Polylysine-Induced ^{2}H NMR-Observable Domains in Phosphatidylserine/Phosphatidylcholine Lipid Bilayers

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ABSTRACT The interaction of three polylysines, Lys_5 ($N = 5$), Lys_{30} ($N = 30$), and Lys_{100} ($N = 100$), where N is the number of lysine residues per chain, with phosphatidylserine-containing lipid bilayer membranes was investigated using ^{2}H NMR spectroscopy. Lys_{30} and Lys_{100} added to multilamellar vesicles composed of (70:30) (mol:mol) mixtures of choline-deuterated 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) + 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS) produced two resolvable ^{2}H NMR spectral components under conditions of low ionic strength and for cases where the global anionic lipid charge was in excess over the global cationic polypeptide charge. The intensities and quadrupolar splittings of the two spectral components were consistent with the existence of polylysine-bound domains enriched in POPS, in coexistence with polylysine-free domains depleted in POPS. Lys_5, however, yielded no ^{2}H NMR resolvable domains. Increasing ionic strength caused domains to become diffuse and eventually dissipate entirely. At physiological salt concentrations, only Lys_{100} yielded ^{2}H NMR-resolvable domains. Therefore, under physiological conditions of ionic strength, pH, and anionic lipid bilayer content, and in the absence of other, e.g., hydrophobic, contributions to the binding free energy, the minimum number of lysine residues sufficient to produce spectroscopically resolvable POPS-enriched domains on the ^{2}H NMR millisecond timescale may be fewer than 100, but is certainly greater than 30.

INTRODUCTION

An abiding question in membrane science concerns the minimum protein charge density sufficient to induce electrostatic domain formation, given a particular complementary membrane surface charge density. A domain is defined as a region of the membrane having a distinct lipid and/or protein composition, and having significant size and duration. Domains are known to influence membrane functioning, as reviewed extensively in a series of recent articles (Edidin, 1992; Glaser, 1992; Jesaitis, 1992; Thompson et al., 1992; Vaz, 1992; Wolf, 1992; Tocanne et al., 1994; Raudino, 1995).

Theory informs us that domains are produced when electrostatic attraction draws relatively mobile acidic phospholipids toward a relatively immobile membrane-bound basic peptide or protein (Denisov et al., 1998; May et al., 2000). The protein's cationic amino acid residues, like lysine or arginine, are often clustered together in one region of the protein. Theory indicates, further, that the most highly compositionally differentiated domains are produced when a highly charged protein binds to a weakly charged membrane at far below saturating levels of binding (May et al., 2000). Another key point is that domains are predicted to dissipate when the global cationic charge from the protein is present in excess over the global anionic charge from the bilayer lipids.

Experimentally, domains have been observed using fluorescence digital imaging microscopy (Luan et al., 1995; Denisov et al., 1998), fluorescence recovery after photobleaching (FRAP) (Jovin and Vaz, 1989; Polozova and Litman, 2000), fluorescence spectroscopy (Davenport et al., 1989), fluorescence resonance energy transfer (FRET) (Stillwell et al., 2000), electron spin resonance (ESR) spectroscopy (Zachowski and Devaux, 1983), atomic force microscopy (Gliss et al., 1998), vibrational spectroscopy (Mendelsohn and Moore, 1998), and ^{2}H NMR spectroscopy (Mitrakos and Macdonald, 1996, 1997, 2000; Crowell and Macdonald, 1997, 1998). Few of these techniques are capable of defining domain composition. Consequently, few of the predictions of theories of domain formation have been tested rigorously.

^{2}H NMR of choline-deuterated phosphatidylcholine is one technique capable of observing domains and defining domain compositions, as demonstrated with a series of synthetic polyelectrolytes, homopolynucleic acids, and homopolypeptides (Macdonald et al., 1998, 2000). These ^{2}H NMR studies confirm many predictions of the theory of domain formation and provide certain guidelines for conditions under which domain formation can be observed in ^{2}H NMR spectra. Although other factors are important, first, and foremost, is the stricture that in order to observe domains there must be a global excess of lipid bilayer surface charge over added polyelectrolyte charge, in accord with theory (Denisov et al., 1998; May et al., 2000). Otherwise, the bilayer surface becomes homogeneously covered with polyelectrolyte, so that no regions of distinct composition can exist.

The essentially identical ^{2}H NMR technique has been used in many laboratories to great advantage in examining peptide and protein interactions with charged lipid bilayers. Examples include pentalysine (Roux et al., 1988), $K_2GL_{20}K_2A$, which is a synthetic membrane-spanning pep-

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tide capped at either end with a pair of lysines (Roux et al., 1989), the MARCKS (151–175) effector region peptide (Victor et al., 1999), and PMP1 (18–36), a yeast plasma membrane protein fragment rich in arginine (Roux et al., 2000). No ^{2}H NMR evidence of domain formation was reported in any of these studies. However, in all of these cases the peptide or protein charge was present in excess over the lipid charge. Therefore, the question of whether such cationic peptides are capable of electrostatic induction of ^{2}H NMR-observable domains in bilayers containing anionic amphiphiles remains indeterminate.

Polylysine is often used as a proxy for highly charged proteins, because it binds avidly to acidic phospholipid bilayers (Hartmann and Galla, 1978; de Kruijff et al., 1985; Kim et al., 1991; Ben-Tal et al., 1996; Kleinschmidt and Marsh, 1997). Long polylysines (MW $>$ 14,000) in particular bind tightly to the bilayer surface (Carrier and Pézolet, 1986; Laroche et al., 1988) and induce domain formation via lateral segregation of acidic lipids from mixtures with phosphatidylcholine, as demonstrated using Raman, fluorescence, and ESR spectroscopies (Hartmann and Galla, 1978; de Kruijff et al., 1985; Carrier and Pézolet, 1986; Laroche et al., 1988). However, shorter polylysines, such as pentalysine or tetralysine, bind only atmospherically to lipid bilayers; which is to say that their binding is superficial with little or no penetration into the bilayer (Roux et al., 1988). Such oligolysines have never been reported to induce lateral domain formation. It is clear, therefore, that there is some minimal length requirement for polylysine-induced domain formation, but it is not clear what that minimal length might be. This is significant because oligolysines resemble the charged regions of many membrane-associating peptides and proteins in terms of their absolute number of cationic charges. Where they differ is in an oligolysine's complete lack of hydrophobic amino acids or hydrophobic modifications.

Here we examine the relationship between polylysine length and the ability to induce domains in mixed phosphatidylserine plus phosphatidylcholine bilayers as determined using ^{2}H NMR of choline-deuterated phosphatidylcholine. Three polylysines, of length $N = 5$, 30, and 100 lysine units, are examined under circumstances in which there is an excess of lipidic over peptidic charge, so that conditions for domain formation are most propitious. We demonstrate that when this guideline is adhered to, under conditions of physiological ionic strength, pH, and surface charge density, only the longest of the polylysines investigated produces ^{2}H NMR-observable domains.

MATERIALS AND METHODS

Materials

Nondeuterated 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoric acid (POPA), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS) were purchased from Avanti Polar Lipids (Alabaster, AL). Sodium acetate, tris (hydroxymethyl) aminomethane (Tris), 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS), and ^{2}H-depleted water were purchased from Sigma-Aldrich, Canada (Oakville, Ontario, Canada). Pentalysine (Lys_5), poly(L-lysine) (MW = 7,300; $N = 30$) (Lys_{30}) and poly(L-lysine) (MW = 20,000; $N = 100$) (Lys_{100}) were also obtained from Sigma-Aldrich, Canada.

Synthesis of choline-deuterated phosphatidylcholine

POPC, specifically deuterated at either the choline α (POPC-α-d_2) or β (POPC-β-d_2) methylene segment, was synthesized as described previously by Marassi et al. (1993). The purity of the synthesized lipids was confirmed by thin layer chromatography and by ^{1}H, ^{2}H, and ^{31}P NMR.

Preparation of MLVs

The desired molar composition of POPC (70 mol %) plus POPG or POPS (30 mol %) was attained by mixing appropriate volumes of chloroform/methanol lipid stock solutions, followed by removal of solvent under a stream of nitrogen and overnight desiccation under vacuum. The dried lipids were dispersed in Lys_5-, Lys_{30}-, or Lys_{100}-containing buffer (typically 40 mM Tris, pH 7.4) and the mixtures were vortexed and freeze-thawed in liquid nitrogen five times. The resulting multilamellar vesicle (MLV) preparation was transferred to an NMR sample tube for measurement.

^{2}H NMR spectroscopy

^{2}H NMR spectra were recorded on a Chemagnetics CMX300 NMR spectrometer operating at 45.98 MHz, using a Chemagnetics broadline deuterium probe equipped with a 5-mm-diameter solenoid coil. The quadrupolar echo pulse sequence, (90_x - τ - 90_y - τ - *acq*) (Davis et al., 1976), was employed using quadrature detection with complete phase cycling of the pulse pairs. The 90° pulse length was 2 μs, the interpulse delay was 30 μs, the recycle delay was 100 ms, the spectral width was 50 kHz, and the data size was 2K. Quadrupolar transverse relaxation (T_2^e) times were obtained from the dependence of the signal intensity on the interpulse delay in the standard quadrupolar echo pulse sequence. All spectra were recorded at room temperature.

^{31}P NMR spectroscopy

^{31}P NMR spectra were recorded on the same Chemagnetics CMX300 solid-state NMR spectrometer operating at 121.25 MHz, using a Chemagnetics double-resonance magic-angle spinning probe but without sample spinning. The Hahn echo sequence (90_x - τ - 180_y - τ - *acq*) with complete phase cycling of the pulses and proton decoupling during acquisition was employed as described by Rance and Byrd (1983). The 90° pulse length was 6 μs, the echo spacing was 30 μs, the recycle delay was 5 s, the spectral width was 100 kHz, and the data size was 2K. All spectra were recorded at room temperature.

