DNA-Induced Lateral Segregation of Cationic Amphiphiles in Lipid Bilayer Membranes as Detected via ^{2}H NMR

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ABSTRACT: ^{2}H NMR spectroscopy was used to investigate the response of specifically choline-deuterated 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) to changes in surface electrostatic charge in membranes consisting of mixtures of POPC plus various cationic amphiphiles plus polyadenylic acid (polyA). Three different cationic amphiphiles were investigated: cetyltrimethylammonium bromide (CTAB), dioleoyldimethylaminopropane (DODAP), and 3β[*N*-(*N*′,*N*′-dimethylaminoethane)carbamoyl]-cholesterol (DC-CHOL). Each of the cationic amphiphiles elicited a concentration-dependent decrease (increase) in the quadrupolar splitting from POPC-α-d_2 (POPC-β-d_2), as expected for the accumulation of cationic charges at the surface of a lipid bilayer. However, the strength of the response varied with the cationic amphiphile in the order CTAB > DODAP > DC-CHOL. When polyA was added to the cationic amphiphile-containing lipid bilayers, the ^{2}H NMR spectrum consisted of two overlapping Pake patterns, indicating the presence of two lipid domains with different effective surface charges and only slow exchange of lipids between the two domains. There was no evidence of any non-bilayer lipid arrangements. Analysis of the quadrupolar splittings of the two ^{2}H NMR spectral components demonstrated that the polyA-containing domain was enriched with respect to cationic amphiphiles while the polyA-free domain was depleted with respect to cationic amphiphiles. We conclude that polyA is able to laterally segregate cationic amphiphiles into long-lived lipid domains of distinct composition.

Lipofection, the use of cationic liposomes in the transfer of genes, has become a popular method of gene therapy (Felgner & Rhodes, 1991; Remy *et al.,* 1995; Debs, 1995). In this transfection technology the genetic material is "packaged" together with a cationic amphiphile in order to enhance its transport across the plasma membrane into the cell's interior (Fraley *et al.,* 1981). The success of this strategy is in part due to the compression of the DNA when neutralized by the cationic amphiphiles, which aids its transmembrane transfer. In addition, successful transfer requires an excess of cationic over anionic charges within the "package" since this encourages binding to the typically anionic surface of a biomembrane. Moreover, the presence of a modicum of fusogenic bilayer-destabilizing amphiphile, such as phosphatidylethanolamine, further enhances the transfection potential.

Many of the important details regarding the mechanism of transfection remain obscure. For instance, a number of different cationic amphiphiles have been developed and examined for their efficiencies as agents of transfection (Leventis & Silvius, 1990; Gao & Huang, 1991; Behr *et al.,* 1989; Felgner *et al.,* 1987). It is by no means obvious, however, why different cationic amphiphiles display different propensities as transfer agents, nor is it known whether, or to what degree, the DNA remains complexed with the cationic amphiphiles after arriving at the lipid bilayer membrane surface, nor is it apparent how, or in what form, the genetic material is transferred to the opposite face of the membrane, or how the DNA is released and becomes available to the cell's genetic machinery. It is certain that this lack of knowledge inhibits the process of designing improved transfection agents and transfection strategies.

The role played by electrostatic interactions in current transfection techniques is pivotal. Electrostatic attraction dictates that DNA and cationic amphiphiles will complex with one another. Electrostatic attraction draws the DNA−cationic amphiphile complex toward the membrane surface and decrees that binding will occur. Classically, to investigate surface electrostatic charge in such situations one might turn to electrophoretic mobility measurements of the zeta potential of either the DNA−cationic amphiphile complexes themselves, or of liposomes exposed to the complexes. However, such an approach suffers from the fact that one is measuring a global property of an entire particle. Consequently, interesting details of particle architecture and molecular distribution are averaged out. ^{2}H NMR of choline-deuterated phosphatidylcholine is an alternative method for monitoring membrane surface electrostatics (Akutsu & Seelig, 1981; Altenbach & Seelig, 1984; Seelig & Macdonald, 1987; Seelig *et al.,* 1987), and has been used to characterize the surface binding of charged ligands, to examine the lateral phase separation of charged lipids, and even to resolve differences between the two surfaces of a lipid bilayer membrane [for a recent review see Macdonald (1996)]. The ^{2}H NMR technique possesses a particular advantage over classical means of measuring surface electrostatic charge in that it is sensitive to the detailed topography of surface charge distribution.

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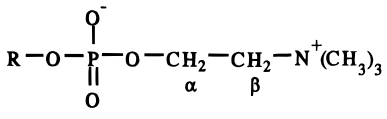
In this article we describe ^{2}H NMR studies of the membrane surface electrostatic response to the presence of three different cationic amphiphiles, all of which have been used as transfection agents. These include the single-chain cationic amphiphile CTAB (cetyltrimethylammonium bromide), the double-chain cationic lipid DODAP (dioleoyldimethylaminopropane) and the cholesterol-based cationic species DC-CHOL (3β[*N*-(*N*′,*N*′-dimethylaminoethane)carbamoyl] cholesterol). In addition, we report the effects of adding polyadenylic acid (polyA) to lipid membranes consisting of a mixture of each such cationic amphiphile plus phosphatidylcholine. PolyA is a single-stranded substitute for DNA in our model system. We demonstrate that polyA addition produces the appearance of a separate lipid domain, laterally segregated within the plane of the lipid bilayer membrane, and long-lived on the ^{2}H NMR time scale. The ^{2}H NMR spectra indicate that this DNA-containing domain retains an overall bilayer architecture and is composed of an equivalent number of anionic DNA and cationic amphiphile charges plus trapped phosphatidylcholine. These findings suggest that DNA and cationic amphiphiles remain complexed with one another even after a transfection "package" binds to and fuses with a lipid bilayer membrane.

MATERIALS AND METHODS

Materials. 1-Palmitoyl-2-oleoyl phosphatidic acid (POPA) was purchased from Avanti Polar Lipids (Alabaster, AL). Polyadenylic acid (polyA, MW 7 000 000) and oleoyl chloride were obtained from Sigma (St. Louis, MO). 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) and CTAB were purchased from BDH (Toronto, ON). 3-Dimethylamino-1,2-propanediol, 2,3,4- triisopropylbenzenesulfonyl chloride (TPS), tetraphenylboron (TPB), cholesteryl chloroformate and *N*,*N*-dimethylethylenediamine were purchased from Aldrich (Milwaukee, WI).

Synthesis of Choline-Deuterated Phosphatidylcholine. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was selectively deuterated at either the α or β positions of its choline head group using a strategy based on a combination of the methods of Harbisson and Griffin (1984) and Aloy and Rabout (1913), and described in detail elsewhere (Marassi & Macdonald, 1993). The structure of the choline head group of POPC and the nomenclature of the deuterolabeling positions are indicated below.



