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Deuterium NMR and the Topography of Surface Electrostatic Charge

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Biological membranes play a pivotal role in cellular structure and function, where they act to define compartments, to control communication between the inside and outside, and to catalyze biochemical reactions.[1,2] Their structure is a "fluid-mosaic" of lipids and proteins.[3] The lipids are arranged into a two-dimensional planar bilayer with their hydrophobic acyl chains sequestered toward the bilayer interior and their hydrophilic polar head groups facing the aqueous exterior. The membrane proteins associate with the polar membrane surface, or else insert into, and even traverse, the lipid bilayer proper.

Surface electrostatic charge is an important property of membranes, capable of influencing a broad range of membrane-associated functions.[4−6] Membrane surface charges can arise from the presence of charged lipids and proteins intrinsic to the membrane, or from the adventitious binding of extrinsic charged species such as ions, drugs, anaesthetics, peptides, or proteins.

The accepted starting point for describing membrane surface electrostatics and its effects is the Gouy−Chapman (GC) theory.[4−7] GC theory is intended to deal with an infinitely-planar surface bearing a smeared surface charge acting upon point charges in solution. In many situations relevant to biological membranes such assumptions are patently inappropriate. Since membranes are a fluid-mosaic, local details of surface geometry and surface charge distribution can diverge significantly from the ideal of a planar surface with a homogeneous charge distribution. Furthermore, if the charged species of interest is a polyelectrolyte, such as a protein or a nucleic acid, then a point-charge assumption is inadequate. In addition, biological membranes possess two surfaces, an inside and an outside, and these typically differ with respect to their surface charge.

There exist a host of techniques for measuring surface electrostatic charge, and these include electrophoretic mobility, conductance, ionizing electrode, NMR, and ESR methods.[3−6] However, most such methods measure the net surface charge and fail, therefore, to differentiate between homogeneous and inhomogeneous charge distributions. Those methods capable of resolving lateral[8] or transbilayer[9] compositional inhomogeneities in membranes are often either perturbing, or opaque to interpretation in terms of molecular-level details.

A true understanding of the relationship between membrane surface charge and membrane function requires a grasp of the lateral and transbilayer inhomogeneities of surface charge distribution, and how these arise. In short, one seeks to appreciate the detailed topography of surface charge location and distribution. One technique which has proved useful for detailed molecular-level studies of membrane surface charge topography is ^{2}H NMR spectroscopy of choline-deuterated phosphatidylcholine (PC).[10] This Account describes how ^{2}H NMR is employed to measure surface charge in membranes, and its particular benefits for assessing surface charge topography.

^{2}H NMR Fundamentals in Lipid Bilayers

The fundamental physical observable in the ^{2}H NMR spectrum from specifically choline-deuterated PC incorporated into model lipid bilayer vesicle membranes is the quadrupolar splitting, $\Delta\nu$, corresponding to the separation between the two maxima or "horns" in the ^{2}H NMR spectrum shown in Figure 1. The overall line shape of such a ^{2}H NMR spectrum is a Pake doublet.[11,12] The Pake doublet line shape is a consequence of the fact that in aqueous solution PC spontaneously assembles into lipid bilayer vesicles. These are usually multilamellar vesicles (MLVs), having an onionskin-like architecture of concentric lipid bilayer vesicles. If special preparation techniques are employed,[13] then one may produce unilamellar vesicles (ULVs), having the architecture shown in cross-section in Figure 1. Within the two-dimensional lipid bilayer sheet, whether in MLVs or ULVs, individual PC molecules orient with their polar head groups facing the aqueous bathing medium and their fatty acyl chains sequestered toward the hydrophobic membrane interior. Motions experienced by PC tend to reduce the size of the ^{2}H NMR quadrupolar splitting, provided they are fast on the ^{2}H NMR time scale (10^{-5} to 10^{-6} s). If the lipid vesicles are large (diameter >500 nm), then overall vesicle translation or rotation is slow relative to this time scale. Likewise, lateral diffusion of individual PC molecules about the radius of curvature of the vesicle is too slow to influence the quadrupolar splitting. However, rotation of individual

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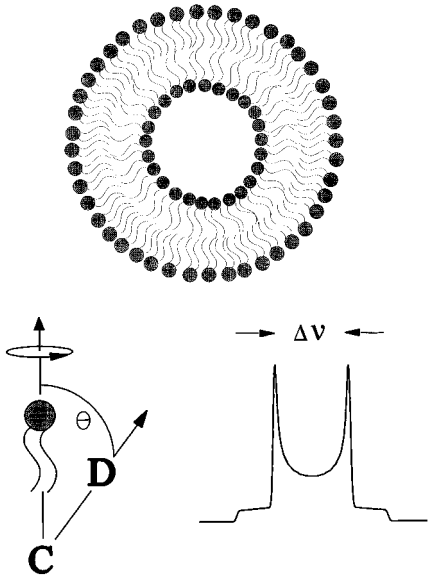