De-Pake-ing of ^{2}H NMR spectra

^{2}H NMR spectra were de-Paked according to Bloom and coworkers (Bloom et al., 1981; Sternin et al., 1983). The original experimental line shapes were all axially symmetric, and de-Pake-ing produced the 90° oriented-sample spectra from which the intensity and quadrupolar splitting of each spectral component could be obtained.

RESULTS AND DISCUSSION

POPC + POPS mixtures are liquid-crystalline bilayers in the presence of polylysine

Fig. 1 shows ^{31}P NMR spectra for MLVs of molar composition POPC + POPS (70:30) in 40 mM Tris (pH 7.4) with or without Lys_{30}, a polylysine ~30 lysine residues in length. There is abundant evidence that polylysine, in the course of binding to anionically charged lipid bilayers, influences the thermotropic and/or macroscopic phase behavior of lipids (Takahashi et al., 1996, and references therein). ^{31}P NMR is a recognized means of determining whether any macroscopic changes in bilayer lipid thermotropic phase or lipid morphology occur in phospholipid bilayers (Seelig, 1978; Cullis and de Kruijff, 1979).

Spectrum A in Fig. 1 corresponds to MLVs in the absence of Lys_{30}. This line shape is indicative of liquid-crystalline lipids in a bilayer arrangement. The residual chemical shift anisotropy (csa) of −39 ppm, corresponding to the frequency difference between the upfield and downfield shoulders in the spectrum, is typical of lipid bilayers containing mixtures of POPC plus an anionic lipid (Wohlgemuth et al., 1980; Tamm and Seelig, 1983).

Spectra B and C demonstrate that the overall bilayer-type ^{31}P NMR line shape is retained upon addition of Lys_{30} in lys/ser charge ratios of 0.625 and 2.0, respectively. Hence, under these circumstances polylysine does not induce any nonbilayer lipid architecture such as the hexagonal H_{II}, isotropic, or cubic phases. Nor do the ^{31}P NMR spectra show any indication of a Lys_{30}-induced conversion of lipids from the liquid-crystalline to a gel state, although an increase in T_m to a temperature below room temperature cannot be excluded. The gel state is characterized by restricted molecular mobility, which is manifest as broadening of the ^{31}P NMR spectra. In the presence of excess Lys_{30} (relative to POPS charges), there is an increase in the magnitude of the csa to −46 ppm, a value typical of a neutralized membrane surface (Tamm and Seelig, 1983). Similar ^{31}P NMR results are obtained when anionic phospholipid bilayers consisting of mixtures of POPC with POPG are exposed to an excess of the cationic polyelectrolyte poly(vinylbenzyltrimethylammonium chloride) (PVTA) (Crowell and Macdonald, 1998).

There is no evidence in the ^{31}P NMR spectra of the presence of Lys_{30}-induced domains. However, for several reasons, ^{31}P NMR spectroscopy is a less-than-ideal means for detecting electrostatically induced domains, and particularly so under the current circumstances. First, the ^{31}P NMR csa of POPC is less sensitive to surface charge, by nearly a factor of 10 in absolute terms, than the ^{2}H NMR quadrupolar splitting from POPC-α-d_2 or POPC-β-d_2 (Scherer and Seelig, 1989). Second, anions in general produce a spectroscopic response that is weaker by roughly a factor of 2 relative to the response produced by cations (Seelig et al., 1987; Scherer and Seelig, 1989; Beschiaschvili and Seelig, 1990), whereas POPS in particular produces one of the weakest responses of any anionic phospholipid, as demonstrated below. (The sensitivity to POPS is relevant because the NMR domain-detection techniques used here will report on inhomogeneities in POPS distribution induced by polylysine (Mitrakos and Macdonald, 1996, 1997, 2000; Crowell and Macdonald, 1997, 1998).) Third, the ^{31}P NMR spectra in Fig. 1 consist of a superposition of subspectra contributed by POPC and POPS, two phospholipids that do not differ greatly in either isotropic or anisotropic chemical shifts, as is obvious from the fact that separate POPC and POPS components cannot be resolved even in the absence of polylysine. If polylysine-induced domains were present, up to four overlapping subspectral components would be anticipated. Because one does not anticipate tremendous differences between these subspectra, then one cannot expect to resolve individual components. In sum-

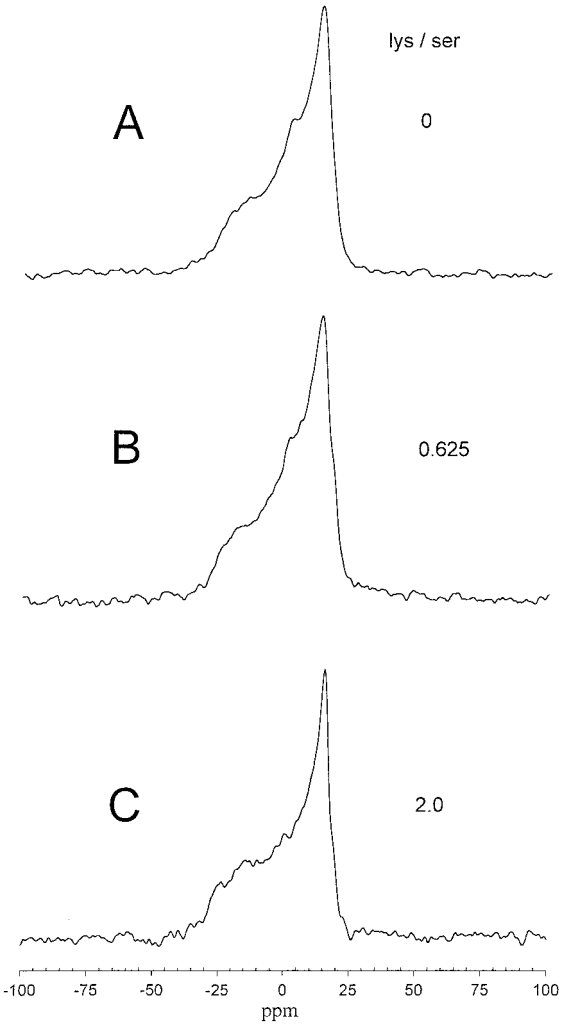

FIGURE 1 Experimental ^{31}P NMR spectra of POPC + POPS (70:30) MLVs in 40 mM Tris (pH 7.4) as a function of added Lys_{30}. (*A*) No Lys_{30}; (*B*) Lys/ser charge ratio = 0.625; (*C*) Lys/ser charge ratio = 2.

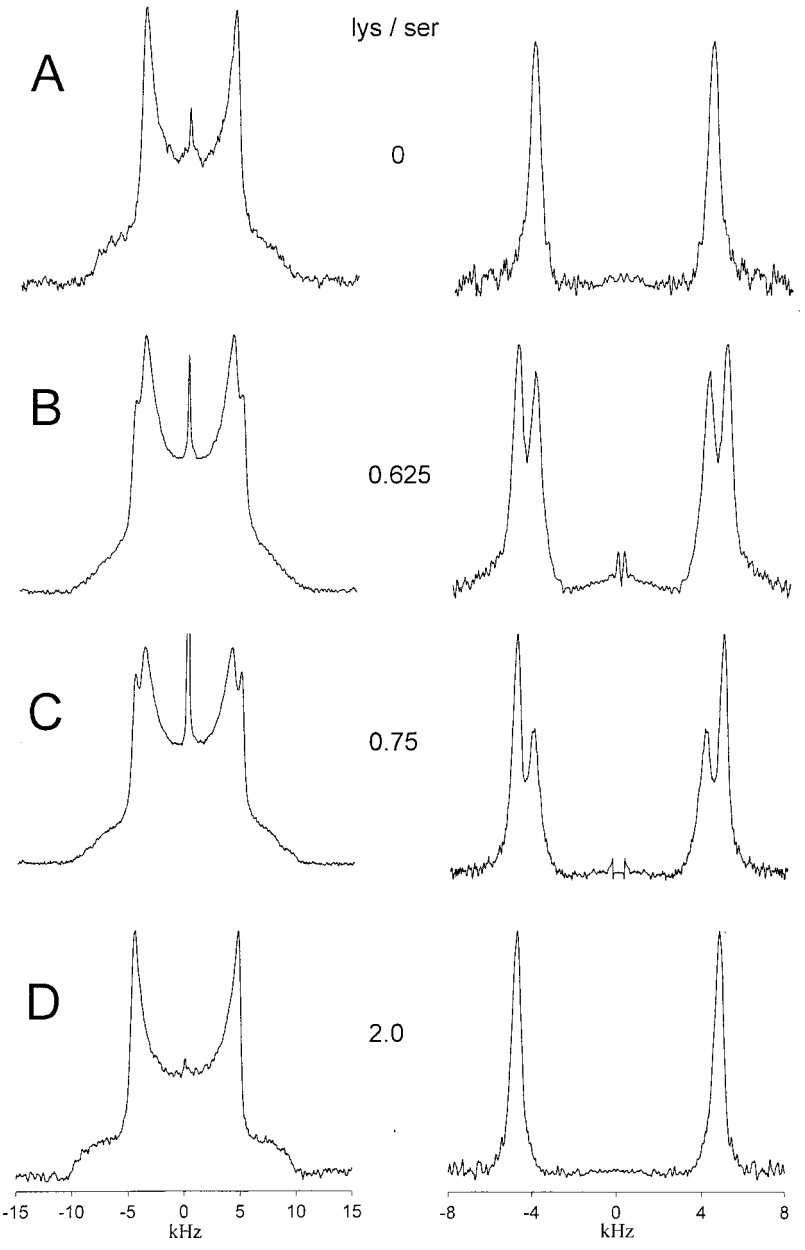

FIGURE 2 ^{2}H NMR spectra (*left*) and corresponding de-Paked spectra (*right*) of POPC-α-d_2 + POPS (70:30) MLVs in 40 mM Tris (pH 7.4) as a function of added Lys_{30}. (*A*) No Lys_{30}; (*B*) Lys/ser charge ratio = 0.625; (*C*) Lys/ser charge ratio = 0.75; (*D*) Lys/ser charge ratio = 2.0.

mary, ^{31}P NMR spectroscopy is inferior as a means to detect polylysine-induced domains in POPS + POPC mixtures. As will be shown below, ^{2}H NMR is much better suited to this task.

