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Cationic amphiphile interactions with polyadenylic acid as probed via ^{2}H-NMR

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Abstract

^{2}H-NMR spectroscopy was used to investigate the effects of polyadenylic acid (PolyA) on three aminomethyl-deuterated cationic amphiphiles: specifically, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*′,*N*′,*N*′-trimethylammonium chloride (DOTAP-γ-d_3), 3β-[*N*-(*N*′,*N*′,*N*′-trimethylaminoethane)carbamoyl] cholesterol (TC-CHOL-γ-d_3), and cetyltrimethylammonium bromide (CTAB-γ-d_9). When mixed with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and incorporated into lipid bilayer membranes, each of the cationic amphiphiles yielded ^{2}H-NMR spectra consisting of a motionally averaged Pake powder pattern. The ^{2}H-NMR quadrupolar splitting generally increased with increasing mole fraction of cationic amphiphile in the lipid bilayer with the exception of CTAB-γ-d_9. Adding PolyA caused the quadrupolar splitting to increase progressively in every case, until a 1:1 cation:anion charge ratio was achieved, after which the quadrupolar splitting changed no further. Deuterium NMR relaxation time measurements showed a parallel increase in T_2^{qe} with increasing PolyA. The size of these changes produced by PolyA increased in the order: TC-CHOL < DOTAP < CTAB. NaCl addition reversed much, but not all, of the PolyA-related changes in ^{2}H-NMR quadrupolar splittings and T_2^{qe} relaxation times. A UV-based PolyA-membrane binding assay showed that salt addition caused PolyA desorption, and that the salt concentration required to do so increased in the order: TC-CHOL < DOTAP < CTAB. The results are consistent with an electrostatic binding of PolyA to the cationic lipid bilayer surface, accompanied by formation of a stoichiometric charge complex between PolyA and the cationic amphiphile, in which the cationic amphiphile retains considerable motional freedom. The strength of the complex increases in the order: TC-CHOL < DOTAP < CTAB. 0005-2736/98/$ – see front matter © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Cationic lipid; Polyadenylic acid; Ionic strength; Deuterium nuclear magnetic resonance

1. Introduction

Interactions between polyelectrolytes and oppositely charged amphiphiles are of fundamental scientific, as well as applied biomedical, interest. When the polyelectrolyte is a cationic peptide, binding to membrane surfaces can lead to segregation of anionic phospholipids into domains, and this can constitute a mechanism for control of cell signaling pathways [1–3]. When the polyelectrolyte is an anionic species,

Abbreviations: CTAB, cetyltrimethylammonium bromide; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*′,*N*′,*N*′-trimethylammonium chloride; ^{2}H, deuterium; MLV, multilamellar vesicle; NMR, nuclear magnetic resonance; PACA, polyacrylic acid; PolyA, polyadenylic acid; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; T_1, longitudinal relaxation time; T_2^{qe}, transverse relaxation time; TC-CHOL, 3β-[*N*-(*N*′,*N*′,*N*′-trimethylaminoethane)carbamoyl] cholesterol

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like DNA, binding to cationic amphiphiles is essential to successful gene transfer in gene therapy applications [4–7].

A variety of cationic amphiphiles have been proposed and tested for use in gene transfer technologies [8–11]. The role of the cationic species is twofold: it confers a net cationic charge on the DNA package, thereby ensuring binding to anionic target membrane surfaces, and it condenses the anionic DNA into a small package, thereby lowering the energy barrier to transmembrane transport. It is well established that different cationic amphiphiles display markedly different efficiencies as gene transfer agents, and that different cell lines respond differently to different cationics [12–14]. However, the physical basis for these differences remains uncertain.

The choice of cationic amphiphile can influence the structural morphology of the 'packages' formed when DNA is complexed with mixtures of cationic plus neutral lipids. For instance, multilamellar structures of alternating DNA monolayers and lipid bilayers have been reported for plasmid DNA interacting with the cationic lipid DOTAP (*N*-[1-(2,3-dioleoyloxy)propyl]-*N*′,*N*′,*N*′-trimethylammonium chloride) plus either DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine) or DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine) [15]. Similar lamellar structures were reported for DNA complexed with the cationic amphiphiles CTAC (cetyltrimethylammonium chloride), DODAB (dioctadecyldimethylammonium bromide), or DOTAP, all in mixtures with DOPE [16,17]. On the other hand, spaghetti-like structures, in which individual DNA strands are surrounded with a bilayer of mixed cationic plus neutral lipids, have been reported to arise when DNA is exposed to liposomes consisting of DOPE plus the cationic lipid DC-CHOL (3β-[*N*-(*N*′,*N*′-dimethylaminoethane)carbamoyl] cholesterol) [18,19]. At higher DOPE contents there is a conversion to honeycomb-like structures, in which individual DNA strands are surrounded with a monolayer of mixed cationic plus neutral lipid and the DNA-monolayer units pack into hexagonal bundles. Molecular level theories confirm the thermodynamic stability of both the spaghetti-like and honeycomb-like architectures [20]. It is not clear, however, that any of these three different DNA/lipid morphologies consistently lead to greater transfection efficiencies.

The choice of cationic amphiphile can also influence the fate of the DNA/lipid 'packages' once they encounter the target membrane surface. ^{2}H-NMR studies show that, once present at a lipid bilayer surface, the DNA and cationic amphiphile remain together as a complex, forming laterally segregated domains containing a stoichiometric anionic-cationic charge ratio [21]. Such polyelectrolyte-induced domain formation can be observed with any number of different anionic [21,22], or cationic [23,24], polyelectrolytes binding to oppositely charged membrane surfaces. However, the details of domain composition and size depend on the particular cationic amphiphile [19]. Between the three cationic amphiphiles CTAB (cetyltrimethylammonium bromide), DODAP (*N*-[1-(2,3-dioleoyloxy)propyl]-*N*′,*N*′-dimethylammonium chloride), and DC-CHOL, the size of the DNA-dependent domains increased in the order CTAB < DODAP < DC-CHOL, and their degree of enrichment with cationic amphiphile increased in the order DC-CHOL < DODAP < CTAB. Again, it is not clear whether, or how, such differences relate to efficiencies of gene transfection.