Synthesis of DODAP. 1,2-Dioleoyl-3-dimethylaminopropane (DODAP) was synthesized and purified as described by Leventis and Silvius (1990). Briefly, 3-dimethylamino-1,2-propanediol was acylated with oleoyl chloride to produce DODAP. The product was dissolved in 99:1 (v/v) hexane/acetic acid, applied to a silicic acid column, and eluted with 20% diethyl ether in hexane, 100% chloroform, and, finally, 5% methanol in chloroform to remove DODAP in the form of its acetic acid salt. The latter was converted to the hydrochloride by adding an equimolar amount of HCl to a methanolic solution of DODAP. The methanol was removed under a stream of argon gas and the product was dried under vacuum overnight. The purity of the lipid was monitored by thin-layer chromatography (TLC) and proton NMR. TLC yielded a single spot with $R_f = 0.70$ when chromatographed in chloroform:methanol (90/10, v/v). Proton NMR (200 MHz): δ 0.9 ppm, triplet, 6H, oleoyl methyls; δ 1.3 ppm, broad singlet, 40H, oleoyl methylenes; δ 1.65 ppm, multiplet, 4H, oleoyl β-methylenes; δ 2.05 ppm, multiplet, 8H, α-methylenes to double bond; δ 2.28 ppm, singlet, 6H, amino methyls; δ 2.35 ppm, triplet, 4H, oleoyl α-methylenes; δ 2.47 ppm, triplet, 2H, C1-propyl protons; δ 4.1 and 4.35 ppm, doublet of doublets, 2H,geminal coupling ($J = 12$ Hz) and vicinal coupling with C2 proton ($J = 3.6$ Hz and 6.1 Hz), C3-propyl protons (inequivalent H_a and H_b); δ 5.2 ppm, multiplet, 1H, C2-propyl proton; δ 5.35 ppm, triplet, 4H, oleoyl olefinic protons.

Synthesis of DC-CHOL. 3β[*N*-(*N*′,*N*′-dimethylaminoethane)carbamoyl] cholesterol (DC-CHOL) was synthesized and characterized according to the method of Gao and Huang (1991) by coupling *N*,*N*-dimethylethylenediamine to cholesteryl chloroformate. The final product was recrystallized twice from absolute ethanol at -20 °C and dried under vacuum, yielding a compound which chromatographed as a single spot on TLC. Proton NMR (200 MHz): δ 2.18 ppm, singlet, 6H, amino methyls; δ 2.36 ppm, triplet, 2H, C1-ethyl protons; δ 3.21ppm, multiplet, 2H, C2-ethyl protons; δ 5.18 ppm, broad triplet, 1H, amido proton; δ 5.35 ppm, broad triplet, 1H, olefinic cholesterol proton.

Preparation of MultiLamellar Vesicles (MLVs). Lipid mixtures of the desired composition were prepared by combining the appropriate volumes of chloroform stock solutions of either POPC-α-d_2 or POPC-β-d_2 with those of either DODAP, CTAB, or DC-CHOL. Typically, the lipid mixtures contained 10 mg of either POPC-α-d_2 or POPC-β-d_2. The solvent was removed under a stream of argon and the residue was dried overnight under vacuum. The dried lipids were then resuspended in approximately 150 μL of aqueous buffer (20 mM HEPES, pH 7.4). The lipids were hydrated by gentle warming and vortexing, and were then freeze−thawed five times to ensure homogeneous mixing.

Preparation of MLVs Containing PolyA. Dried lipid mixtures were prepared as described above but were hydrated using an aqueous stock solution containing the desired quantity of polyA in deuterium-depleted water plus sufficient deuterium-depleted water to bring the final volume to 150 μL. The lipids plus polyA were again gently warmed and vortexed and subjected to five freeze−thaw cycles to ensure total mixing of polyA in the MLV dispersions.

^{2}H NMR Spectroscopy. ^{2}H NMR spectra were recorded on a Chemagnetics CMX300 NMR spectrometer operating at 45.98 MHz, using a Chemagnetics wideline probe. The quadrupolar echo sequence (Davis *et al.*, 1976) was employed using quadrature detection with complete phase cycling of the pulse pairs and a 90° pulse length of 2.0 μs, an interpulse delay of 30 μs, a recycle delay of 100 ms, a spectral width of 100 kHz, and a 2K data size.

Pake Pattern Spectral Line Shape Simulations. ^{2}H NMR Pake pattern line shapes were simulated using a computer program, written in our laboratory, based on the tiling method introduced by Alderman *et al.* (1986). The simulation variables include the quadrupolar splitting and the line width parameter T_2.

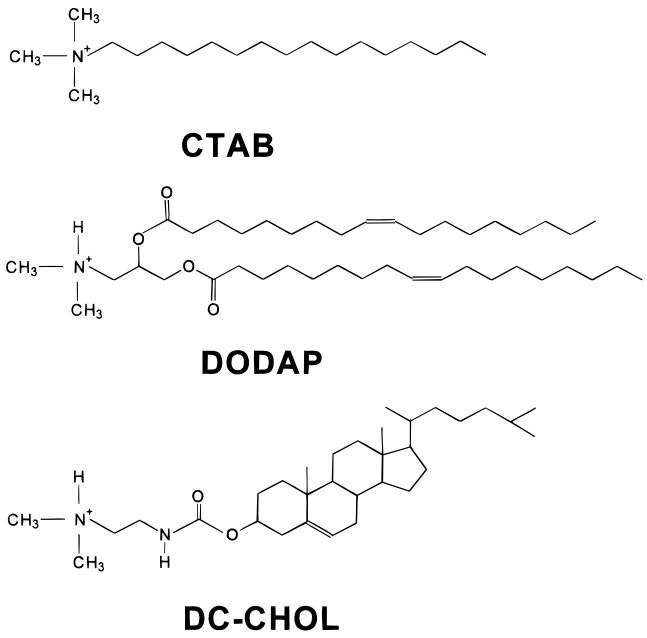


FIGURE 1: Structure of the three cationic amphiphiles employed here. From top to bottom, CTAB, DODAP, and DC-CHOL.

RESULTS AND DISCUSSION

^{2}H NMR Response to Different Cationic Amphiphiles. The structures of the three cationic amphiphiles examined in this study are illustrated in Figure 1. Each contains a cationic di- or trimethylamino group attached to a large hydrophobic moiety. In the case of CTAB, the hydrophobic group is a single C16-alkyl chain. In the case of DODAP the hydrophobic group is 1,2-dioleoylpropane, rather reminiscent of the 1,2-diacylglycerol moiety of naturally-occuring lipids. In the case of DC-CHOL the hydrophobic group is a cholesterol ring. In terms of their hydrophile-lipophile balance (HLB) the three cations rank in the order CTAB > DC-CHOL > DODAP. The HLB (Griffin, 1949) is an estimate of the polarity of complex amphiphiles and is obtained by adding together hydrophilic versus lipophilic contributions from individual chemical groups as discussed by Davies and Rideal (1963). Clearly, CTAB is the most polar of the three amphiphiles.