Polylysine induces domain formation in mixed POPC + POPS membranes

Figs. 2 and 3 show ^{2}H NMR spectra from MLVs composed of mixed POPC-α-d_2 + POPS (70:30) and POPC-β-d_2 + POPS (70:30), respectively, in 40 mM Tris (pH 7.4), with or without Lys_{30}. The experimental spectra appear in the left-hand column, whereas the corresponding de-Paked spectra appear in the right-hand column. Spectrum A in both figures was obtained in the absence of Lys_{30}. The narrow resonance at 0 Hz is due to the natural abundance of HDO. The ^{2}H NMR signal from the deuterated lipids yields a distinctive line shape consisting of a motionally averaged Pake-doublet powder pattern, typical of lipids undergoing rapid anisotropic motional averaging about their long molecular axes, as expected in a liquid-crystalline bilayer.

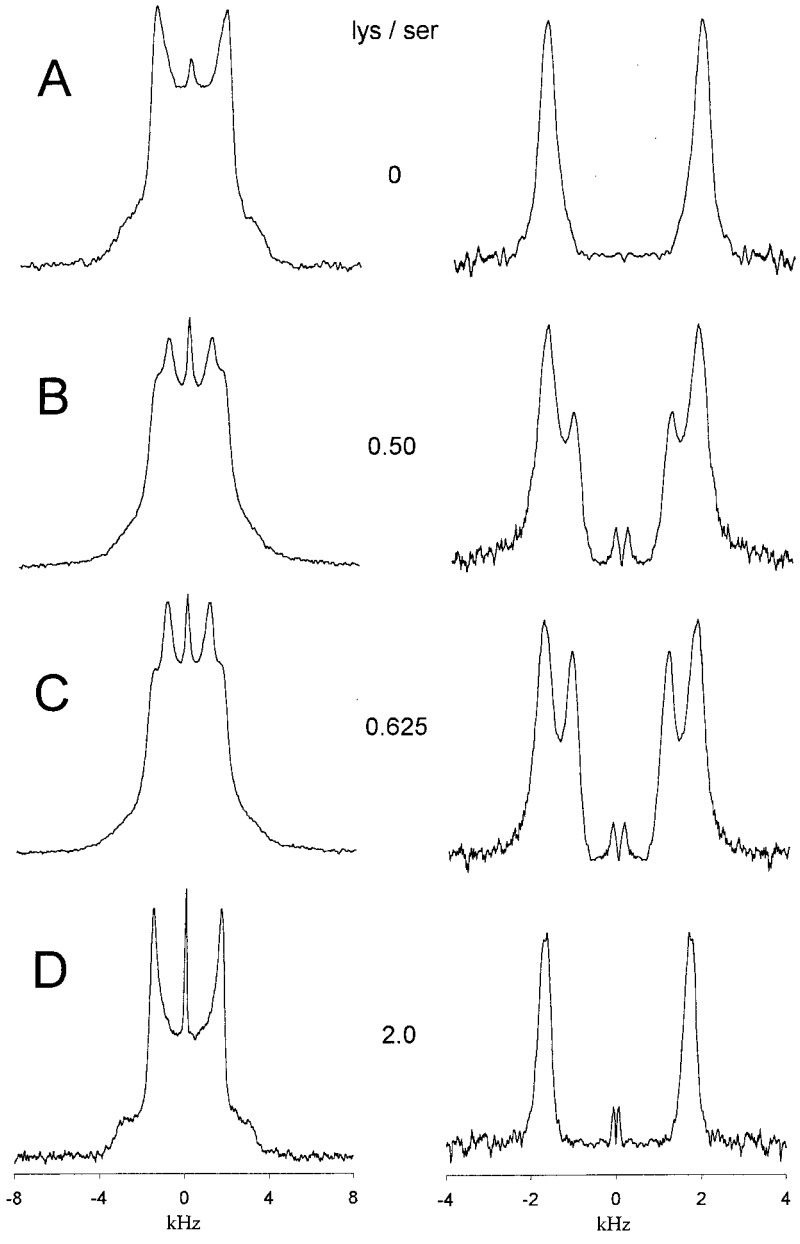

FIGURE 3 ^{2}H NMR spectra (*left*) and corresponding de-Paked spectra (*right*) of POPC-β-d_2 + POPS (70:30) MLVs in 40 mM Tris (pH 7.4) as a function of added Lys_{30}. (*A*) No Lys_{30}; (*B*) Lys/ser charge ratio = 0.50; (*C*) Lys/ser charge ratio = 0.625; (*D*) Lys/ser charge ratio = 2.0.

The quadrupolar splitting, $\Delta\nu_Q$, corresponds to the separation in hertz between the two maxima in each spectrum and equals 8.2 kHz for POPC-α-d_2 + POPS (70:30) and 3.2 kHz for POPC-β-d_2 + POPS (70:30) MLVs. The effect of POPS is to increase the quadrupolar splitting from POPC-α-d_2 and decrease that from POPC-β-d_2, relative to their values for 100% POPC MLVs (6.2 kHz and 5.1 kHz for POPC-α-d_2 and POPC-β-d_2, respectively). Fig. 4 demonstrates that this effect is progressive with increasing mole fraction of POPS. The influence of POPS on $\Delta\nu_Q$ is a manifestation of the well-known conformational response of the phosphocholine group of phosphatidylcholine to surface charge (Seelig et al., 1987), which permits one to monitor surface charge and surface charge distribution. For the case at hand, the changes in the quadrupolar splitting are those expected for an anionic surface charge, consistent with the presence of POPS. The linear relationship between the mole fraction of POPS and the quadrupolar splitting may be characterized in terms of the slope m_{PS}, which equals $+5.9$ kHz mol^{-1} for POPC-α-d_2 and -6.0 kHz mol^{-1} for POPC-β-d_2. The magnitude of such calibration slopes gives an indication of the location of the charge with

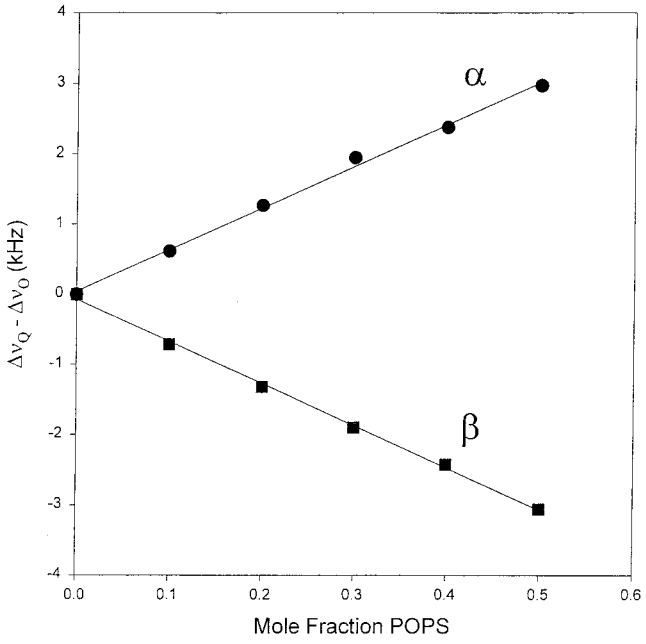

FIGURE 4 Calibration of the quadrupolar splitting of POPC-α-d_2 and POPC-β-d_2 as a function of POPS mol % in mixtures of the two. Quadrupolar splittings are reported as a difference between POPC in the presence of a particular amount of POPS ($\Delta\nu_Q$) and the control quadrupolar splitting in the absence of POPS ($\Delta\nu_O$ = 6.2 and 5.1 kHz for POPC-α-d_2 and POPC-β-d_2, respectively). All MLVs contained 40 mM Tris, pH 7.4. ●, POPC-α-d_2; ■, POPC-β-d_2. The slope m_{PS} = 5.9 kHz mol^{-1} for POPC-α-d_2 and −6.0 kHz mol^{-1} for POPC-β-d_2.

respect to the plane of the phosphocholine group (Beschiaschvili and Seelig, 1990; Rydall and Macdonald, 1992). The values obtained here for POPS are significantly smaller than those found with other anionic amphiphiles, such as POPG, and more closely resemble those reported for aqueous anions, such as thiocyanate, which cannot penetrate as deeply into the bilayer. This is consistent with the fact that the charge center for POPG is focused at the phosphate group, whereas the charge center for POPS is distributed over the phosphoserine moiety.

Spectrum A in Figs. 2 and 3 shows a single quadrupolar splitting. This indicates that, in the absence of Lys_{30}, there is a homogeneous distribution of POPS anionic charge across the entire plane of the lipid bilayer. This is a natural consequence of the rapid lateral diffusion of lipids within the plane of the bilayer. The quadrupolar splittings report on the time and ensemble average charge environment experienced by the lipids. Rapid lateral diffusion averages out any local, instantaneous compositional fluctuations so that, over the timescale of the experiment, all lipids experience the same global mean charge environment. This point is born out by the de-Paked spectra shown on the right. In the absence of Lys_{30}, the de-Paked spectra each consist of a single quadrupolar doublet, confirming that all lipids experience a uniform surface charge environment over the timescale of the experiment.

Spectra B and C in Figs. 2 and 3 were obtained upon adding Lys_{30} to POPC + POPS (70:30) MLVs at a lys/ser charge ratio less than unity. It is apparent that the ^{2}H NMR spectra consist of a superposition of two subspectra and that two quadrupolar splittings can be resolved, whether one observes POPC-α-d_2 or POPC-β-d_2. The two spectral components are most readily apparent in the corresponding de-Paked spectra. These spectra indicate, therefore, that adding Lys_{30} produces two distinct POPC populations in slow exchange with one another on a timescale defined by the inverse in the difference in their quadrupolar splittings.