The goal of the studies reported here is to examine directly three different cationic amphiphiles via ^{2}H-NMR, and to discover the consequences of their complexation with anionic polyelectrolytes. The three are CTAB, DOTAP, and TC-CHOL (3β-[*N*-(*N*′,*N*′,*N*′-trimethylaminoethane)carbamoyl] cholesterol), each deuterated in its quaternary methyls. In our previous study, ^{2}H-NMR of choline-deuterated phosphatidylcholine was employed to examine the consequences of DNA-cationic amphiphile interactions on lipid bilayer surface charge and surface charge distribution [21], and so did not probe the cationic amphiphiles directly. Furthermore, previous ^{2}H-NMR studies were performed at low ionic strength, below physiological in fact [21–24]. The influence of ionic strength on such interactions, or on domain formation, is expected to be critical. In the studies described here the three specifically deuterated cationic amphiphiles are mixed into lipid bilayer membranes containing phosphatidylcholine, and allowed to interact with an anionic polyelectrolyte, specifically polyadenylic acid (PolyA). In addition, a much simpler anionic polyelectrolyte, polyacrylic acid (PACA), is examined. PACA is expected to experience primarily electrostatic, and no hydrophobic,

interactions with lipid bilayers. The ^{2}H-NMR spectra of the three deuterated cationic amphiphiles are examined and compared as a function of their mole fraction in mixtures with phosphatidylcholine, the amount of added anionic polyelectrolyte, and the ionic strength.

2. Materials and methods

2.1. Materials

Non-deuterated POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) was purchased from Avanti Polar Lipids (Alabaster, AL). PolyA and oleoyl chloride were obtained from Sigma (St. Louis, MO). PACA, iodomethane-d_3, hexadecylamine, cholesteryl chloroformate, *N*,*N*-dimethylethylenediamine, 3-dimethylamino-1,2-propanediol, and deuterium-depleted water were purchased from Aldrich (Milwaukee, WI).

2.2. Synthesis of quaternary methyl deuterated DOTAP

First, DODAP was synthesized and purified as described by Leventis and Silvius [9], by coupling oleoyl chloride to 3-dimethylamino-1,2-propanediol. The product was purified by eluting the acetic acid salt of DODAP from a silicic acid column with 10% methanol in chloroform. Its purity was assayed by TLC and ^{1}H-NMR as described elsewhere [21]. Second, the amino functionality of DODAP was quaternized by reaction with a tenfold molar excess of methyl-d_3 iodide (24 h in dry diethyl ether) to produce DOTAP with one deuterated aminomethyl group (i.e. DOTAP-γ-d_3). The product was purified by two acetone precipitations and then chromatographed on Bio-Rad AG 1-X4 anion exchange resin prepared in the chloride form (Bio-Rad, Mississauga, ON) in order to convert from the iodide form. The purity was determined as described for DODAP.

2.3. Synthesis of quaternary methyl deuterated TC-CHOL

First, DC-CHOL was synthesized and characterized according to the method of Gao and Huang [10] by coupling *N*,*N*-dimethylethylenediamine to cholesteryl chloroformate. The final product was recrystallized twice from absolute ethanol at −20°C, dried under vacuum, and then further purified and characterized as described previously [21].

DC-CHOL was then reacted with methyl-d_3 iodide in the same manner as for DODAP to produce TC-CHOL with one of its aminomethyls deuterated (i.e. TC-CHOL-γ-d_3). The product was then purified by two acetone precipitations and counterion-exchanged to produce the chloride form as described above for DOTAP. Finally, TC-CHOL-γ-d_3 was recrystallized from absolute ethanol and dried under vacuum. Its purity was monitored using TLC, ^{1}H-NMR, and ^{2}H-NMR.

2.4. Synthesis of quaternary methyl deuterated CTAB

CTAB-γ-d_9 was synthesized by the methylation of hexadecylamine with methyl-d_3 iodide as described by Semchyschyn et al. [25].

2.5. Preparation of multilamellar vesicles (MLVs)

Lipid mixtures of a desired composition were prepared by combining appropriate volumes of chloroform stock solutions of either CTAB-γ-d_9, TC-CHOL-γ-d_3 or DOTAP-γ-d_3 with POPC. Typically, the lipid mixtures consisted of 10 mg of POPC plus the prescribed amount of aminomethyl-deuterated cationic amphiphile. The solvent was removed under a stream of argon and the mixture dried under vacuum. The dried lipids were then hydrated in 160 μl of buffer with or without salt solution in deuterium-depleted water. The hydration process consisted of gentle warming and vortexing, followed by five cycles of freeze-thaw to ensure homogeneous mixing.

2.6. Preparation of MLVs containing PolyA or PACA

Dried lipid mixtures were prepared as described above, but were hydrated by adding the desired quantity of PolyA or PACA dissolved in deuterium-depleted water plus sufficient deuterium-depleted water, and/or NaCl in deuterium-depleted water, to bring the final volume to 200 μl. The mix-

tures were once again gently warmed and vortexed and subjected to five freeze-thaw cycles to ensure homogeneous mixing.

2.7. ^{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 deuterium probe. The quadrupolar echo sequence [26] was employed using quadrature detection with complete phase cycling of the pulse pairs, a 90° pulse length of 2.1 μs, an interpulse delay of 30 μs, a recycle delay of 100 ms, a spectral width of 50 kHz, and a 2K data size. Transverse relaxation times (T_2^{qe}) were obtained from the echo intensity as a function of the separation τ between the two 90° pulses in the quadrupolar echo sequence. $1/T_2^{qe}$ corresponds to the slope in a semilogarithmic plot of the normalized intensity at the peak of the echo versus the time $t = 2\tau$. All measurements were performed at room temperature.

2.8. Difference assay of PolyA binding to cationic lipid bilayer membranes

Dried lipid samples were prepared as described above, and sufficient PolyA and NaCl were added from separate aqueous stock solutions to achieve a stoichiometric anion:cation ratio between PolyA and the particular cationic amphiphile, along with the desired final NaCl concentration. The final volume of the mixture was brought to 300 μl, and the samples were hydrated and equilibrated as described above. The dispersions were then centrifuged at 13 000 rpm for 1 h to pellet the lipid bilayers. The supernatant was removed, diluted, and passed through a Centricon-500 microconcentrator (Amicon, Oakville, ON) to remove any unpelleted lipid. The filtrate was then further diluted until its UV absorbance, measured using a Hewlett Packard 8452A Diode Array spectrophotometer, fell into the concentration regime where Beer's law was obeyed for PolyA. The PolyA concentration in the original supernatant was then calculated from a standard curve.