Each of these three cationic amphiphiles produces a cationic surface electrostatic charge when mixed with POPC that is detected using ^{2}H NMR of specifically choline-deuterated POPC. The fundamental spectroscopic observation is shown if Figure 2. The left-hand column of ^{2}H NMR spectra were obtained with POPC-α-d_2 and the right-hand column of ^{2}H NMR spectra were obtained with POPC-β-d_2. In the absence of cationic amphiphiles (top spectra) the ^{2}H NMR spectra consist of a Pake doublet line shape, characteristic of liquid-crystalline lipids arranged in a bilayer architecture. The quadrupolar splitting ($\Delta\nu$) corresponds to the separation, in Hz, between the two maxima or "horns" of the spectrum. The narrow resonance line appearing at the chemical shift position of deuterated water (0 Hz) arises

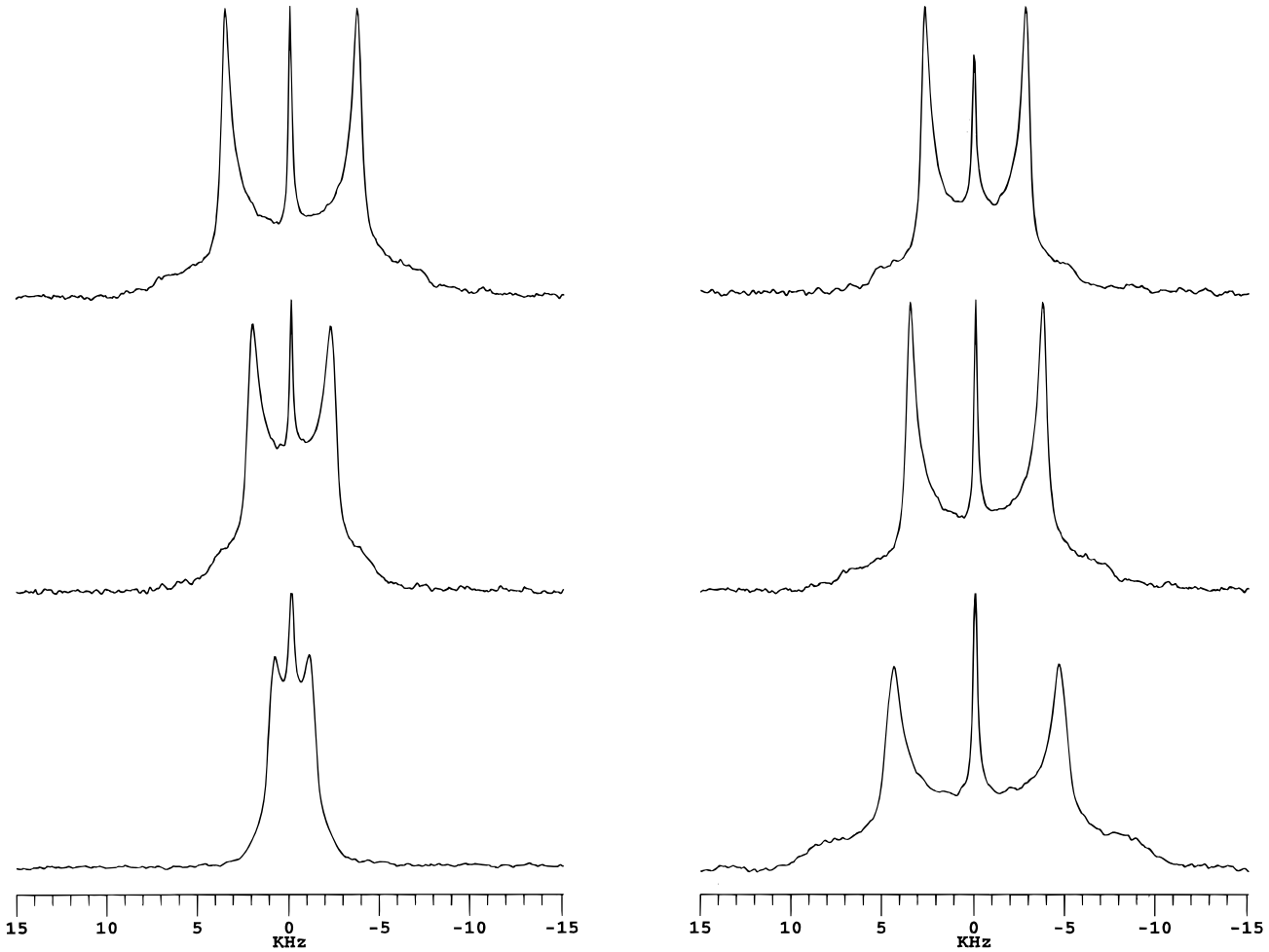


FIGURE 2: Effect of cationic surface charge on the ^{2}H NMR spectrum of POPC-α-d_2 (left) and POPC-β-d_2 (right) in the presence of (proceeding from the top) 0% CTAB, 7.5 mol % CTAB, and 15 mol % CTAB.

from the natural abundance deuterium present in the water in the MLV preparation.

For the case of 100% POPC membranes the quadrupolar splittings from POPC-α-d_2 and POPC-β-d_2 are rather similar, as expected for a neutral membrane surface. When increasing amounts of CTAB are mixed with the POPC (7.5 mol % in the middle and 15 mol % in the bottom spectra) the quadrupolar splitting from POPC-α-d_2 decreases, while that from POPC-β-d_2 increases. This counterdirectional change in the quadrupolar splittings from the two deuterolabeling positions is characteristic of the "molecular voltmeter" response of phosphatidylcholines to the presence of surface charge (Seelig *et al.*, 1987). This behavior is believed to originate with a concerted conformational change undergone by the phosphocholine group in response to surface charge interactions. The direction of the change observed here is diagnostic of the accumulation of positive surface charges. Since only a single quadrupolar splitting is observed in each instance, one may conclude that CTAB is homogeneously distributed amongst all the MLVs in the entire sample and within the plane of a given bilayer membrane. Consequently, all POPC molecules experience the same average surface charge environment. Qualitatively similar effects are recorded in the ^{2}H NMR spectra of POPC-α-d_2 and POPC-β-d_2 mixed with either of the three different cationic amphiphiles investigated here.