Progressive additions of Lys_{30} cause one spectral component to increase in intensity at the expense of the other. Thus, one readily identifies which spectral component arises from the POPC population associated with Lys_{30} bound at the bilayer surface.

For the case of POPC-α-d_2 (Fig. 2, spectra B and C), the spectral component associated with Lys_{30} has a quadrupolar splitting greater than the control value of 8.2 kHz, whereas that of the remaining spectral component is smaller than the control value. For the case of POPC-β-d_2 (Fig. 3, spectra B and C), the opposite is true; that is, the spectral component associated with Lys_{30} has a quadrupolar splitting less than the control value of 3.2 kHz, whereas that of the other spectral component is greater than the control value. This inverse effect of Lys_{30} on the quadrupolar splittings from the two deutero-labeling positions indicates, therefore, that the two POPC populations observed in any given spectrum differ with respect to their local surface charge. Moreover, the direction of the changes in quadrupolar splitting indicate that the Lys_{30}-associated POPC population experiences an environment enriched with POPS, whereas the remaining POPC population experiences an environment depleted of POPS, relative to the global average. In other words, Lys_{30} has induced a POPS-enriched domain.

The ability to induce ^{2}H NMR-observable lipid bilayer domains is a characteristic common to a host of other polyelectrolytes upon their being added to oppositely charged lipid bilayers, whether cationic polyelectrolytes added to anionic lipid bilayers (Crowell and Macdonald, 1997, 1998) or anionic polyelectrolytes added to cationic lipid bilayers (Mitrakos and Macdonald, 1996, 1997, 2000).

When excess Lys_{30} cationic charge is added, relative to the POPS anionic charge, as shown in spectrum D in Figs. 2 and 3, the ^{2}H NMR spectra revert to single-component spectra and exhibit a quadrupolar splitting close (but not identical) to the value measured in the absence of Lys_{30}. This is consistent with both theoretical predictions regarding domain dissipation at excess polyelectrolyte charge (Denisov et al., 1998) and previous experimental observations (Crowell and Macdonald, 1997, 1998; Mitrakos and Macdonald, 1996, 1997, 2000).

TABLE 1 Lys_{30}-induced domain compositions in POPC + POPS (70:30) mixtures

		$\Delta\nu_Q$ (kHz)		Fractional composition				Lys-bound domain	
	Global Lys/Ser	Free	Bound	X^f_{PC}	X^b_{PC}	X^f_{PS}	X^b_{PS}	Lys/Ser*	Enrichment†
POPC-α-d_2									
	0	8.2		0.70		0.30			
	0.375	7.7	9.9	0.48	0.22	0.16	0.14	0.8	1.5
	0.5	7.7	9.5	0.39	0.31	0.13	0.17	0.9	1.3
	0.625	7.7	9.4	0.32	0.38	0.11	0.19	1.0	1.2
	0.75	7.8	9.4	0.29	0.41	0.11	0.19	1.2	1.1
	2.0		9.1		0.70		0.30		
POPC-β-d_2									
	0	3.2		0.70		0.30			
	0.5	3.4	2.0	0.39	0.31	0.15	0.15	1.0	1.1
	0.625	3.4	2.1	0.31	0.39	0.12	0.18	1.1	1.1
	2.0		3.1	0	0.70	0	0.30		

The uncertainty in the values of $\Delta\nu_Q$ is on the order of $\pm$0.1 kHz. The standard deviation of the slope m_{PS} is on the order of 0.06 (i.e., 1%). Error propagation analysis through Eq. 5 indicates that the error in the values of X^f_{PS} and X^b_{PS} is on the order of $\pm$0.04. Conditions were 40 mM Tris (pH 7.4), 0 mM NaCl.
*Calculated according to Eq. 5.
†Corresponds to the ratio $(X^b_{PS}/X^b_{PC})/(X^t_{PS}/X^t_{PC})$.

Quantifying domain composition from ^{2}H NMR spectra

The ^{2}H NMR spectra can be analyzed quantitatively to obtain the composition of the two domain types as well as the degree of domain separation. One begins by defining the global composition of POPC and POPS in the lipid bilayers in terms of mole fractions as per equation Eq. 1,

$$X^t_{PC} + X^t_{PS} = 1, \tag{1}$$

and then subdividing the POPC and POPS populations into those that are polylysine-bound (superscript *b*) and polylysine-free (superscript *f*) as per Eq. 2,

$$\begin{aligned} X^t_{PC} &= X^b_{PC} + X^f_{PC} \\ X^t_{PS} &= X^b_{PS} + X^f_{PS}. \end{aligned} \tag{2}$$

The values of X^f_{PC}/X^t_{PC} and X^b_{PC}/X^t_{PC} are obtained from the intensity ratios of the two components in de-Paked spectra such as shown in Figs. 2 and 3.

The POPS composition of the polylysine-free domain is obtained from the quadrupolar splitting of the corresponding ^{2}H NMR spectral component, $\Delta\nu^f$, as per Eq. 3,

$$\Delta\nu^f - \Delta\nu_0 = m_{PS}\frac{X^f_{PS}}{X^f_{PS} + X^f_{PC}}, \tag{3}$$

where m_{PS} is the slope of the calibration curve shown in Fig. 4, and $\Delta\nu_0$ is the quadrupolar splitting for 100% POPC. Values of X^f_{PS} obtained in this fashion are listed in Table 1. Note that the compositions obtained independently from POPC-α-d_2 and POPC-β-d_2 ^{2}H NMR spectra under otherwise identical conditions agree with one another. The compositions so calculated demonstrate that the polylysine-free domains are depleted in POPS relative to the global composition.

One may calculate the POPS content of the polylysine-bound domain in one of two ways. First, X^b_{PS} may be calculated by simple subtraction using Eqs. 1 and 2, because three of the four unknowns have been established. Values of X^b_{PS} calculated in this fashion are likewise listed in Table 1. The compositions indicate enrichment of POPS in the polylysine-bound domains relative to the global composition.

Alternately, X^b_{PS} may be calculated directly from the quadrupolar splitting of the polylysine-bound ^{2}H NMR spectral component. Here one is dealing with a ternary mixture of POPC + POPS + polylysine (ignoring the water and the buffer), so that the observed quadrupolar splitting is the net result of perturbations introduced by the cationic plus the anionic species (Marassi and Macdonald, 1992). In principle, to extract compositional information one requires slopes from calibration curves relating the quadrupolar splitting to the mole fraction of charged species for both POPS and polylysine independently. However, polylysine does not bind in the absence of anionic charge, so this is not possible. Instead, one must resort to judicious assumptions regarding the calibration constants.

Experience with a host of different polyelectrolytes indicates that the best choice for the polyelectrolyte ^{2}H NMR calibration constant is zero (Crowell and Macdonald, 1997, 1998; Mitrakos and Macdonald, 1996, 1997, 2000). In other words, POPC does not directly sense the presence of bound polyelectrolyte charges. Rather, they are sensed indirectly through their effect on the lateral distribution of the charged amphiphile. In the case of polylysine, it may be rationalized that its atmospheric binding does not permit a sufficient penetration of the lysine cations into the bilayer proper to be sensed directly by the choline group of POPC (Beschiaschvili and Seelig, 1990; Rydall and Macdonald, 1992).

Consequently, the POPS composition of the polylysine-bound domain may be calculated via an expression analogous to Eq. 3,

$$\Delta\nu^{b} - \Delta\nu_0 = m_{PS}\frac{X^{b}_{PS}}{X^{b}_{PS} + X^{b}_{PC}}, \quad (4)$$

where $\Delta\nu^{b}$ is the quadrupolar splitting and X^{b}_{PC} is the fraction of the ^{2}H NMR intensity assigned to the polylysine-bound spectral component. To bring the values of X^{b}_{PS} calculated by the two methods into accord with one another, it was found necessary, further, to adjust the calibrated slope m_{PS} to a value of 9.5 kHz mol^{-1} for POPC-α-d$_2$ and -9.5 kHz mol^{-1} for POPC-β-d$_2$. In this regard, polylysine behaves exactly like the cationic polyelectrolyte PVTA (Crowell and Macdonald, 1997, 1998). The increased calibration constants indicate an increased sensitivity of POPC to POPS within the polylysine-bound domain. A simple increase in overall orientational order due to polylysine binding, although likely to occur, cannot explain this effect. Instead, the fact that the sensitivity increases for both POPC-α-d$_2$ and POPC-β-d$_2$ suggests that a conformation change occurs within the POPS headgroup in response to polylysine binding, resulting in a deeper penetration of the POPS headgroup into the bilayer proper. The phosphatidylserine headgroup is in fact known to undergo a conformation change in response to surface charge in a manner analogous to that displayed by the phosphatidylcholine headgroup (Roux et al., 1989).

The number of POPS molecules within the polylysine-bound domain on a per-polylysine-chain basis is determined according to the following equation:

$$N_{POPS} = \frac{X^{b}_{PS}}{X^{t}_{PS}}\frac{N_{Lys}}{Q}, \quad (5)$$

where Q is the global polylysine cation to POPS anion charge ratio, assuming 100% protonation of the lysine residues, $X^{t}_{PS} = X^{f}_{PS} + X^{b}_{PS}$ is the global POPS mole fraction, and N_{Lys} is the length of the polylysine chain. Note that the lys/ser ratio within the polylysine-bound domain is then simply N_{Lys}/N_{POPS}. Similarly, the number of POPC molecules within the polylysine-bound domain, on a per-polylysine-chain basis, is calculated according to Eq. 6:

$$N_{POPC} = \frac{X^{b}_{PC}}{X^{t}_{PS}}\frac{N_{Lys}}{Q}. \quad (6)$$

As detailed in Tables 1, 3, 4, and 5, calculation shows that within the polylysine-bound domain the lys/ser ratio is ~1:1, assuming that Lys_{30} binding is quantitative when there is a global excess of anionic surface charge over added polylysine cationic charge. This finding has many precedents in the literature. Carrier and Pézolet (1986), for instance, concluded that there was a 1:1 stoichiometry between lysine side chains and lipidic molecules in their investigations of polylysine interactions with dipalmitoylphosphatidylglycerol bilayers. Charge neutralization within a polyelectrolyte-bound domain is commonly observed with other polyelectrolytes whether synthetic or naturally occurring (Crowell and Macdonald, 1997, 1998; Mitrakos and Macdonald, 1996, 1997, 2000), and is predicted by theories of polyelectrolyte domain induction in lipid bilayers (Denisov et al., 1998; May et al., 2000).