3. Results

3.1. ^{2}H-NMR of quaternary methyl-deuterated cationic amphiphiles in lipid bilayers

The structures of the three cationic amphiphiles used in this study are shown in Fig. 1. Each contains a single cationic charge positioned at its quaternary amino head group. Each is deuterated in at least one of its three aminomethyls. CTAB is a surfactant with a single C16 alkyl chain. DOTAP contains two C18:*cis*Δ9 acyl chains. The hydrophobic portion of TC-CHOL is its cholesterol ring.

The ^{2}H-NMR spectra obtained upon incorporating these deuterated cationic amphiphiles into POPC bilayer membranes are shown in Fig. 2. Each of the cationic amphiphiles produces a Pake pattern spectral line shape, indicative of lipids undergoing rapid anisotropic motional averaging. Previous ^{31}P-NMR and ^{2}H-NMR measurements of choline-deuterated

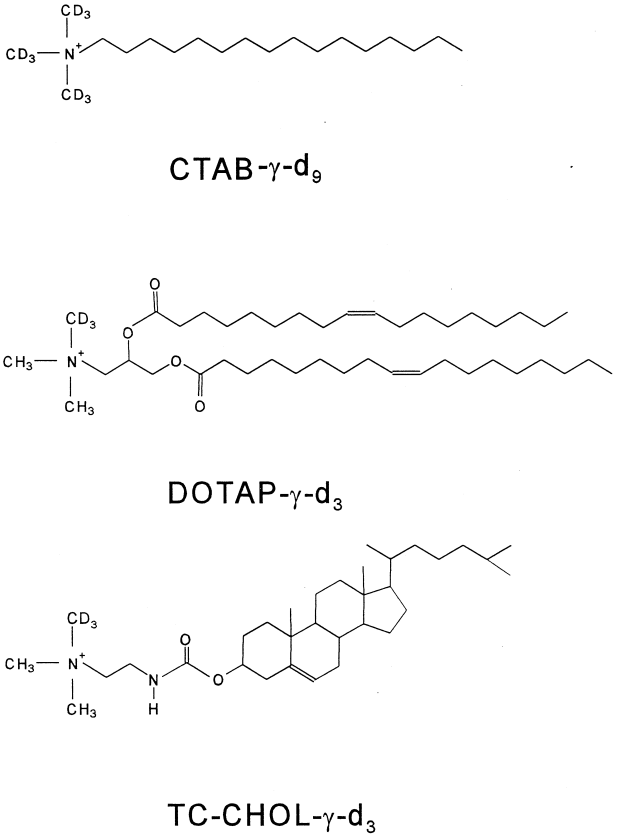

Fig. 1. Structures of the three cationic amphiphiles employed here. From top to bottom, CTAB-γ-d_9, DOTAP-γ-d_3, and TC-CHOL-γ-d_3.

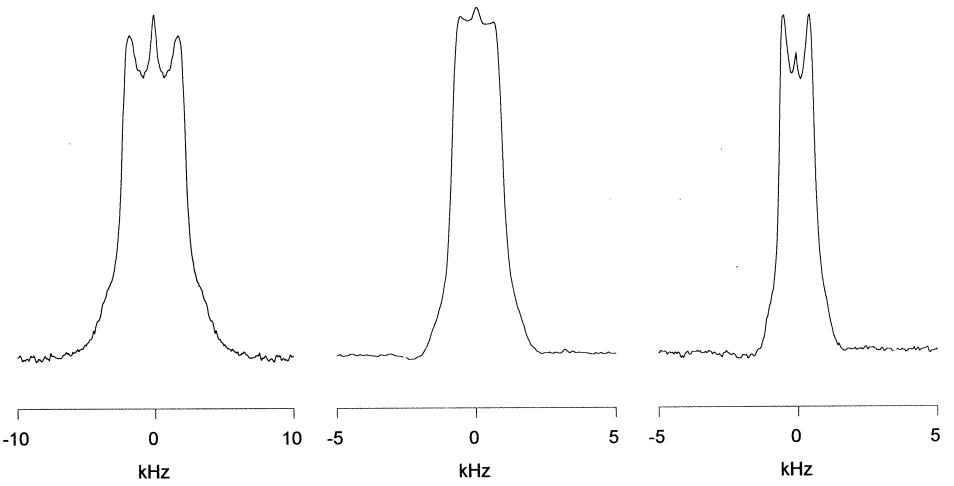

Fig. 2. ^{2}H-NMR spectra of mixed deuterated cationic amphiphile+POPC in the absence of PolyA. From left to right, spectra correspond to: CTAB-γ-d_9+POPC (15/85), DOTAP-γ-d_3+POPC (70/30), and TC-CHOL-γ-d_3+POPC (70/30).

POPC in mixtures with either of these three cationic amphiphiles at these mole fractions were all consistent with an overall fluid bilayer lipid arrangement. We interpret the spectra in Fig. 2 as indicating that each of the cationic amphiphiles incorporate into the membrane proper, that they experience considerable motional averaging, but that the axis of motional averaging does not correspond to the magic angle, and that in each case the orientational order parameter at the position of the quaternary methyl deuteron labels is not so small that the motional averaging is effectively isotropic. The quadrupolar splitting ($\Delta\nu$) in such a spectrum corresponds to the separation, in Hz, between the two maxima or 'horns' in the Pake pattern line shape. Qualitatively, the quadrupolar splittings decrease in the order CTAB > DOTAP ≈ TC-CHOL. The different quadrupolar splittings measured for these three cationics reflects some combination of differences in configuration and order at the level of the trimethylammonium group. Note that if one arranges these three cationics such that the boundaries between their respective hydrophobic and hydrophilic regions are aligned, then the CTAB quaternary methyls lie close to this boundary, while the polar regions of DOTAP, which include the fatty ester groups, and TC-CHOL, which include the (trimethylaminoethane)carbamoyl moiety, extend far from this boundary. If these three cationics adopt equilibrium locations within the lipid bilayer such that the boundary between their hydrophobic and hydrophilic regions are similar, then the extension of the cationic trimethylamino groups into the aqueous bathing medium increases in the order CTAB < DOTAP ≈ TC-CHOL. The difference in orientational ordering that one expects to observe as a consequence would be sufficient to explain the differences in quadrupolar splittings observed in Fig. 2.

The quadrupolar splitting measured for each of the three cationic amphiphiles depends on the composition of the lipid bilayer membrane, as shown in Fig. 3. In the case of CTAB-γ-d_9 the quadrupolar splitting is large initially, but decreases progressively with increasing mole fraction of CTAB relative to POPC. CTAB can only be added in amounts below approx. 15–20 mole%, above which its surfactant properties produce micellization of the membrane structure, and isotropically narrow ^{2}H- and ^{31}P-NMR spectra. So incipient micellization is a factor in determining the concentration dependence of the quadrupolar splittings for CTAB-γ-d_9.