In order to relate a given quadrupolar splitting with a particular surface charge density it is necessary to calibrate the relationship between the two, as shown in part A of Figure 3. Here, quadrupolar splittings for both POPC-α-d_2 and POPC-β-d_2 are plotted as a function of the mole fraction of added cationic amphiphile for each of CTAB, DODAP, and DC-CHOL. In every case the relationship between the quadrupolar splitting and the mole fraction of cationic amphiphile is approximately linear. CTAB induces the greatest response on a mole-to-mole basis (slopes of −29.1 kHz/mol and +25.6 kHz/mol for POPC-α-d_2 and POPC-β-d_2, respectively). However, mole fractions of CTAB greater than approximately 15% lead to disruption of the lipid bilayer architecture through CTAB's surfactant action. DC-CHOL induces the least response on a mole-to-mole basis (slopes of −12.8 and +4.0 kHz/mol for POPC-α-d_2 and POPC-β-d_2, respectively). Moreover, levels of DC-CHOL greater than about 50 mol % produce broad, ill-defined ^{2}H NMR spectral line shapes, suggesting an inhomogeneous distribution of DC-CHOL within the plane of the lipid bilayer. DODAP, on the other hand, induces an intermediate response (slopes of −21.2 and +11.5 kHz/mol for POPC-α-d_2 and POPC-β-d_2, respectively). In addition, the ^{2}H NMR spectra indicate that DODAP remains homogeneously mixed with POPC, and retains an overall bilayer architecture, over a wide range of molar ratios.

Further evidence of the differences between the ^{2}H NMR response to these three cationic amphiphiles is obtained when one correlates the quadrupolar splittings from POPC-α-d_2 versus POPC-β-d_2 for a given level of added charge, as shown in part B of Figure 3. The linearity of such α−β correlations suggest that the phosphocholine group undergoes a concerted conformation change in response to the presence of surface charges. This conformational change probably involves a tilting of the P-N dipole relative to the membrane surface (Macdonald *et al.*, 1991), as well as changes in the internal torsion angles within the choline group (Konstant

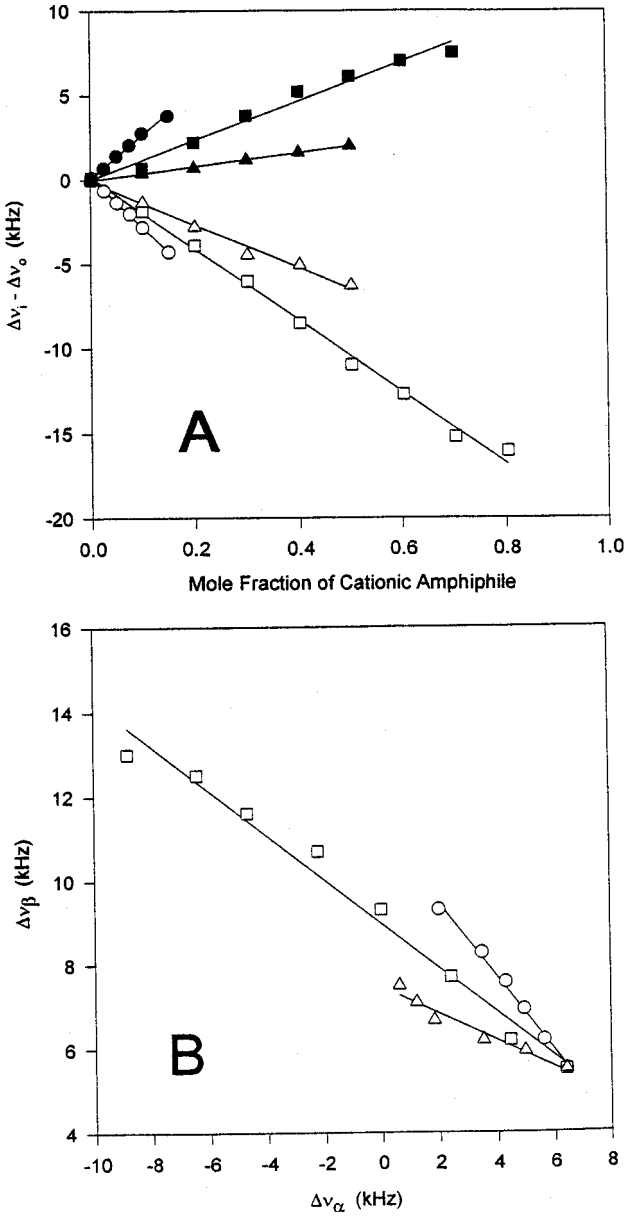


FIGURE 3: (A) Calibration curves relating the ^{2}H NMR quadrupolar splittings from POPC-α-d_2 (open symbols) and POPC-β-d_2 (closed symbols) to the mole fraction of added cationic amphiphile: CTAB (circles), DODAP (squares), DC-CHOL (triangles). The quadrupolar splittings are plotted as the difference between the value measured for a given mixture and the value measured for 100% POPC. (B) α−β Correlation plots of the quadrupolar splittings from POPC-α-d_2 and POPC-β-d_2. The quadrupolar splittings from the two deuteron labelling positions, obtained under identical conditions of cationic amphiphile concentration are plotted with respect to one another for CTAB (circles), DODAP (squares), and DC-CHOL (triangles). The best linear fit to a given data set is shown as a solid line.

et al., 1994). Note that such calibration plots provide an empirical relationship between the quadrupolar splitting and the mole fraction of charged species which is independent of the precise details of the conformational change giving rise to the altered quadrupolar splittings. It is generally found that cationic species yield an α−β correlation with a slope equal to −0.50 (Scherer & Seelig, 1989; Beschiaschvili & Seelig, 1990). DODAP, for instance, exhibits a slope of −0.53. However, CTAB produces a slope of −0.88, while DC-CHOL displays a slope equal to −0.32. There appears to be a correspondence between the slope in such an α−β