The degree of enrichment of POPS within the polylysine-bound domain is obtained from the ratio $(X^{b}_{PS}/X^{b}_{PC})/(X^{t}_{PS}/X^{t}_{PC})$, which is greater than unity for enrichment and less than unity for depletion. Tables 1, 3, 4, and 5 list the degrees of enrichment for the various situations investigated here. Generally, it is found that the degree of enrichment decreases with increasing polylysine load at the surface, in accord with the predictions of electrostatic domain formation theory (Denisov et al., 1998; May et al., 2000).

Limits to observation of polyelectrolyte-induced domains by ^{2}H NMR

There exist two major spectroscopic limits to the ability to observe polyelectrolyte-induced domains via ^{2}H NMR spectroscopy. First, the polyelectrolyte-free and -bound domains must differ sufficiently in composition that they produce a resolvable difference in quadrupolar splitting in the ^{2}H NMR spectrum. This will depend primarily on the sensitivity of the quadrupolar splitting to the particular charged species involved, as exemplified by the calibration constants extracted from calibration curves such as those in Fig. 4. Second, there must be sufficient signal intensity arising from a minority domain type to be observable against the background of the signal intensity from the majority domain type. This will depend largely on the spectral signal-to-noise ratio. Hence, optimal conditions for observing polyelectrolyte-induced lipid domains via ^{2}H NMR would involve an approximately equal distribution of deuterated POPC between bound and free domains, having the greatest possible difference in their composition of a charged lipid of maximum sensitivity in the ^{2}H NMR calibration experiment.

Theoretical depictions of polyelectrolyte-induced domain formation provide further guidelines for optimal domain observation (Denisov et al., 1998; May et al., 2000). First, domains will dissipate at excess polyelectrolyte versus amphiphile charge. Second, the greatest compositional difference between bound and free regions of the membrane occurs for the case of a weakly charged membrane and a highly charged polyelectrolyte at low polyelectrolyte loading.

The theoretical maximum compositional difference between domains does not necessarily coincide with the optimal spectroscopic condition for domain observation. In practice, S/N limitations rarely permit one to observe a minority domain constituting less than 20% of the total,

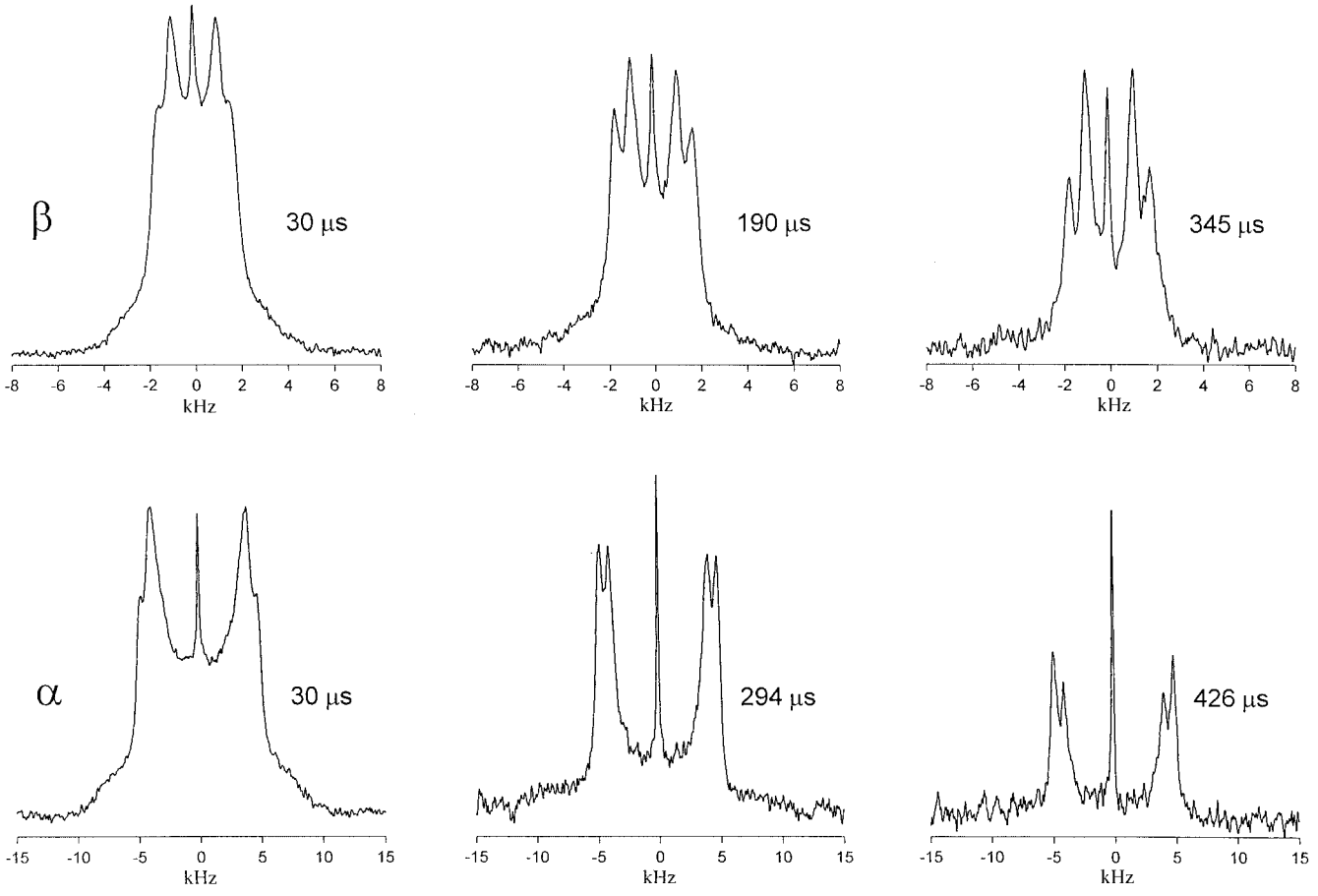

FIGURE 5 The effect of increasing the interpulse delay τ in the quadrupolar echo sequence on signal intensity and resolution in experimental ^{2}H NMR spectra of POPC + POPS (70:30) MLVs plus Lys_{30} in a lys/ser charge ratio of 0.625 (40 mM Tris, pH 7.4). The spectra in the top row are from MLVs containing POPC-β-d_2, and the bottom spectra are from MLVs containing POPC-α-d_2. (*Upper row*) τ = 30, 190, and 345 μs; (*Lower row*) τ = 30, 294, and 426 μs. The transverse relaxation times (T_2^{qe}) are listed in Table 2.

whereas differences in quadrupolar splitting of less than 1 kHz become problematic to resolve. Consequently, it is generally possible to observe domains only in lipid bilayers containing between 10 and 50 mol % charged amphiphile and for global polyelectrolyte/amphiphile charge ratios above 0.3. For the specific case of POPC + POPS + Lys_{30}, these compositional limits are narrowed further by the fact that POPS is less sensitively detected by the ^{2}H NMR technique than most other charged amphiphiles (Beschiaschvili and Seelig, 1990; Rydall and Macdonald, 1992).

Nevertheless, in marginal situations it is possible to enhance domain resolution by exploiting the fact that the spin-spin relaxation time, T_2^{qe}, is not necessarily symmetric across a ^{2}H NMR powder pattern, nor identical for the two domain types. Fig. 5 shows a series of ^{2}H NMR spectra from mixtures of composition POPC + POPS (70:30), in the presence of Lys_{30} at a global lys/ser ratio of 0.625, where the echo-spacing tau in the quadrupolar echo sequence has been increased progressively from left to right. First, because the spin-spin relaxation time is longer at the spectral horns than at the shoulders, the intensity of the horns survives longer and resolution of the two spectral components is enhanced. Second, the spin-spin relaxation time is longer in the Lys_{30}-bound than in the Lys_{30}-free domain, paralleling previous observations with a variety of polyelectrolytes (Crowell and Macdonald, 1997, 1998; Mitrakos and Macdonald, 1996, 1997, 2000). This can provide a means of enhancing the relative contribution of the polyelectrolyte-bound domain when it is in the minority, as may be ascertained from Fig. 5.

Values of T_2^{qe} as measured by this experiment are listed in Table 2 for a variety of relevant situations. In all such measurements, single-exponential decays of individual spectral components are observed. It is found that the T_2^{qe} relaxation time for POPC-α-d_2 is always shorter than that for POPC-β-d_2 and that the T_2^{qe} relaxation time for both is shorter in the presence of POPS compared with pure POPC. In the presence of polylysine, the T_2^{qe} is always longer in the polyelectrolyte-bound domain. The origin of this effect can be traced back to the influence of bound polyelectrolytes on

TABLE 2 Effect of Lys_{30} on ^{2}H NMR T_2^{qe} relaxation times of choline-deuterated POPC in POPC + POPS (70:30) mixtures

	Transverse relaxation T_2^{qe} (ms)			
	POPC-α-d_2		POPC-β-d_2	
Global Lys/Ser	Lys_{30} free	Lys_{30} bound	Lys_{30} free	Lys_{30} bound
0	0.73		1.21	
0.625	0.62	0.95	1.12	1.31
2.0		1.01		2.16

Conditions were 40 mM Tris (pH 7.4), 0 mM NaCl.

local collective bilayer oscillations and overall lipid vesicle curvature, both of which are dampened upon polyelectrolyte binding. Lateral diffusion of phospholipids around points of curvature is a major contributor to the transverse relaxation of deuterated phospholipids in membranes (Köchy and Bayerl, 1993). Our T_2^{qe} observations imply that the lipid lateral diffusion is hindered within the polyelectrolyte-bound domains, or the bound polylysine dampens the large-scale collective motions of the phospholipid ensemble, or bound polylysine increases the vesicle's radius of curvature, or some combination of the above.