Increasing the mole fraction of DOTAP-γ-d_3 or TC-CHOL-γ-d_3 relative to POPC increases the quadrupolar splitting from a value of about 500 Hz at low mole fractions to over 1000 Hz at higher mole fractions. These values are similar to the quadrupolar splittings reported for the trimethylaminocholine deuterons in DMPC [27]. The head group of phosphatidylcholine bears a large dipole moment, and consequently undergoes a concerted conformational

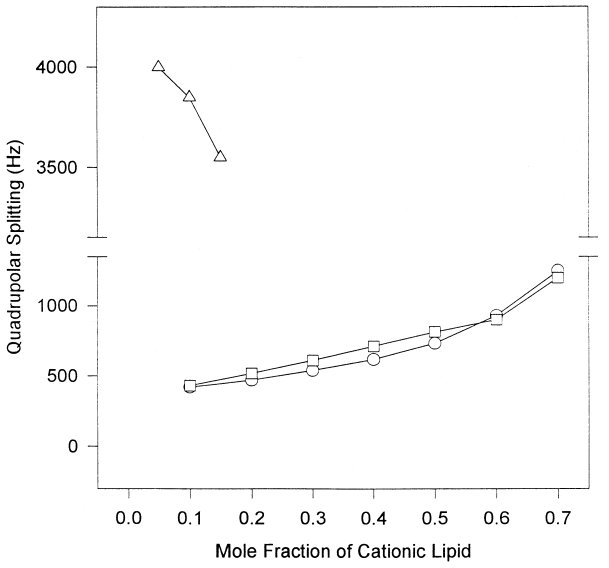

Fig. 3. Relationship between the ^{2}H-NMR quadrupolar splittings from the three deuterated cationic lipids and the mole fraction of added cationic amphiphile in POPC membranes: CTAB-γ-d_9 (triangles), DOTAP-γ-d_3 (circles), TC-CHOL-γ-d_3 (squares).

response to changes in surface charge density [28,29]. Since the cationic charge in DOTAP and TC-CHOL is a monopole, one does not anticipate that there should be any concerted conformational response of the trimethylammonium head group of these cationic amphiphiles to changes in surface charge density. Therefore, the effect of added cationic amphiphile on the quadrupolar splittings as reported in Fig. 3 is more likely due to changes in orientational ordering and dynamics.

The values of T_2^{qe} obtained from quadrupolar echo decay curves for different proportions of the various cationic amphiphiles mixed with POPC are listed in Table 1. For all three cationic amphiphiles the echo intensity decay was mono-exponential. At comparable molar compositions, T_2^{qe} increases in the order: CTAB < TC-CHOL. For CTAB, T_2^{qe} increases with increasing CTAB content. For DOTAP and TC-CHOL, T_2^{qe} decreases with increasing mole fraction of cationic amphiphile. The relative values of T_2^{qe} for these three cationic amphiphiles have two likely origins. First, they could reflect differences in orientational order associated either with differences in the location of the trimethylamino deuterons as discussed above, or with an ordering effect of the sterol ring of TC-CHOL versus a disordering effect of the incipient micellization by CTAB. Second, they could reflect differences in the lateral diffusion coefficients of the three cationic amphiphiles within the plane of the two-dimensional bilayer, since Reinl and Bayerl [30] have demonstrated the relationship between reduced lateral diffusion of lipids and increased T_2^{qe}. If so, this suggests that the diffusivity of these three cationics decreases in the order: CTAB > DOTAP > TC-CHOL. Interestingly, this is the reverse of the order of cross-sectional area occupied by these three when incorporated into lipid bilayers. However, both lipid packing and head group interactions with water and with other lipids are major determinants of diffusivity in lipid bilayers [31,32]. Since these three cationic amphiphiles are anticipated to differ from one another in both respects, it would be preferable to measure their diffusion coefficients rather than to speculate further as to the origin of their different values of T_2^{qe}.

3.2. *Effect of PolyA and PACA in ^{2}H-NMR of quaternary methyl-deuterated cationic amphiphiles*

The anionic polyelectrolytes PolyA and PACA are both known to induce domain formation in mixed cationic amphiphile+POPC lipid bilayers, as demonstrated using ^{2}H-NMR of choline-deuterated POPC

Table 1
^{2}H-NMR T_2^{qe} relaxation times for cationic amphiphiles mixed with POPC

POPC/X (mole/mole)	CTAB-γ-d_9 (ms)	DOTAP-γ-d_3 (ms)	TC-CHOL-γ-d_3 (ms)
95/5	0.88	–	–
90/10	0.65	1.25	1.90
85/15	1.07	–	–
70/30	–	1.12	1.54
50/50	–	0.93	1.36