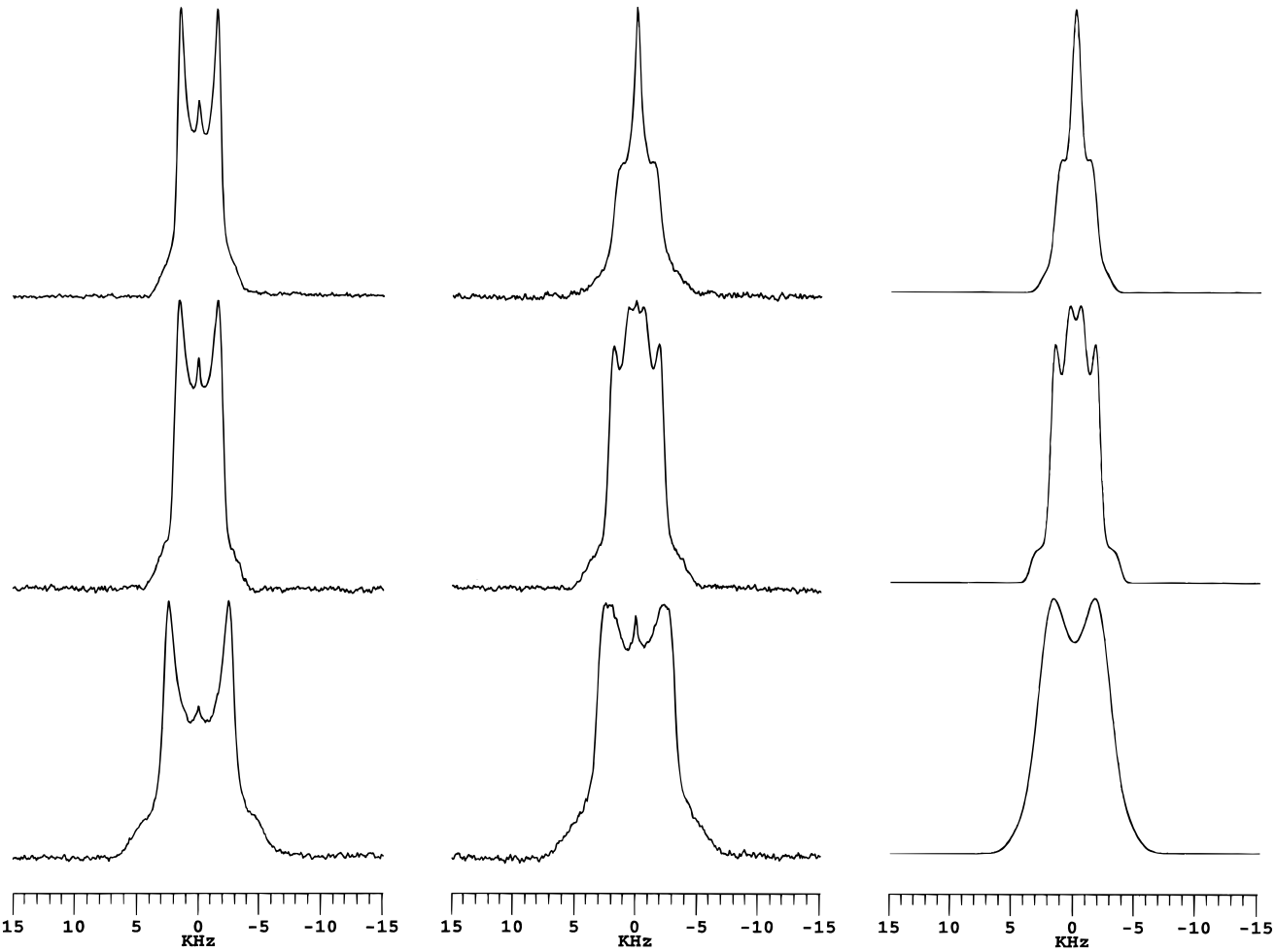


FIGURE 4: ^{2}H NMR spectra of POPC-α-d_2 in mixtures with (from top to bottom) CTAB, DODAP, and DC-CHOL, respectively. The left column represents control spectra obtained in the absence of polyA. The middle column shows the results of adding polyA. The right column represents simulated ^{2}H NMR spectra for the corresponding spectra in the middle. The membrane compositions, amounts of added polyA, and simulation results are listed in Tables 1 and 2.

plot and the location of a given charged species relative to the plane of the phosphocholine group of POPC (Beschiaschvili & Seelig, 1990; Rydall & Macdonald, 1992). For instance, hydrophobic ions, which are known to penetrate well into the bilayer proper, induce a stronger response per unit bound charge than do aqueous ions, which bind more superficially (Beschiaschvili & Seelig, 1990; Rydall & Macdonald, 1992).

The sensitivity of the "molecular voltmeter" response actually depends on a number of factors in addition to the location of the charged group relative to the plane occupied by the phosphocholine group of POPC. One factor is whether the charge is cationic or anionic. Another is the statistical probability that POPC and the charged species will encounter one another. Since all three amphiphiles under consideration are cationic, they each display calibration constants greater than comparable anionic species (Beschiaschvili & Seelig, 1990). Likewise, all three are sufficiently hydrophobic that one expects them to penetrate deeply into the membrane proper. However, the location of their charged groups relative to that of phosphocholine will depend on the length of any spacer between the predominantly hydrophobic portion of the molecule and the charge carrier. Broadly, any charged group which fails to penetrate into the polar region of the membrane is not sensed by the "molecular voltmeter" (Beschiaschvili & Seelig, 1990; Rydall & Macdonald, 1992). The results of the $\alpha-\beta$ correlations drawn in Figure 3b suggest that differences exist in the actual location of the charged group between the three cationic amphiphiles. However, the three cations differ in several other respects. One major difference is that only CTAB will be fully charged at physiological pH. DODAP and DC-CHOL, having dimethylamino groups with pK_a's in the region of 8.5, will only be 90% charged at physiological pH. We note that the ^{2}H NMR method provides one means of determining the membrane-bound pK_a of such ionizable functional groups (Lau & Macdonald, 1995). Another difference between the three is the cross-sectional area they occupy when intercalated into the lipid bilayers. For CTAB this quantity will be about half that expected for DODAP or DC-CHOL. Consequently, a given molar ratio of CTAB produces a higher surface charge density than that produced by DODAP or DC-CHOL. Finally, cholesterol and its derivatives are notoriously prone to demixing (Vist & Davis, 1990; McMullen & McElhaney, 1995). This suggests the possibility that, even at lower molar ratios, POPC might not have the same statistical encounter probability with DC-CHOL as it does with cationic species which mix ideally, such as CTAB and DODAP. Without further experimentation it would be injudicious to attempt to decide between these various possibilities. We note, however, that charge location can profoundly influence the efficacy of transfection agents. For