Dependence of domain formation on polylysine chain length

Fig. 6 shows de-Paked ^{2}H NMR spectra from POPC-α-d_2 + POPS (70:30) MLVs (40 mM Tris, pH 7.4) in the presence of three different lengths of polylysine: Lys_5, Lys_{30}, and Lys_{100}. Two different lys/ser charge ratios were investigated as indicated in the figure. Lys_5 produces no change in the ^{2}H NMR spectrum at any concentration investigated. A single quadrupolar splitting is observed with a value identical to that measured in the absence of Lys_5. This result is consistent with the findings of Roux et al. (1988) that pentalysine does not alter the quadrupolar splittings from choline-deuterated phosphatidylcholine. However, because these researchers used amounts of pentalysine that were in a large excess with respect to serine charge, domain formation could not have been observed in any case, not that Roux et al. (1988) were seeking to observe domains. Our ^{2}H NMR measurements permit us to conclude that Lys_5 is too short to induce domains even under conditions otherwise favorable to domain formation. It is likely that this is due to the fact that pentalysine binds only atmospherically and fails to penetrate into the bilayer surface (Roux et al., 1988; Kim et al., 1991; Kleinschmidt and Marsh, 1997).

Lys_{30} and Lys_{100} both produce two-component spectra, indicating domain formation. This is in accord with previous findings that longer polylysines bind more closely to the membrane surface (Kleinschmidt and Marsh, 1997), so that some side chains penetrate into the bilayer (Hammes and Schullery, 1970; Carrier and Pézolet, 1986; Carrier et al., 1990).

The de-Paked spectra for Lys_{30} and Lys_{100} demonstrate, however, that at a given lys/ser global charge ratio, a greater proportion of the POPC is trapped within a Lys_{100}-bound domain than within a Lys_{30}-bound domain. Nevertheless, quantitative analysis reveals that in all cases the lys/ser charge ratio within the polylysine-bound domain is close to 1:1 regardless of the polylysine chain length, as detailed in Table 3. Hence, the degree of enrichment of POPS within the polylysine-bound domains decreases with increasing polylysine chain length; i.e., the domains become less compact and less differentiated in composition relative to the global average.

These compositional effects of polylysine chain length are contrary to our previous findings on chain-length effects in domains induced by the anionic polyelectrolyte polystyrenesulfonate (PSSS) in cationic lipid bilayers (Mitrakos and Macdonald, 2000). With PSSS, increasing the chain length produced more compact, more highly enriched domains.

Domain compositional differences may be understood in terms of the thermodynamics of polyelectrolyte interactions with lipid bilayers (Denisov et al., 1998). Binding and domain formation are driven largely by the favorable free energy of electrostatic attraction between the polyelectrolyte and the amphiphiles, but balanced by the unfavorable entropy of demixing charged amphiphiles into domains. The polyelectrolyte extracts an additional entropic fee arising from the loss of configurational freedom upon binding at the surface and then organizing laterally into a domain. The entropic expense of organizing laterally may be ameliorated if there is a favorable free energy of lateral interaction between polyelectrolytes. In the case of PSSS, this was rationalized to occur as a result of aromatic ring interactions. Consequently, longer chains favor lateral interactions, thereby partially offsetting the unfavorable entropy of lipid demixing and permitting a more compact, enriched domain to form.

In the case of higher-molecular-weight polylysine, there is a conformational change from a random coil to an α-helix upon binding to anionic bilayers containing phosphatidylserine (Hammes and Schullery, 1970) or phosphatidylglycerol (Carrier and Pézolet, 1986). The 1:1 lys/ser stoichiometry within the polylysine-bound domain argues in favor of the bridge model proposed by Carrier and Pézolet (1986) on the basis of small-angle x-ray diffraction results. Here, the lysine residues on one face of the α-helical polylysine interact with one bilayer surface whereas those on the opposing face interact with, and bridge to, a second neighboring bilayer. Previous ^{2}H NMR studies with synthetic polyelectrolytes indicate that such domains contain multiple polyelectrolyte chains. A potential source of inter-chain attraction for polylysine bound at the POPC + POPS surface would be the helix macrodipole (Wada, 1976), leading to either head-to-tail or side-by-side antiparallel arrangements. However, experimental studies of the statistics of cylinder stacking in two dimensions show that the porosity,

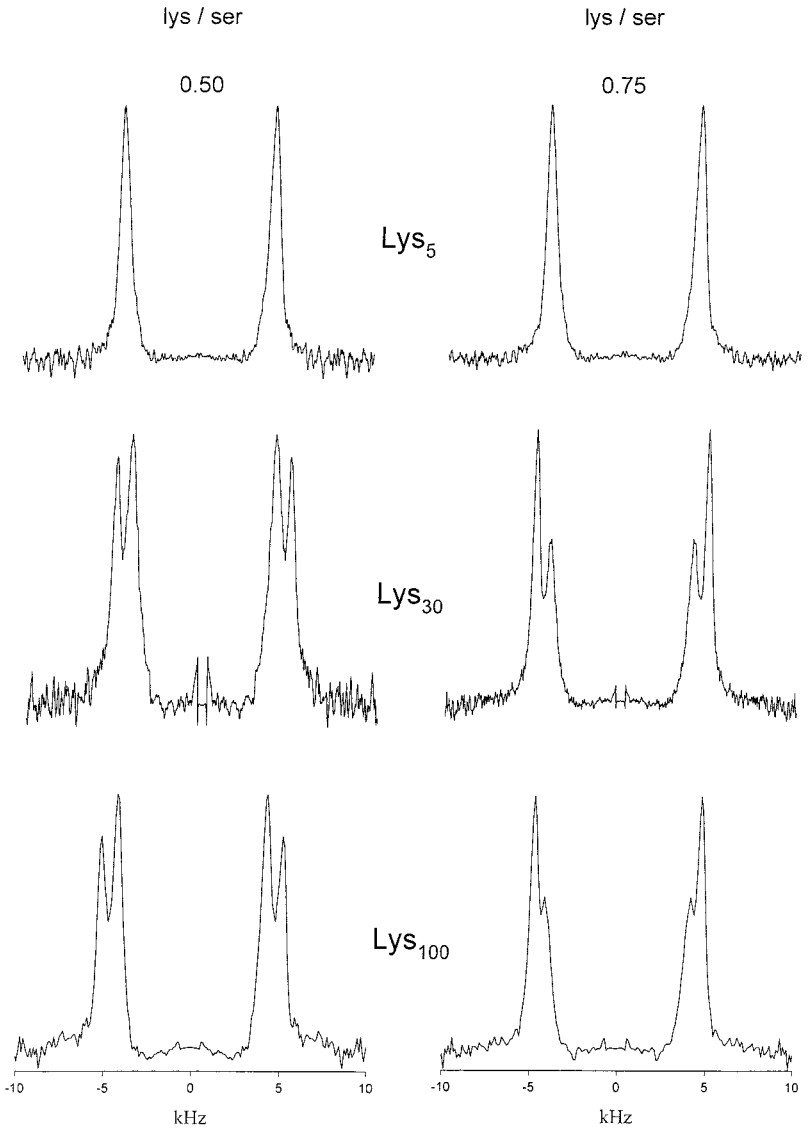

FIGURE 6 De-Paked ^{2}H NMR spectra from POPC-α-d_2 + POPS (70:30) MLVs in 40 mM Tris (pH 7.4) for different polylysine chain lengths, and particular lys/ser charge ratios, as indicated in the figure.

i.e., the space entrapped between loosely stacked cylinders, follows a porosity law derived from the excluded volume model elaborated by Onsager (1948) wherein increasing cylinder length leads to an increase in porosity (Novellani et al., 2000). Thus, longer polylysine chains would tend to form more porous domains, entrapping more POPC, leading to less enrichment with POPS in the polylysine-bound domain.

High-molecular-weight polylysine upon binding to phosphatidic-acid-containing lipid bilayers induces lateral separation of the anionic lipid, but itself adopts a β-sheet conformation in the bound state (Laroche et al., 1988, 1990). Because both intra- and inter-chain hydrogen bonding is possible, one expects that the domains formed by polylysine in a β-sheet conformation would be more compact and highly enriched with respect to the anionic lipid than for the case of an α-helical conformation. Moreover, with increasing chain length the opportunities for inter-chain hydrogen bonding increase, suggesting that as the polylysine molecular weight increases, more compact domains should result in phosphatidic-acid-containing bilayers, in contrast to the current results with phosphatidylserine. These questions will be the subject of future investigations.