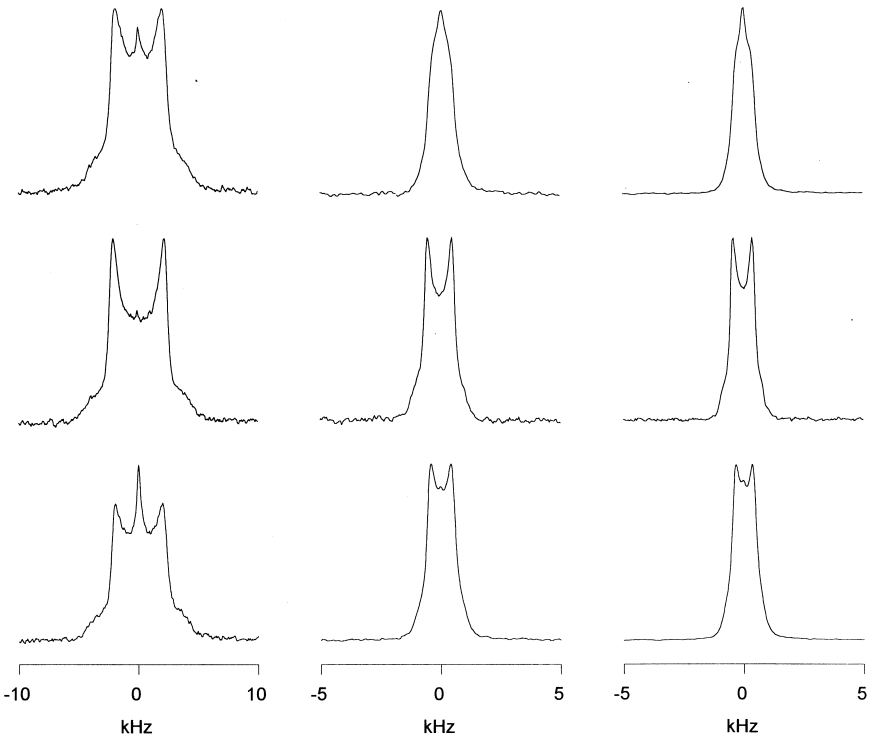

Fig. 4. ^{2}H-NMR spectra of mixed cationic amphiphile + POPC lipid bilayers in the absence (top row) and presence of PolyA (middle row), and in the presence of PolyA plus salt (bottom row). From left to right the spectra correspond to: CTAB-γ-d_9+POPC (10/90), DOTAP-γ-d_3 + POPC (30/70), and TC-CHOL-γ-d_3 + POPC (30/70). The bottom row of spectra show the effect of 250 mM NaCl in the case of CTAB-γ-d_9 and 800 mM NaCl for both DOTAP-γ-d_3 and TC-CHOL-γ-d_3 mixtures.

[21,22]. One observes two components in the ^{2}H-NMR spectrum, corresponding to polyelectrolyte-bound and polyelectrolyte-free POPC populations. The two POPC populations are, therefore, in slow exchange with one another on the time scale of the difference in their quadrupolar splittings.

It is highly desirable to establish whether domain formation can be observed directly with deutero-labeled cationic amphiphiles. Fig. 4 shows ^{2}H-NMR spectra of the three quaternary methyl-deuterated cationic amphiphiles in the absence (top row) and in the presence (middle row) of polyA. The various spectra were obtained from lipid bilayers containing POPC mixed with (from left to right): 10% CTAB-γ-d_9, 30% DOTAP-γ-d_3, and 30% TC-CHOL-γ-d_3. In every case, the effect of adding PolyA (or PACA, data not shown) is to increase the quadrupolar splitting. There is no evidence of separate PolyA-free and PolyA-bound spectral components. At all proportions of PolyA anionic charge to amphiphile cationic charge, only a single Pake pattern component could be observed in the ^{2}H-NMR spectrum from any of the methyl-deuterated amphiphiles. Thus, it is not possible to observe directly the coexistence of PolyA-bound and PolyA-free cationic amphiphiles in their ^{2}H-NMR spectra, at least when the deuteron labels are located on the quaternary methyls. Even low temperature measurements, which often help to resolve such differences, are of no avail in these cases.

Why is it not possible to observe distinct populations of free and bound cationic amphiphiles in these ^{2}H-NMR spectra, when free and bound POPC are so readily differentiated? It seems unlikely that the cationic amphiphiles, which are held by electrostatic attraction in the vicinity of the polyelectrolyte, should be in fast exchange when the zwitterionic POPC is in slow exchange. A likely explanation is that the difference in quadrupolar splitting between the free and bound cationic amphiphile populations is so small that they cannot be resolved spectroscopically, even in the slow exchange regime. In the case

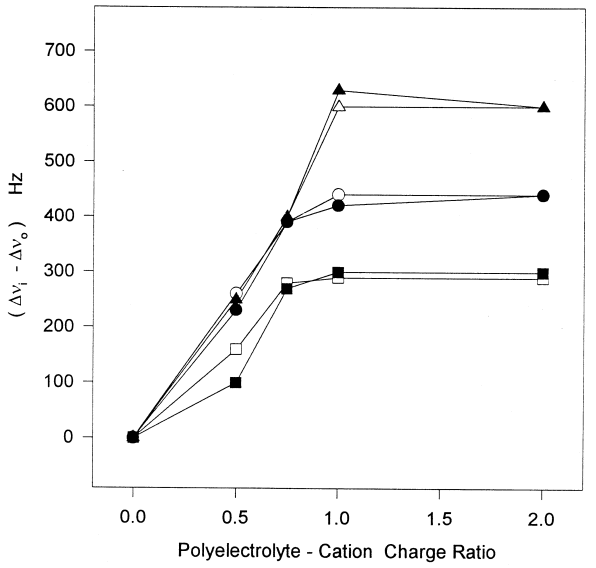

Fig. 5. ^{2}H-NMR quadrupolar splittings of mixed cationic amphiphile + POPC lipid bilayers as a function of the amount of added polyelectrolyte: CTAB-γ-d_9 + POPC (10/90) (triangles), DOTAP-γ-d_3 + POPC (30/70) (circles), TC-CHOL-γ-d_3 + POPC (30/70) (squares). Open symbols refer to the addition of PolyA while closed symbols refer to the addition of PACA. The quadrupolar splittings are plotted as the difference between the values measured for a given mixture of the cationic membranes with polyelectrolyte and the value measured in the absence of polyelectrolyte.

of choline-deuterated POPC, the quadrupolar splittings respond very sensitively to the local surface electrostatic charge, a quantity which is vastly different in polyelectrolyte-free and polyelectrolyte-bound environments. In the case of quaternary methyl-deuterated cationic amphiphiles, the quadrupolar splittings reflect primarily local ordering and configurational effects. These do not appear to vary widely between polyelectrolyte-free and polyelectrolyte-bound environments, according to the data in Fig. 3 and Table 1.

Adding progressively increasing amounts of PolyA or PACA produces a progressive increase in the quadrupolar splitting for all the cationic amphiphiles, as shown in Fig. 5. In all three cases no further change is observed beyond a polyelectrolyte anion:amphiphile cation ratio of 1:1. The absolute change in quadrupolar splitting at apparent saturation decreases in the order: CTAB > DOTAP > TC-CHOL. These results suggest the formation of a 1:1 stoichiometric complex between cationic and anionic charged groups, as deduced previously from studies with deuterated POPC [21,22]. TC-CHOL is an exception to this generalization, as will be discussed further shortly.

Fig. 5 also shows that PolyA and PACA have virtually identical effects on the quadrupolar splitting of each cationic amphiphile, implying that the precise structure of the polyelectrolyte is less important than the fact of its charge, as one might expect given that electrostatics is the dominant force. In a similar vein, Buser et al. [33] report that polyelectrolyte binding is essentially independent of the chemical nature of lipids and polyelectrolytes.