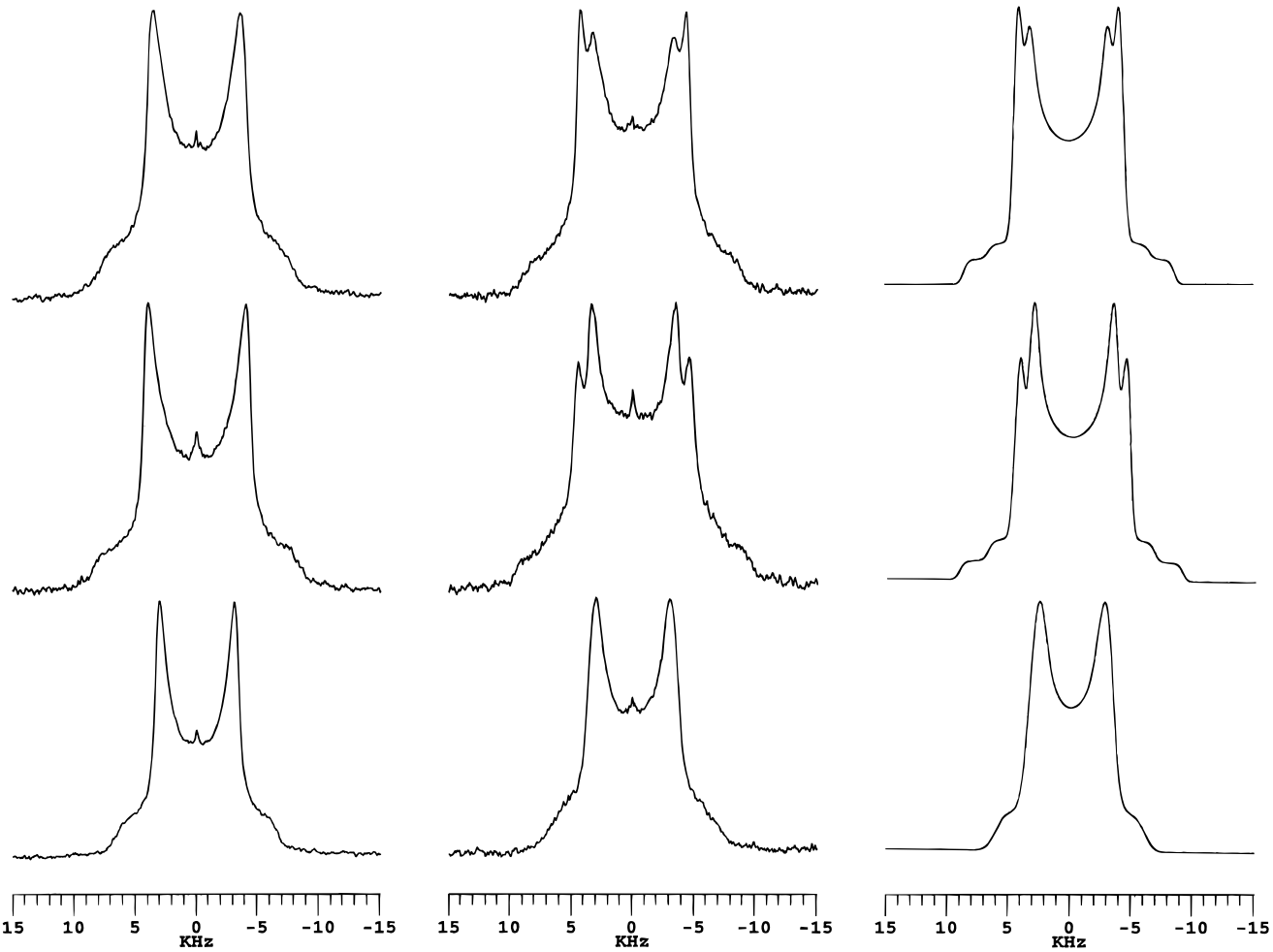


FIGURE 5: ^{2}H NMR spectra of POPC-β-d_2 in mixtures with (from top to bottom) CTAB, DODAP, and DC-CHOL, respectively. The left column represents control spectra obtained in the absence of polyA. The middle column shows the results of adding polyA. The right column represents simulated ^{2}H NMR spectra for the corresponding spectra in the middle. The membrane compositions, amounts of added polyA, and simulation results are listed in Tables 1 and 2.

Table 1: Experimental ^{2}H NMR Data for POPC + Cationic Amphiphile Lipid Bilayers + PolyA

membrane composition	calibration constant m_i (kHz/mol)	polyA/cation charge ratio	free domain $\Delta\nu^f$ (kHz)	bound domain $\Delta\nu^b$ (kHz)
90/10 POPC-α-d_2/CTAB	−35.0	0.50	3.20	1.00
90/10 POPC-β-d_2/CTAB	25.6	1.00	6.60	8.60
90/10 POPC-α-d_2/DODAP	−31.0	0.50	3.70	1.60
80/20 POPC-β-d_2/DODAP	12.5	0.75	6.80	9.00
80/20 POPC-α-d_2/DC-CHOL	−8.5	0.50	5.00	4.10
70/30 POPC-β-d_2/DC-CHOL	20.0	0.75	6.00	6.30

example, Farhood *et al*., (1992) investigated a series of cationic cholesterol derivatives similar to DC-CHOL. Of the two tertiary amino derivatives, that containing a succinyl spacer arm between the substituted ethylenediamine and cholesterol was the more effective transfection agent.

PolyA Induces Domain Formation in Mixed Cationic Amphiphile + POPC Membranes. CTAB, DODAP, and DC-CHOL are of interest here because of their role as agents of transfection of genetic material. Since their interactions with DNA or RNA will be primarily electrostatic in nature, although hydrophobic contributions to the overall interaction energy cannot be dismissed out of hand, it is of interest to examine the ^{2}H NMR response of mixed cationic amphiphile + POPC lipid bilayer membranes to the addition of DNA. The effects of added polyA on the ^{2}H NMR spectra of choline-deuterated POPC mixed with different cationic amphiphiles are illustrated in Figures 4 and 5. Figure 4 shows a series of ^{2}H NMR spectra for POPC-α-d_2 mixed with CTAB (top row), DODAP (middle row), or DC-CHOL (bottom row). Figure 5 shows the corresponding series of ^{2}H NMR spectra for POPC-β-d_2. Details regarding the composition of the lipid mixtures and the amounts of added polyA are given in the figure captions and in Tables 1 and 2. In both Figures 4 and 5 the left column of spectra represent controls obtained in the absence of added polyA. The quadrupolar splittings are altered relative to 100% POPC lipid bilayers in the manner expected for the presence of cationic surface charges, as discussed above. The middle column of spectra shows the effect of adding polyA to such cationic lipid bilayers. The most obvious change is the appearance of a second spectral component, although this is least apparent for the case of DC-CHOL. We note that when polyA is added to 100% POPC-α-d_2 or POPC-β-d_2 MLVs it has no discernible effect on the quadrupolar splittings.

Table 2: Simulated ^{2}H NMR Data for POPC + Cationic Amphiphile + PolyA

membrane composition	fraction POPC: "free" X^{f}_{z}/X^{t}_{z}	fraction POPC: "bound" X^{b}_{z}/X^{t}_{z}	mole fraction cationic amphiphile: "free" $X^{f}_{+}/(X^{f}_{+}+X^{f}_{z})$	mole fraction cationic amphiphile: "bound" $X^{b}_{+}/(X^{b}_{+}+X^{b}_{z})$	predicted $\Delta\nu^{b}$ (kHz)	cation/anion charge ratio in polyA "bound" domain
90/10 POPC-α-d_2/CTAB	0.75	0.25	0.09	0.13	2.00	0.90
90/10 POPC-β-d_2/CTAB	0.28	0.72	0.04	0.12	8.60	0.87
90/10 POPC-α-d_2/DODAP	0.75	0.25	0.09	0.14	2.20	0.86
80/20 POPC-β-d_2/DODAP	0.43	0.57	0.10	0.26	8.80	0.99
80/20 POPC-α-d_2/DC-CHOL	0.69	0.31	0.16	0.27	4.10	0.83
70/30 POPC-β-d_2/DC-CHOL	0.55	0.45	0.25	0.35	6.20	0.71

Consequently, there is minimal DNA−lipid bilayer interaction in the absence of an electrostatic attraction.