Ionic strength effects on polylysine-induced domains

Fig. 7 shows de-Paked ^{2}H NMR spectra for POPC-α-d_2 + POPS (70:30) MLVs in the presence of Lys_{30} at a lys/ser

TABLE 3 Polylysine molecular weight and polylysine-induced domain compositions in POPC-α-d_2 + POPS (70:30) mixtures

		$\Delta\nu_Q$ (kHz)		Fractional composition				Lys-bound domain	
	Global Lys/Ser	Free	Bound	X^f_{PC}	X^b_{PC}	X^f_{PS}	X^b_{PS}	Lys/Ser*	Enrichment†
Lys_5									
	0.50	8.0		0.70		0.30			
	0.75	8.1		0.70		0.30			
Lys_{30}									
	0.50	7.7	9.5	0.39	0.31	0.13	0.17	0.9	1.3
	0.75	7.8	9.4	0.29	0.41	0.11	0.19	1.2	1.1
Lys_{100}									
	0.50	7.8	9.5	0.36	0.34	0.13	0.17	0.9	1.2
	0.75	7.7	9.0	0.22	0.48	0.07	0.23	1.0	1.1

Conditions were 40 mM Tris (pH 7.4), 0 mM NaCl.
*Calculated according to Eq. 5.
†Corresponds to the ratio $(X^b_{PS}/X^b_{PC})/(X^t_{PS}/X^t_{PC})$.

charge ratio of 0.625 and Lys_{100} at a lys/ser charge ratio of 0.50 as a function of the concentration of NaCl, as indicated in the figure. Theory states that if polyelectrolyte-induced domain formation is largely electrostatic in origin, then increasing ionic strength should eventually screen the electrostatic attraction between the polyelectrolyte and the charged amphiphiles, resulting in polyelectrolyte desorption and domain dissipation. This is evident for the case of Lys_{30}. At 75 mM NaCl it is still possible to resolve distinct Lys_{30}-bound and -free domains. However, the difference in the quadrupolar splittings of the two domain types decreases as both revert toward the control value, whereas the intensity of the Lys_{30}-bound domain actually increases. Quantitative analysis, as detailed in Table 4, demonstrates that the Lys_{30}-bound domains are less enriched with POPS, relative to the values measured in the absence of NaCl. Hence, the polylysine domains have become more diffuse and less enriched with respect to POPS. Note that the calculation of the degree of enrichment tabulated in Table 4 is predicated on the assumption of quantitative polylysine binding, a situation unlikely to pertain at high ionic strength. Hence, it is possible to obtain calculated degrees of enrichment that are less than unity, and are clearly erroneous, given the fact that the ^{2}H NMR spectra demonstrate the presence of a POPS-enriched domain.

At 150 mM NaCl only a single quadrupolar splitting is observed, and its value is close to that measured in the absence of Lys_{30}. Hence, this ionic strength appears sufficient to cause total domain dissipation. Interestingly, on a macroscopic level, Lys_{30} nevertheless causes flocculation and precipitation of MLVs, indicating that the surface electrostatic phenomena of flocculation can be decoupled from that of domain induction.

For Lys_{100} similar effects are observed in the presence of NaCl with the important difference that distinct polylysine-bound and -free domains remain resolvable even at 150 mM NaCl. Thus, long polylysines retain the ability to laterally segregate POPS under conditions of physiological ionic strength, whereas shorter polylysines do not.

These general effects of ionic strength on polyelectrolyte-induced domain size and composition are in accord with theoretical predictions (Denisov et al., 1998; May et al., 2000) and have been observed by ^{2}H NMR with other polyelectrolytes (Mitrakos and Macdonald, 1998, 2000). However, for polyadenylate and polystyrenesulfonate binding to cationic lipid bilayers, ionic strengths of 500 mM NaCl were required to cause desorption and domain dissipation (Mitrakos and Macdonald, 1998, 2000). Hence, relatively speaking, polylysine does not adsorb with nearly the affinity of these other polyelectrolytes.

pH effects on polylysine-induced domains

Fig. 8 *A* shows de-Paked ^{2}H NMR spectra for POPC-α-d_2 + POPS (70:30) MLVs in the presence of Lys_{30} (lys/ser = 0.5), as a function of bulk pH. Table 5 details the results of quantitative analysis. In the presence of a negative surface potential such as produced by POPS, the surface pH is lower than the bulk pH. The apparent pKa is then higher than the intrinsic pKa of a given ionizable group. The intrinsic pKa of the POPS carboxyl group is ~3.5 whereas that of the POPS amino group is in the region of 9.8 (Tsui et al., 1986). The intrinsic pKa of the amino groups of polylysine in solution is ~9.5 (de Kruijff et al., 1985).

At pH 4.0, given the prevailing low salt and moderately high surface charge conditions, the POPS carboxyl groups are expected to be largely protonated, so that most POPS molecules carry a net neutral charge. Consequently, polylysine binding will be curtailed and domain formation will not occur. This expectation is borne out by the ^{2}H NMR spectrum, which shows only a single quadrupolar splitting with a value unchanged from the control.

At pH 7.4 the POPS carboxyl group is deprotonated and POPS carries a net single negative charge. Polylysine binding produces domain formation as evident in the ^{2}H NMR spectrum in the figure and as discussed throughout this report.

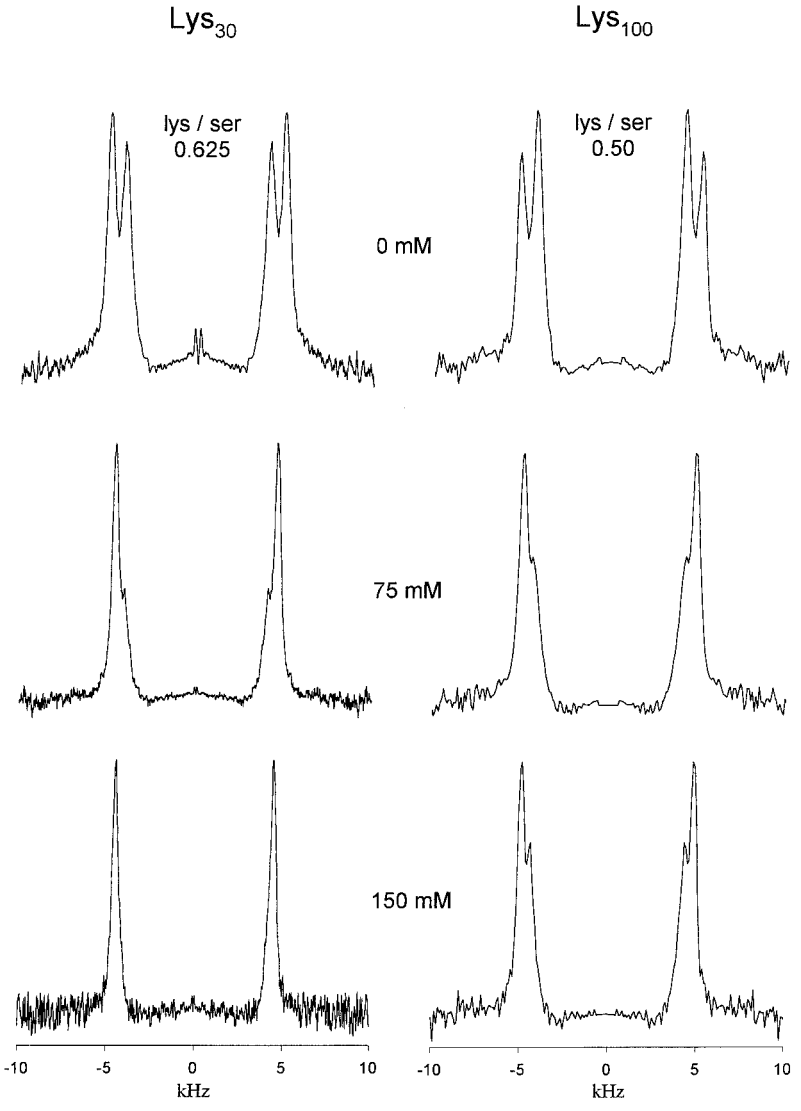

FIGURE 7 Ionic strength effects on Lys_{30}- and Lys_{100}-induced domains as manifest in de-Paked ^2H NMR spectra from POPC-α-d_2 + POPS (70:30) MLVs. The relevant NaCl concentration and the lys/ser charge ratio are indicated in the figure.

At pH 9.5 the POPS amino groups will still be largely protonated so that POPS will still carry a net single negative charge. Polylysine, however, will have a decreased charge density so that its membrane surface binding will be reduced but not eliminated entirely. This situation is reflected in the fact that the ^2H NMR spectrum shows a reduced, but not absent, Lys_{30}-bound component. Overall, the effects of pH on polylysine-induced domain formation in POPS-containing bilayers conform to expectations derived from knowledge of the relevant pKa values and the effects of surface charge on surface pH versus bulk pH.

It was not expected, however, that a bulk pH of 4.0 would have no influence on the ^2H NMR quadrupolar splitting of POPC-α-d_2 + POPS mixtures in the absence of polylysine. POPS will be largely charge neutral at this pH, given the low salt conditions. Thus, one anticipates a quadrupolar splitting near that of a neutral membrane surface. However, it must be borne in mind that the conformational response undergone by the phosphocholine headgroup in the presence of charged amphiphiles is not due directly to the general surface charge but rather is the result of one-on-one interactions with a particular charged species (Marassi and Macdonald, 1992). The fact that the quadrupolar splitting at pH 4.0 remains virtually unchanged relative to its value at neutral pH suggests that the conformational change resulting from the interaction between the phosphocholine and phosphoserine headgroups is not due solely to a Coulombic attraction of the quaternary amine of the former to the serine carboxyl of the latter but also involves interactions with other moieties such as the phosphate group, which remains charged at pH 4.0.

TABLE 4 Ionic strength effects on polylysine-induced domain compositions in POPC-α-d_2/POPS (70:30) mixtures

		$\Delta\nu_Q$ (kHz)		Fractional composition				Lys-bound domain	
	NaCl (mM)	Free	Bound	X^f_{PC}	X^b_{PC}	X^f_{PS}	X^b_{PS}	Lys/Ser*	Enrichment†
Lys_{30} (global Lys/Ser = 0.625)									
	0	7.7	9.4	0.32	0.38	0.11	0.19	1.0	1.2
	75	8.0	8.9	0.15	0.55	0.07	0.23	0.8	1.0
	150		8.6		0.70		0.30		
Lys_{100} (global Lys/Ser = 0.50)									
	0	7.8	9.5	0.36	0.34	0.13	0.17	0.9	1.2
	75	7.8	9.1	0.19	0.51	0.07	0.23	0.7	1.1
	150	8.1	9.1	0.24	0.46	0.12	0.18	0.8	0.9

Conditions were 40 mM Tris (pH 7.4), 0 mM NaCl.
*Calculated according to Eq. 5.
†Corresponds to the ratio $(X^b_{PS}/X^b_{PC})/(X^t_{PS}/X^t_{PC})$.