Another potential means of resolving polyelectrolyte-bound and polyelectrolyte-free populations of cationic amphiphiles is to examine their relaxation behavior. Fig. 6 shows the type of T_2^{qe} intensity decays obtained with DOTAP-γ-d_3+POPC bilayers to which have been added various amounts of PolyA. In all cases, a single exponential suffices to describe the decay, within the limits of experimental error. Saturating amounts of PolyA cause T_2^{qe} to roughly double relative to the value obtained in its absence. Even when only 50% of the initial cationic charge is

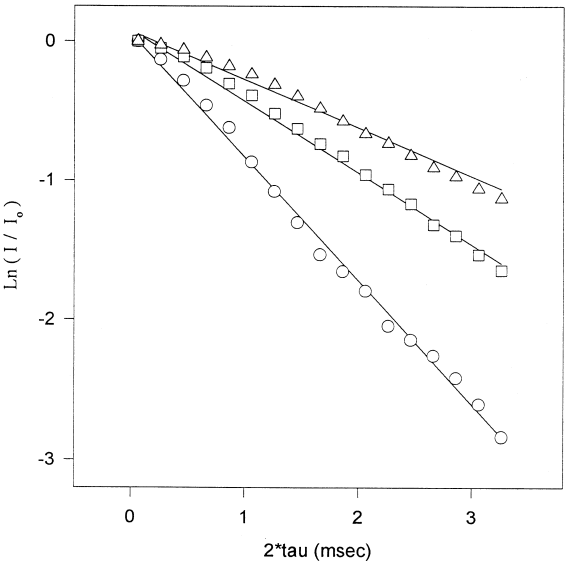

Fig. 6. T_2^{qe} echo intensity decay curves plotted as the normalized intensity at the top of the echo versus 2τ. The example shown here is for a mixture of DOTAP-γ-d_3 + POPC (30/70) with and without PolyA. The linear decay plots result from MLVs formed with the above ratio in the absence of PolyA (circles), in the presence of 50% neutralizing charge from the addition of PolyA (squares) and in the presence of a 100% added neutralizing charge from PolyA (triangles).

neutralized by added PolyA, one still observes only a single exponential decay of the echo intensity, and the value of the T_2^{qe} falls approximately halfway between the values measured in the absence versus the presence of saturating PolyA. We conclude that there is not a sufficient difference in relaxation rate between the PolyA-bound and PolyA-free environments to permit resolution of two components in the intensity decays.

Similar increases in T_2^{qe} are observed when PolyA is added to either DOTAP-γ-d_3, or TC-CHOL-γ-d_3, or CTAB-γ-d_9. Details are provided in Table 2. It is interesting that no measurable difference in the longitudinal relaxation time T_1 could be obtained upon PolyA addition. Similar trends have been reported previously. Crowell and Macdonald [24], for instance, observed that the T_2^{qe} of choline-deuterated POPC increases when trapped within polyelectrolyte-bound domains. Reinl and Bayerl [30] reported that electrostatic association of phosphatidylglycerol with myelin basic protein increases T_2 while leaving T_1

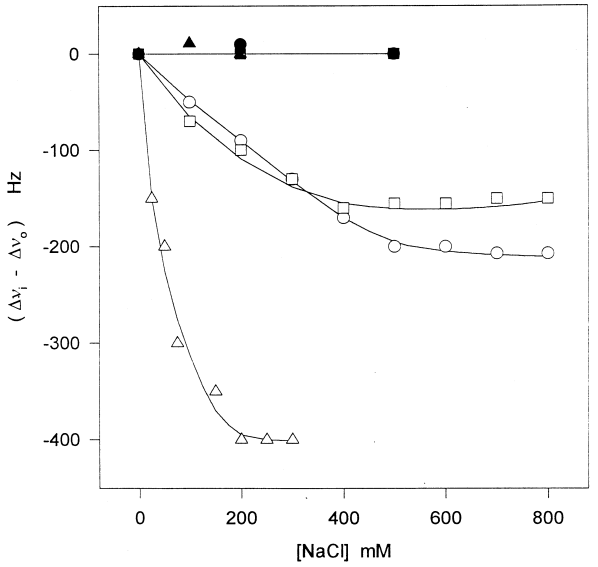

Fig. 7. ^{2}H-NMR quadrupolar splittings resulting from the addition of NaCl to mixtures consisting of CTAB-γ-d_9 + POPC (10/90) (triangles), DOTAP-γ-d_3 + POPC (30/70) (circles), and TC-CHOL-γ-d_3 + POPC (30/70) (squares). Closed symbols refer to the addition of NaCl to these lipidic samples in the absence of PolyA and the open symbols correspond to the addition of salt to 1:1 charge complexes of the cationic lipid mixtures + PolyA. The quadrupolar splittings are plotted as the difference between the value measured when PolyA is added in a 1:1 charge ratio to the cationic amphiphile with salt and the value measured for the same situation in the absence of salt.

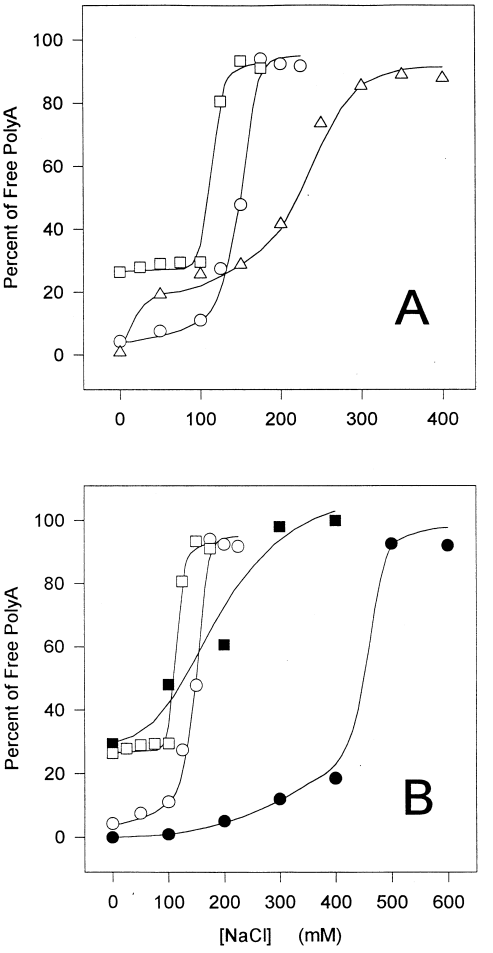

Fig. 8. Ultraviolet (UV) difference binding assay of the effects of salt on PolyA binding to lipid bilayers containing cationic amphiphiles. Each of the preparations contained a 1:1 ratio of anionic charge from PolyA to the cationic charge from the particular amphiphile. (A) CTAB + POPC (10/90) (triangles), DOTAP + POPC (10/90) (circles), and TC-CHOL + POPC (10/90) (squares). (B) DOTAP + POPC (10/90) (open circles) and (30/70) (closed circles), TC-CHOL + POPC (10/90) (open squares) and (30/70) (closed squares).

unchanged. This would indicate that the changes in relaxation times produced by polyelectrolytes are due to changes in the spectral density of slow motions, such as lateral diffusion or bilayer ensemble fluctuations.