The right column of spectra are simulations of the ^{2}H NMR spectra in the middle column. The simulations each consist of a superposition of two spectral components, each of which has a quadrupolar splitting and line width factor corresponding to that observed experimentally. Hence, the only variable is the intensity contributed by a particular component to the overall spectrum. Clearly, this strategy successfully reproduces the experimental ^{2}H NMR spectra obtained in the presence of polyA. One concludes that polyA addition produces two distinct POPC populations which are in slow exchange with one another on the time scale delimited by the difference in their quadrupolar splittings. As will be described below, analysis of the ^{2}H NMR quadrupolar splittings and spectral intensities provides complete information regarding the degree of phase separation and the composition of each of these separate lipid domains.

The spectra in Figures 4 and 5 were obtained with one particular amount of anionic charge from added polyA, yielding the overall anion/cation ratio listed in Table 1. In separate experiments it was found that increasing the polyA concentration increased the intensity of the spectral component with the smaller quadrupolar splitting for POPC-α-d_2 and the spectral component with the larger quadrupolar splitting for POPC-β-d_2. This observation identifies which spectral component corresponds to polyA-associated POPC versus polyA-free POPC. Hence, the spectral simulations may be used to obtain the fraction of the total POPC contained within either domain, assuming no differences in $T1$ or $T2$ relaxation behavior. Our preliminary $T1$ and $T2$ measurements (data not shown) indicate that no significant differences in relaxation times exist between the two spectral components.

Focusing on the polyA-free POPC component, one observes that its quadrupolar splitting always reports a surface charge density less cationic (i.e., more neutral) than the initial control values, regardless of whether one examines ^{2}H NMR spectra from POPC-α-d_2 or POPC-β-d_2. One concludes that the polyA-free lipid domain is depleted with respect to the cationic amphiphile.

For the polyA-associated POPC component, one observes that its quadrupolar splitting always reports a surface charge more cationic than the initial control values, again regardless of whether one examines ^{2}H NMR spectra from POPC-α-d_2 or POPC-β-d_2. This result is contrary to our previous experience with ternary mixtures of cationic + anionic + zwitterionic charged species (Marassi & Macdonald, 1992) wherein the quadrupolar splittings report the expected neutralization of net surface charge. The present contrarian finding, that polyA addition produces an apparently more cationic domain as reported by ^{2}H NMR, we have dubbed the "anti voltmeter" response and is substantiated by our mathematical model. In fact, similar effects are observed with a variety of large anionic polyelectrolytes added to cationic amphiphile-containing lipid bilayers (Mitrakos and Macdonald, unpublished results). Moreover, one may perform the electrostatic mirror image experiment, adding cationic polyelectrolytes to anionic amphiphile-containing lipid bilayers, and obtain a similar "anti voltmeter" response (Crowell and Macdonald, unpublished results). Therefore, this would appear to be a property common to polyelectrolytes of sufficient size interacting with oppositely-charged lipid bilayer surfaces under conditions of low salt concentration.

Quantifying Phase Separation and Phase Composition from ^{2}H NMR Spectra. The total populations of zwitterionic (X^{t}_{z}) or cationic (X^{t}_{+}) lipids present globally in the entire lipid bilayer preparation are conveniently defined in terms of mole fractions such that

$$X^{t}_{z} + X^{t}_{+} = 1 \tag{1}$$

Each of these populations may be subdivided into those which are polyA-bound (superscript b) and those which are polyA-free (superscript f), according to eq 2.

$$X^{t}_{z} = X^{b}_{z} + X^{f}_{z} \qquad X^{t}_{+} = X^{b}_{+} + X^{f}_{+} \tag{2}$$

The values of (X^{b}_{z}/X^{t}_{z}) and (X^{f}_{z}/X^{t}_{z}) are obtained from the intensities of the two components in the ^{2}H NMR spectral simulations as described above. The details are listed in Table 2. From the quadrupolar splitting of the polyA-free domain ($\Delta\nu^{f}$) and the calibration constants measured in Figure 3 one may calculate the cationic amphiphile composition of the polyA-free domain according to eq 3,

$$\frac{X^{f}_{z}(\Delta\nu^{f} - \Delta\nu_0)}{(m_i - \Delta\nu^{f} + \Delta\nu_0)} = X^{f}_{+} \tag{3}$$

where $\Delta\nu_0$ is the quadrupolar splitting for 100% POPC and m_i is the calibration constant for either the α or β deuterolabeling position for a particular cationic amphiphile. The results of applying this analysis are listed in Table 2. They demonstrate that the polyA-free domain is depleted with respect to cationic amphiphiles relative to the initial global composition. Further, one may now calculate the mole fraction of cationic amphiphile in the polyA-bound phase (X^{b}_{+} / X^{t}_{+}). The results of such calculations are likewise listed in Table 2. They reveal that the ratio of cationic

(amphiphile) to anionic (polyA) charge within the polyA-bound domain is always close to 1:1, indicating a neutral complex if it is assumed that every single polyA phosphate group is able to interact with a cationic amphiphile.

In a ternary mixture of cationic + anionic + zwitterionic species, the observed quadrupolar splitting ($\Delta\nu_{+/-}$) is perturbed relative to the value measured for 100% POPC ($\Delta\nu_0$) by an amount which is the sum of the perturbations due to the cationic and anionic species individually in binary mixtures (Marassi & Macdonald, 1992), according to eq 4.

$$(\Delta\nu_{+/-} - \Delta\nu_0) = (\Delta\nu_- - \Delta\nu_0) + (\Delta\nu_+ - \Delta\nu_0) \quad (4)$$

$$= m_+X_+ + m_-X_-$$

The quadrupolar splitting in such a ternary mixture may be estimated, therefore, if one has available the relevant calibration constants m_+ and m_- from calibration experiments in binary mixtures. No such calibration data is available for polyA because polyA apparently interacts only very weakly with 100% POPC lipid bilayers. To analyze the quadrupolar splittings from the polyA-bound domains in terms of compositions one must resort, therefore, to certain assumptions.

What if one assumes that POPC has no direct contact with polyA and only detects its presence indirectly through polyA's effects on the local distribution of cationic amphiphiles? If this physical situation really exists then the effective mole fraction of anionic charge (X_-) in eq 4 drops to zero. Simultaneously, the effective mole fraction of cationic charge (X_+) in the polyA-bound domain equals the quantity [$X^{\,b}_{+}/(X^{\,b}_{+} + X^{\,b}_{z})$] which can be evaluated using an expression analogous to eq 3. Thus one can predict the quadrupolar splitting for the case that POPC does not detect polyA directly. The results of such calculations are listed in Table 2 for each circumstance in Table 1. It is evident that the experimental and predicted values of $\Delta\nu^b$ agree closely with one another. This indicates that the pretense of a polyelectrolyte somehow masked from direct detection by POPC, and whose presence is detected only indirectly through its influence on the local lipid composition and rate of exchange with bulk lipid, is capable of explaining the ^{2}H NMR observations. In the following section we propose a model of the polyA−lipid bilayer interaction which explains the physical origin of the polyA "masking" effect.