Phosphatidylglycerol-containing domains are poorly resolved

Fig. 8 *B* shows de-Paked ^{2}H NMR spectra for POPC-α-d_2 + POPG (70:30) MLVs (40 mM Tris, pH 7.4) as a function of the amount of added Lys_{30}. The top spectrum was obtained in the absence of Lys_{30} and consists of a single quadrupolar splitting, indicating, as expected, a uniform charge environment for all POPC molecules. The quadrupolar splitting equals 9.5 kHz, demonstrating that the POPG anionic charge produces the expected conformational response of choline to surface charge but that the response is greater for POPG versus POPS at a given mole fraction of anionic lipid headgroup. This difference can be related back to the deeper location of the anionic charge of POPG relative to POPS within the polar layer at the bilayer surface.

The middle and lower spectra were obtained with lys/PG charge ratios of 0.625 and 0.75, respectively. These amounts of Lys_{30} yield well-resolved domains in POPC + POPS membranes. Lys_{30} addition to POPG-containing MLVs causes macroscopic flocculation of the samples, indicating electrostatic interaction with the bilayer surface. However, although the ^{2}H NMR spectra indicate the presence of overlapping subspectra, they are clearly more poorly resolved than in the corresponding cases with POPS, largely due to increased linewidths. Analysis of the domain compositions after spectral deconvolution of the overlapping subspectra yields degrees of enrichment roughly comparable to those obtained with POPS, but with considerable uncertainty associated with the values obtained due to line-broadening problems.

One potential source of the additional line broadening observed with POPG is the relative weakness of its interaction with polylysine. For polylysine to bind POPG, the lysine cation must penetrate to the POPG phosphate, whereas for POPS the lysine cation need only bind to the serine carboxylate group. The latter extends further outward from the bilayer surface than does the phosphate group. In addition, the carboxylate of the POPS headgroup has less of an orienting effect on the water dipoles than does a phosphate group, which further enhances the affinity of POPS for water-soluble charged molecules (Collins, 1997). For example, calcium binds to both phosphatidylserine and phosphatidylglycerol, but the bond created with phosphatidylserine possesses twice the strength of its bond to phosphatidylglycerol (Cevc, 1990). Also, calcium induces lateral segregation of phosphatidylserine, but not phosphatidyl-

TABLE 5 Bulk pH effects on Lys_{30}-induced domain compositions in POPC-α-d_2 + POPS (70:30) mixtures

		$\Delta\nu_Q$ (kHz)		Fractional composition				Lys-bound domain	
	Global Lys/Ser	Free	Bound	X^f_{PC}	X^b_{PC}	X^f_{PS}	X^b_{PS}	Lys/Ser*	Enrichment†
pH 4.0									
	0	8.1		0.70		0.30			
	0.50	8.6		0.70		0.30			
pH 7.4									
	0	8.2		0.70		0.30			
	0.50	7.7	9.5	0.39	0.31	0.13	0.17	0.9	1.3
pH 9.5									
	0	8.3		0.70		0.30			
	0.50	7.8	10.0	0.52	0.18	0.19	0.11	1.5	1.4

Conditions were 40 mM acetate (pH 4.0) or Tris (pH 7.4) or CAPS (pH 9.5), 0 mM NaCl.
*Calculated according to Eq. 5.
†Corresponds to the ratio $(X^b_{PS}/X^b_{PC})/(X^t_{PS}/X^t_{PC})$.

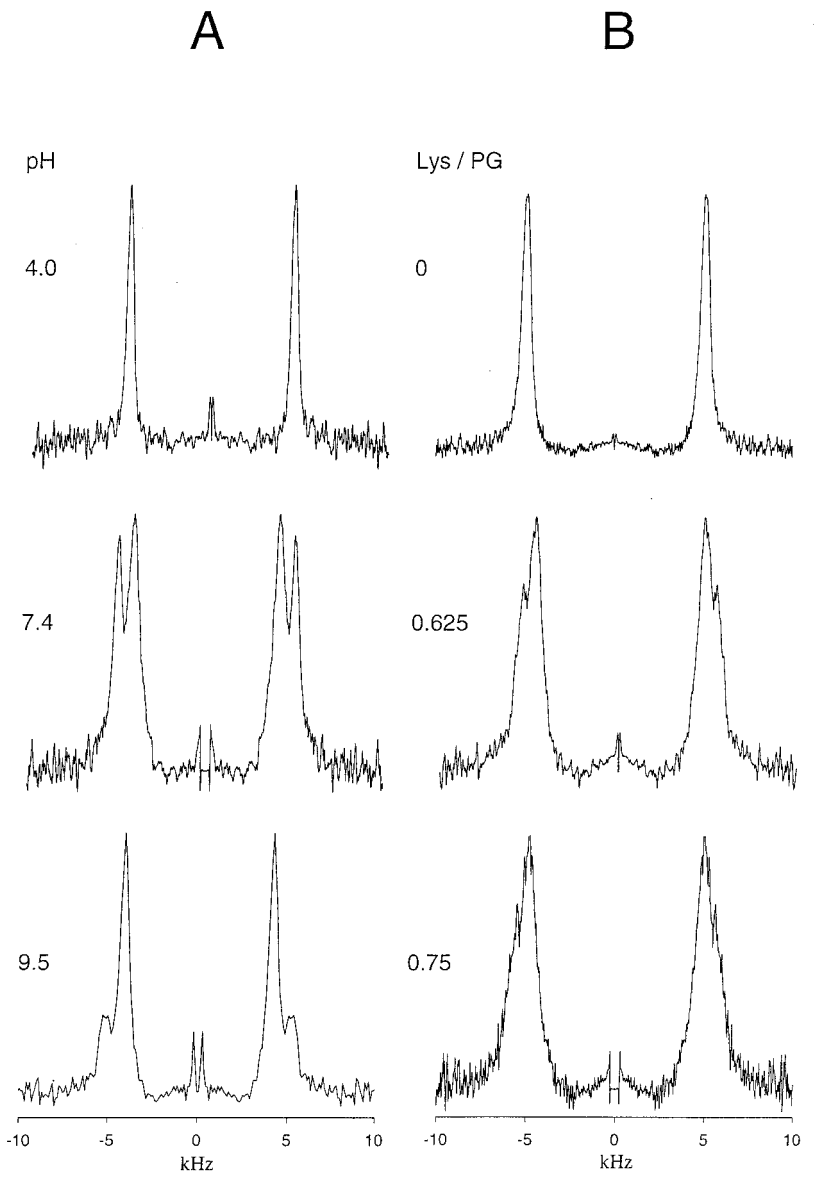

FIGURE 8 (*A*) De-Paked ^{2}H NMR spectra from POPC-α-d_2 + POPS (70:30) MLVs at different bulk pH in the presence of Lys_{30} (lys/ser charge ratio = 0.5). pH 4.0 = 40 mM acetate, pH 7.4 = 40 mM Tris, and pH 9.5 = 40 mM CAPS. (*B*) De-Paked ^{2}H NMR spectra from POPC-α-d_2 + POPG (70:30) MLVs in the presence of Lys_{30} at the indicated lys/ser charge ratio (40 mM Tris, pH 7.4).

glycerol (Huang and Feigenson, 1993; Huang et al., 1993). Phosphatidylserine segregation and domain formation is induced by a number of proteins (Tocanne et al., 1994; Denisov et al., 1998), and a number of proteins and peptides show a similar preference for phosphatidylserine. For example, the binding and internalization of melittin is more efficient when the bilayer contains phosphatidylserine rather than phosphatidylglycerol (Monette and Lafleur, 1995). Another example concerns histone H1, which binds to mixed micelles containing phosphatidylserine but not to those containing phosphatidylglycerol or phosphatidylinositol (Kinnunen et al., 1994). In the case of a weaker association with bound polylysine, rates of exchange of POPG between bound and free states would be faster than for POPS, potentially producing exchange broadening.

Another explanation may be that the domain composition resulting from polylysine addition to phosphatidylglycerol is simply more complex than that resulting with phosphatidylserine. Specifically, Carrier and Pézolet (1986) reported that polylysine induced three types of domains in phosphatidylglycerol bilayers for a lipid-to-lysyl residue ratio greater than one. Unless the domains were quite different in composition, it might prove rather difficult to resolve separate domain subspectra in our ^{2}H NMR spectra. The result would be apparent spectral broadening.

CONCLUDING REMARKS

Polylysines are of interest primarily because they resemble the basic regions of membrane-associating peptides and proteins. Oligolysines (e.g., Lys_5) approximate the cationic charge of peptides like the MARCKS protein effector domain, MARCKS (151–175) (Taniguchi and Manenti, 1993; Kim et al., 1994; Swierczynski and Blackshear, 1995; McLaughlin and Aderem, 1995; Glaser et al., 1996), or PMP1, a regulatory subunit associated with the yeast plasma membrane H(+)-ATPase (Beswick et al., 1998; Roux et al., 2000), or the pseudo-substrate region of protein kinase C (Mosior and McLaughlin, 1991). Longer polylysines (e.g., Lys_{30} and Lys_{100}) approximate the cationic charge of highly basic peripheral membrane proteins such as myelin basic protein (Carnegie, 1971; Eylar et al., 1971) or cytochrome c (Dickerson, 1972; Moore and Pettigrew, 1990).

The major findings reported here are that Lys_{30} and Lys_{100}, but not Lys_5, are able to laterally segregate POPS from mixtures with POPC and form POPS-enriched domains but that under physiological salt conditions only Lys_{100}, and not Lys_{30}, retains this ability. This clearly suggests that, unassisted by other factors such as hydrophobic contributions to the binding energy, only the largest basic peripheral membrane proteins would be expected to laterally segregate acidic lipids. Hydrophobic contributions to binding are, however, ubiquitous, whether arising from hydrophobic amino acid residues or from post-translational modification with hydrophobes. The next obvious step in a systematic physical investigation would be to examine and compare hydrophobically modified basic polypeptides versus control polypeptides for their ability to induce electrostatic domain formation as observed using ^{2}H NMR.

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