The fact that both the quadrupolar splitting and the T_2^{qe} increase upon addition of polyA suggests that a major effect of coupling with PolyA is to increase the orientational order of the cationic amphiphile's head group. PolyA is believed to intercalate between the membrane lipids [34], where its size, rel-

ative rigidity, and electrostatic interaction could all contribute to a decrease in the amplitude of motion of the cationic amphiphile head group. The resulting increase in the orientational order parameter would be manifest in both the quadrupolar splitting and T_2^{qe} [35].

Another potential source of the longer T_2^{qe} relaxation times of cationic amphiphiles in the presence of PolyA is slower lateral diffusion within the plane of the membrane. Reinl and Bayerl [30] have demonstrated that a reduction of the lipid lateral diffusion coefficient by one order of magnitude leads to a two-fold increase in T_2, which, coincidentally, is more or less the effect we observe in the presence of PolyA. Furthermore, on the basis of Monte Carlo simulations Pink et al. [36] have predicted that lateral diffusion of lipids will decrease upon interacting electrostatically with oppositely charged proteins. Therefore, both electrostatic binding to the PolyA anionic sites, and the archipelago effect [37], in which the tortuosity of the diffusion path increases due to the presence of polyelectrolyte, should tend to decrease the diffusion coefficient of the bound cationic amphiphile.

3.3. *Effect of ionic strength on 1:1 cationic amphiphile-PolyA complexes*

The effect of salt on the ^{2}H-NMR spectra of the deuterated cationic amphiphiles in the presence of PolyA is shown in the bottom row of spectra in Fig. 4. These spectra resulted from adding salt to the 1:1 cationic amphiphile-PolyA mixtures shown in the spectra directly above them. For the particular spectra in Fig. 4, in the case of 10/90 (mol/mol) CTAB-γ-d_9/POPC the NaCl concentration was 250 mM, while for 30/70 (mol/mol) DOTAP-γ-d_3/POPC and TC-CHOL-γ-d_3/POPC, the concentration of NaCl was 800 mM. The immediate effect of adding salt is to reduce the size of the quadrupolar splitting, although not to the extent that it returns to the value measured in the absence of PolyA (compare the top and bottom rows of spectra in Fig. 4).

The spectrum of CTAB-γ-d_9 in the presence of PolyA plus 250 mM NaCl shows an additional isotropic resonance, consistent with the presence of isotropically mobile amphiphile. The intensity of this resonance accounts for 6–8% of the total, and it is observed whenever PolyA and high salt are present together. No other cationic amphiphile shows such a resonance under any conditions. This resonance might be due to CTAB which has been removed from the lipid bilayer by PolyA.

Progressively higher salt concentrations lead to a progressive decrease in the quadrupolar splittings towards their values in the absence of PolyA, as shown in Fig. 7. The response is immediate and approximately linear with salt concentration, until eventually leveling off. In the absence of PolyA, added salt has absolutely no effect on the quadrupolar splittings of the deuterated cationic amphiphiles. This means that this salt effect can be attributed solely to its influence on the polyelectrolyte interaction with the membrane surface. Since added salt reverses the initial PolyA effect, it is plain that salt is screening the electrostatic attraction between PolyA and the cationic membrane surface.

The largest absolute salt effect is measured with CTAB-γ-d_9 while the smallest is obtained with TC-CHOL-γ-d_3. This reflects the fact that CTAB-γ-d_9 is initially most sensitive to PolyA, while TC-CHOL-γ-d_3 is least. However, the salt concentration required to achieve the maximum reversal of the original PolyA effect is greater for DOTAP-γ-d_3 than for

Table 2
^{2}H-NMR T_2^{qe} relaxation times for POPC + deuterated cationic amphiphile + PolyA + salt

Anion/cation[a]	90/10 POPC/CTAB-γ-d_9 (ms)	70/30 POPC/DOTAP-γ-d_3 (ms)	70/30 POPC/TC-CHOL-γ-d_3 (ms)
0	0.65	1.13	1.54
0.5	0.53	1.86	2.00
1.0	0.81	2.46	3.32
1.0+250 mM NaCl	0.66	–	–
1.0+800 mM NaCl	–	1.29	1.07

CTAB-γ-d_9 or TC-CHOL-γ-d_3. Keeping in mind that CTAB is present only at 10 mole%, as opposed to the 30 mole% levels for DOTAP and TC-CHOL, these results indicate an order of strength of interaction between the cationic amphiphile and the PolyA as follows: CTAB > DOTAP > TC-CHOL.

A direct measure of the amount of bound polyelectrolyte is obtained via the UV difference binding assay described in Section 2. Fig. 8 displays the results obtained for PolyA binding to membranes containing one of the three different cationic amphiphiles, as a function of the salt concentration in the medium. In Fig. 8A each of the cationic amphiphiles was present at the level of 10%, which eliminates differences due solely to initial differences in surface charge density. PolyA was always added in an amount sufficient to neutralize the initial surface charge if 100% binding occurs. Each case shows a similar sigmoidal increase in the amount of free PolyA with increasing salt concentration. Evidently, the screening effect of salt on both the membrane surface charge and the polyelectrolyte charge decreases their mutual electrostatic attraction to the point that binding of the polyelectrolyte to the membrane surface no longer occurs. One sees further that the amount of salt required to desorb PolyA decreases in the order CTAB > DOTAP > TC-CHOL. Evidently, TC-CHOL binds PolyA with less affinity than does CTAB. This point is also evident from the fact that even at very low salt concentrations, not all added PolyA is adsorbed to membranes containing TC-CHOL.

Similar findings have been reported for the MARCKS protein which desorbs from vesicles composed of 20% acidic lipids at NaCl concentrations between 0.1 M and 0.5 M [38]. Mosior and McLaughlin [39] have shown that the amount of bound polyelectrolyte (polylysine) depends sigmoidally on the amount of oppositely charged amphiphile (phosphatidylglycerol). This is a manifestation of the cooperative nature of the electrostatic binding of a multivalent molecule [33]. By the same token, the screening effects of increasing salt concentration, which lead to desorption of bound polyelectrolyte, can be expected to display a sigmoidal dependence in reversing the cooperative binding.