A Model for Polyelectrolytes at a Mixed Cationic + Zwitterionic Lipid Bilayer Surface. The ^{2}H NMR results reported above permit us to propose a model for the arrangement of polyA at the surface of a lipid bilayer containing a mixture of cationic + zwitterionic amphiphiles, as shown schematically in Figure 6. PolyA is drawn to the membrane surface through electrostatic attraction. Upon binding, the adenosine bases penetrate into the acyl chain region of the lipid bilayer, as indicated by infrared (Mal'tseva *et al.*, 1983) and NMR (Budker, 1990) evidence. This leaves the sugar-phosphate backbone located within the polar interface region. The intercalated polyA is pictured to assume a two-dimensional self-avoiding random-coil configuration wherein individual nucleotide units occupy sites on a lattice, the spacing of which is determined primarily by the cross-sectional areas of the bilayer lipids.

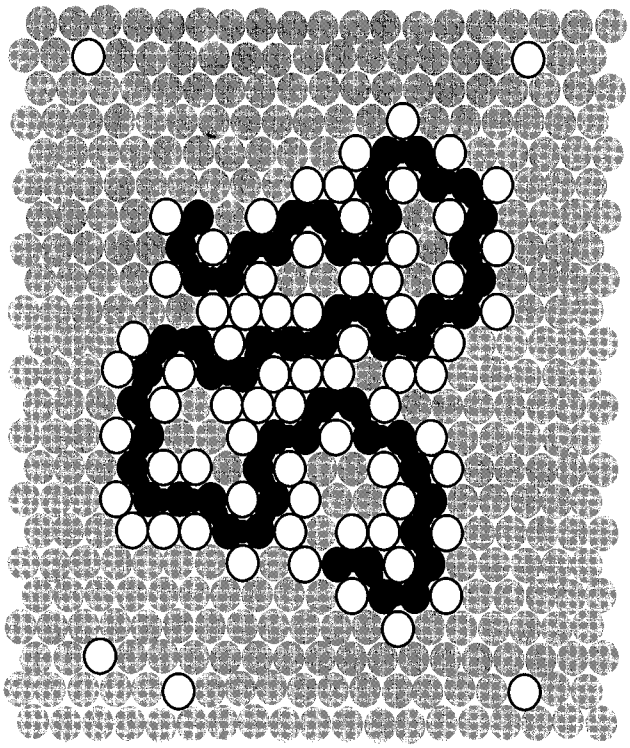


FIGURE 6: Schematic representation of polyA (black circles) forming a domain of distinct composition in mixed POPC (closed circles) plus cationic amphiphile (open circles) lipid bilayers.

The region of the lipid bilayer occupied by polyA defines a distinct domain through a combination of two effects. First, electrostatic attraction between anionic nucleotide units and cationic amphiphiles tends to draw the two together such that the lipid composition in the vicinity of the polyA will be, on average, more cationic than that of the bulk. If these electrostatic forces are sufficiently strong, then the cationic amphiphiles screen the charge of polyA from the POPC. Second, the effective lateral diffusion coefficient of individual lipids within the domain will decrease due to the well-known archipelago effect (Saxton, 1993). This inhibits the ability even of zwitterionic lipids trapped within the polyA-defined domain to exchange with, and average over, the bulk lipid population. The dimensions of such as random-coil polymer permits the inclusion of considerable amounts of zwitterionic lipid, the details of which should depend on a number of factors including the polymer molecular weight, the stiffness of the polymer backbone, the polyelectrolyte's linear charge density, the ionic strength, and the global cationic/zwitterionic amphiphile in the lipid bilayer.

Such a model explains all our ^{2}H NMR observations including the appearance of distinct POPC populations, the depletion of cationic amphiphile from the polyA-free domain, the enrichment of cationic amphiphile in the polyA-bound domain, and the "anti voltmeter" response observed in the latter. In particular, if the cationic amphiphiles remain closely associated with the anionic polyelectrolyte, then POPC has little statistical probability of direct interaction with polyA. Instead, POPC encounters an environment enriched with the cationic amphiphile, thereby leading to the "anti voltmeter" response described above.

There is, however, an alternate explanation that one might conceive in order to explain the ^{2}H NMR "anti voltmeter" results. This second possibility is that the plane of binding of polyA leaves the anionic phosphates located above the

plane of the phosphocholine groups. This could decrease the sensitivity of the "molecular voltmeter" to the presence of polyA, possibly to the extent that the calibration constant (m_-) in a polyA calibration plot becomes so small that the polyA term in eq 4 can be ignored. This second scenario seems less likely than the first, however, given the literature evidence regarding the deep penetration of the nucleotide bases into the membrane interior (Mal'tseva *et al.*, 1983; Budker, 1990). Moreover, it is difficult to envisage that so superficial a polyA binding location would permit domain formation and lateral segregation.

CONCLUDING REMARKS

The studies presented here constitute the first demonstration of the formation of segregated domains of cationic lipids induced by polynucleotides. The results suggest that cationic amphiphiles can remain associated with polynucleotides even after the cationic amphiphile−DNA "package" binds to and fuses with a biological membrane. The three different cationic amphiphiles investigated yielded different responses on the part of the "molecular voltmeter", the sensitivity of which correlated loosely with their transfection efficiencies (Remy *et al.*, 1995). The investigations reported here encompass a narrow set of circumstances and at least four other key experimental variables demand further investigation. The first is the effects of ionic strength. The present measurements were carried out in the absence of salt other than buffer. At physiological salt concentrations charge screening may well decrease electrostatic interactions to the extent that distinct lipid domains can no longer be discerned. The second variable is the molecular weight of the polyelectrolyte. Clearly, the size of the polyelectrolyte will determine the dimensions of any domain, the strength of the electrostatic attractions, and the rate of exchange of lipid in and out of the domain. The third variable is the structure of the polyelectrolyte. PolyA is a very stiff polyelectrolyte relative to, for example, polyacrylate which is highly flexible. PolyA has both a hydrophilic portion (the sugar-phosphate backbone) and a rather more hydrophobic portion (the adenine bases). This amphiphilicity apparently permits polyA to intercalate between the membrane lipids. Other polyelectrolytes, such as polyglutamate with its glutamic acid side chains and peptide backbone, are everywhere hydrophilic and should adopt a binding location different from polyA. A fourth variable would be the linear charge density of the polyelectrolyte, a factor critical to determining the electrostatic attraction to a charged surface. Finally, it will be important to examine the cationic amphiphile itself for its response to the presence of anionic polyelectrolytes. Aminomethyl deutero-labeled CTAB, DODAP, and DC-CHOL, for instance, would provide ^{2}H NMR spectra reflecting differences in local distribution and interactions with polyelectrolytes. Such measurements are currently in progress.

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