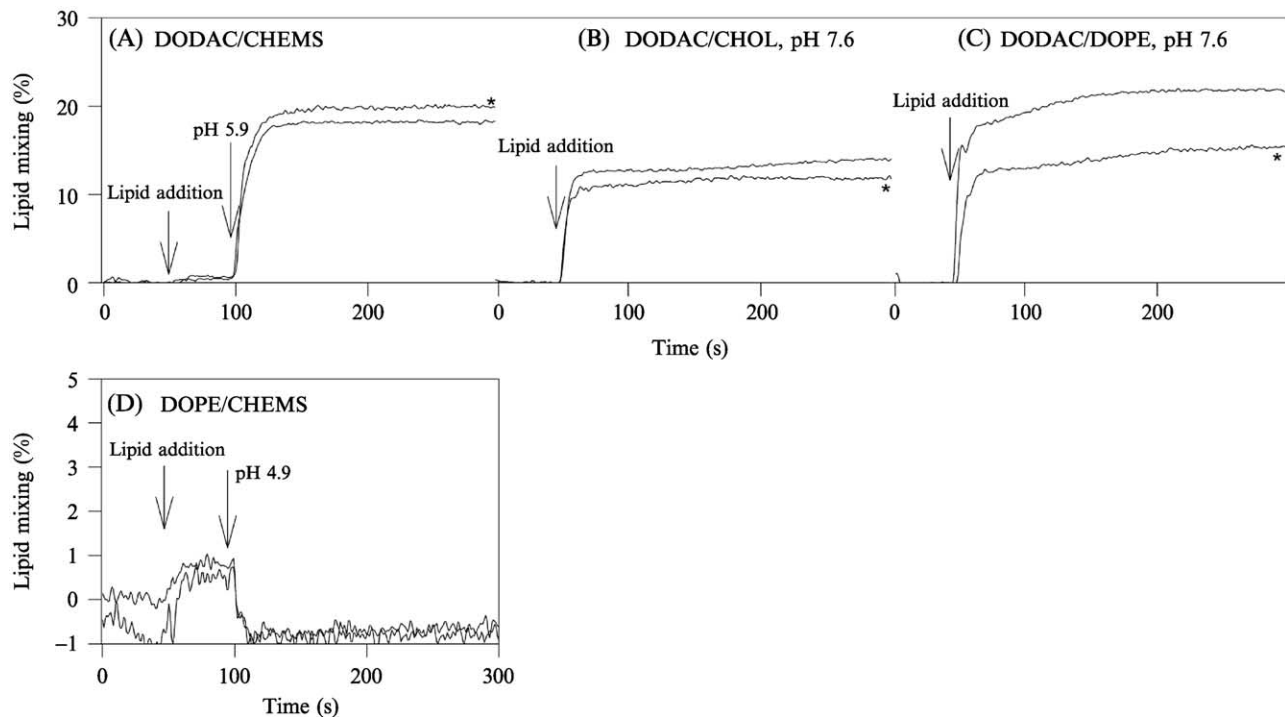


FIG. 8. Fusogenic properties of tunable pH-sensitive, cationic, and conventional pH-sensitive LUVs with anionic target LUVs. Symmetrically or inner monolayer-labeled NBD-PS (both with symmetrically labeled Rh-PE) anionic DOPS/POPC/NBD-PS/Rh-PE LUVs (30/70/1/1 molar ratio) were mixed with unlabeled (A) DODAC/CHEMS (0.85 molar ratio), (B) DODAC/cholesterol (0.85 molar ratio), (C) DODAC/DOPE (0.85 molar ratio), and (D) DOPE/CHEMS (70/30 mol%). Inner monolayer lipid-mixing traces are denoted by an asterisk. Unlabeled fusogenic LUVs were added to labeled target vesicles at 50 s. In the case of pH-sensitive DODAC/CHEMS and DOPE/CHEMS LUVs, the pH was acidified from pH 7.5 as indicated with an aliquot of 1 M acetic acid at 100 s. Lipid mixing (%) was determined as described in the text.

membrane allow for fine-tuning of the pH threshold of membrane instability. Tunable pH-sensitive liposomes are capable of being formulated into vesicles of defined size and are suitable for the entrapment of a variety of water-soluble molecules. Endosomal escape has been defined as a major barrier to successful drug and gene delivery.²³ Tunable pH-sensitive liposomes represent a promising new carrier system with the advantageous property of controllable pH-sensitive fusion with anionic membranes. These systems may be useful as a synthetic molecular device for overcoming the obstacles of endosomal escape and intracellular delivery. Based on a report by Shi *et al.*,²⁴ pH-sensitive liposomes composed of mixtures of cationic and anionic lipids show promise as efficient intracellular delivery systems.

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[8] Sterically Stabilized pH-Sensitive Liposomes

By VLADIMIR SLEPUSHKIN, SÉRGIO SIMÕES, MARIA C. PEDROSO DE LIMA, and NEJAT DÜZGÜNEŞ

Since their introduction in the 1960s,^{1,2} liposomes have been considered as possible vehicles for delivering molecules into cells that would otherwise not be internalized readily. Realization of this potential, however, has been hampered because either (i) the liposomes are removed rapidly from the circulation by the reticuloendothelial system or (ii) they do not deliver large or highly charged molecules into cells.³⁻⁵ The first of these handicaps has been overcome by the inclusion of poly(ethylene glycol)-derivatized phosphatidylethanolamine (PEG-PE) in the liposome membrane, resulting in much longer half-lives *in vivo*, compared to conventional liposomes.⁶⁻⁸

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