In Fig. 8B we compare the effects of added salt on PolyA binding to lipid bilayers containing either TC-CHOL or DOTAP at 10% versus 30%. It is not possible to make this comparison with CTAB since higher amounts of surfactant solubilize the lipid bilayers. One observes the same sigmoidal dependence of PolyA binding on the concentration of added salt, but the curves shift to a higher salt concentration at a higher surface charge density. This is in accord with one's expectations for higher surface charge densities. However, the difference between TC-CHOL and DOTAP is now magnified, to the extent that DOTAP clearly binds PolyA with higher affinity than does TC-CHOL, since a higher salt concentration is required to screen their mutual electrostatic attraction.

It is interesting to contrast the near linear dependence of the ^{2}H-NMR quadrupolar splittings on salt concentration with the sigmoidal dependence displayed in the UV difference binding assay results. The UV difference assay monitors the desorption of entire polyelectrolyte molecules and, consequently, cooperativity of binding and of desorption plays a prominent role. The ^{2}H-NMR results, however, monitor events at the level of individual amphiphiles and/or monomer segments. Increasing salt concentration would be expected to decrease the affinity per pairwise interaction, producing incremental changes capable of being sensed at the level of individual amphiphiles via ^{2}H-NMR. Only when the incremental changes reach a critical level does the entire polyelectrolyte desorb and appear in the supernatant where it can be measured via UV spectrophotometry.

4. Discussion

This study was aimed at characterizing and differentiating aspects of the behavior of several cationic amphiphiles, when present in lipid bilayer membranes, and when interacting with anionic polyelectrolytes such as DNA. In their role as agents of gene transfection, cationic amphiphiles must fulfill two apparently conflicting requirements. They must bind DNA with sufficient avidity that charge neutralization and size reduction are achieved to the extent that the energy barrier to transmembrane transport of the genetic material substantially decreases. Strong binding of cationic amphiphiles ensures that DNA 'packaging' is optimal, in that the maximum possible

amount of DNA is maximally compacted within an overall cationic 'package'. But the cationic amphiphiles must not bind DNA irreversibly, or the genetic material could fail to become utilized by the cell, despite the assistance of factors such as the endosomal pH, anionic membrane lipids, or physiological salt concentrations, in dissociating the complexes. Thus, fine tuning of the strength of interaction between the cationic amphiphile and the DNA would appear to offer a route towards increased efficiency of transfection.

The binding assay results reported here demonstrate that there exist quantitative differences in the strength of interaction between DNA and CTAB versus DOTAP versus TC-CHOL. TC-CHOL, in particular, exhibits a lower affinity for DNA and a lower salt threshold at which electrostatic screening inhibits DNA binding. Of the three cationics examined, CTAB appears to bind DNA with the greatest avidity, in that it is most resistant to the effects of salt.

The ^{2}H-NMR results reported here demonstrate that the consequences of electrostatic binding to DNA for the quadrupolar splitting and the transverse relaxation time T_2^{qe} of methyl deuterons attached to the cationic amphiphile's head group are qualitatively similar for all three cationics. DNA binding always increases the quadrupolar splitting as well as the transverse relaxation time, and these effects saturate at a 1:1 anion:cation ratio. The increase in the quadrupolar splittings and transverse relaxation times may be understood to arise from an increase in local orientational order and/or a decrease in whole molecule lateral diffusion, such as might reasonably be expected to accompany complexation with a macro-ion like DNA. The 1:1 charge stoichiometry in the complex reflects the predominantly electrostatic basis of the binding, and is a general feature demonstrable via any number of different techniques [21,40,41].

Quantitatively, however, the effects of DNA binding on the quadrupolar splittings and the transverse relaxation times of the cationic amphiphile's quaternary methyl deuterons fall off in the order CTAB > DOTAP > TC-CHOL. Evidently, the size of these DNA-induced effects correlates with the strength of DNA binding. Our previous ^{2}H-NMR study of domain formation induced by DNA in lipid bilayers containing one of these three (or closely related) cationic amphiphiles yielded evidence that CTAB produced the most compact domains having the greatest enrichment with cationic amphiphile, while DC-CHOL produced the least compact domains having the least enrichment [21]. It is plausible to suppose that more compact domains, containing a greater enrichment with cationic amphiphile, would produce quantitatively larger effects on the cationic amphiphile's orientational order and lateral diffusion. Thus, these two ^{2}H-NMR perspectives, one from choline-deuterated phosphatidylcholine [21] and the other from quaternary methyl-deuterated cationic amphiphiles, both point to the conclusion that the strength of interaction of these three cationic amphiphiles with DNA decreases in the order CTAB > DOTAP > TC-CHOL.

The physical basis for this difference in the strength of the interaction of the three cationics with DNA is less clear. One possibility is that, for monovalent amphiphiles, the strength of electrostatic binding by polyelectrolytes is greater when the amphiphile's charge moiety is located deeper within the polar region of the lipid bilayer surface. Several lines of evidence indicate that, of the three amphiphiles investigated here, the cationic charge of CTAB penetrates most deeply into the bilayer's polar region, while that of TC-CHOL extends furthest into the aqueous surroundings. First, the quaternary methyl group of CTAB is located at the immediate boundary between the hydrophobic and hydrophilic regions of the molecule, while for DOTAP and TC-CHOL the cationic group is displaced away from this boundary. Second, the larger quadrupolar splitting of membrane-bound quaternary methyl-deuterated CTAB, relative to DOTAP or TC-CHOL, indicates a greater local orientational order, consistent with a greater depth of penetration. Third, the sensitivity of the phosphatidylcholine 'molecular voltmeter' to these three cationic amphiphiles decreases in the order CTAB > DOTAP > TC-CHOL [21]. Such differences are interpreted as arising from different equilibrium depths of penetration of the charged group into the membrane proper [42]. Specifically, the greater the depth of penetration, the more sensitive the calibrated response of the quadrupolar splitting from choline-deuterated phosphatidylcholine to a given surface charge density.

For a polyelectrolyte, like DNA, electrostatic binding to a charged surface implies some loss of waters of hydration of the charged moieties. It may be that this occurs more readily when the targeted charges are located in the lower dielectric constant medium of the lipid bilayer's polar region, lower, that is, relative to the aqueous medium itself. For multivalent cationic amphiphiles, where DNA binding is strong [43] despite the separation of the charges from the membrane surface, this effect may be compensated by multiple charge-charge contacts.

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