FIGURE 1. Origin of the ^{2}H NMR Pake pattern spectral line shape for choline-deuterated POPC in a lipid bilayer membrane vesicle. In water, POPC spontaneously assembles into lipid bilayers, generally assuming a multilamellar architecture but, under special preparation conditions, assuming the form of unilamellar vesicles such as that shown in the schematic cross-section. In lipid bilayers, individual POPC molecules undergo fast rotation about their long molecular axes. The ^{2}H NMR spectral line shape reflects the powder distribution of POPC long-axis orientations relative to the direction of the magnetic field of the NMR spectrometer. The quadrupolar splitting, $\Delta\nu$, corresponds to the separation (Hz) between the two "horns" in the resulting Pake pattern line shape. The size of the quadrupolar splitting scales with the angle θ between the particular C−D bond and the POPC long molecular axis, according to eq 1.

PC molecules about their long molecular axes, perpendicular to the plane of the bilayer, is fast. Since long-axis rotation is anisotropic, the motional averaging is incomplete, leaving a residual quadrupolar splitting as shown in Figure 1. The residual quadrupolar splitting for deutero-labeled PC becomes a function of the orientation θ of the particular C−D bond relative to the lipid's long molecular axis, according to eq 1, where $\Delta\nu_0$ is the static

$$\Delta\nu/\Delta\nu_0 = \langle S_f|(1/2)(3\cos^2\theta - 1)|\rangle \qquad (1)$$

quadrupolar splitting (125 kHz for an alkyl C−D bond) and the angular brackets indicate that one is measuring an average over the time of the experiment. S_f is an order parameter ($0 \le S_f \le 1.0$) quantifying angular fluctuations about the average orientation. When S_f equals zero, the angular fluctuations are so large that there is essentially no preferred orientation. A value of S_f equal to unity signifies an infinitely narrow distribution about the average angle. The significance of eq 1 is that any change in the average internal conformation of PC changes the quadrupolar splitting of the ^{2}H NMR spectrum.

Phosphatidylcholine Behaves Like a "Molecular Voltmeter" for Surface Charge

Surface electrostatic charge produces a pronounced conformation change in the choline head group of (1-palmitoyl-2-oleoylphosphatidyl)choline (POPC) as demonstrated by the ^{2}H NMR spectra in Figure 2. The two deutero-labeling positions of greatest interest in the choline head group, designated as α- and β-deuterons, are indicated as such in Figure 2. For a neutral membrane surface the quadrupolar splittings from these deutero-labeling positions are nearly identical. When an anionic surface charge is produced by mixing POPC with an anionic phospholipid such as (1-palmitoyl-2-oleoylphosphatidyl)glycerol (POPG), the quadrupolar splitting from POPC-α-d_2 increases while that from POPC-β-d_2 decreases. Conversely, when a cationic surface charge is introduced by mixing POPC with the cationic amphiphile DOTAP (2,3-dioleoyl-1-(trimethylammonio)propane), the quadrupolar splittings from the two deutero-labeling positions change, but in a manner opposite that produced by anionic surface charges. From the first discovery of this effect,[14] it was suggested that this inverse change in the size of the quadrupolar splittings from the two deutero-labeling positions is the result of a specific conformational response of the choline head group of PC to the presence of surface charges, ruling out a generalized change in the order parameter S_f as the cause. Between the extremes of highly anionic and highly cationic surface charge the quadrupolar splittings differ by several tens of kilohertz, so these are not small effects. The utility of this ^{2}H NMR response to surface charge resides in the fact that any and all sources of surface charge produce similar changes in the quadrupolar splittings from choline-deuterated PC. It is due to this universal surface charge response that the ^{2}H NMR technique has been dubbed the "molecular voltmeter".

Akutsu and Seelig[15] first proposed that surface charges induce the entire phosphocholine group to tilt with respect to the plane of the membrane surface. This "choline tilt" model is illustrated schematically in Figure 2. Such a tilt would occur most readily via a rotation about the hinge between the gycerol backbone and the phosphocholine head group, corresponding to the glycerol C_3−O−P dihedral angle.[16] Changes in the choline tilt angle could arise as the large dipole moment of the zwitterionic phosphocholine group seeks to align itself with the electrical field lines emanating from the charged membrane surface.[17] Indeed, such a model is capable of reproducing the dependence of the quadrupolar splittings on either cationic or anionic surface charges.[18] Alternately, one may perform a conformational analysis of the choline head group using the quadrupolar splittings obtained with charged versus neutral surfaces. For the case of a negative surface charge, this approach indicates that the P−N vector reorients from a parallel to a perpendicular alignment with respect to the plane of the bilayer,[19] in agreement with the predictions of the choline tilt model.

More recent evidence has called into question the choline tilt concept. For example, in ternary mixtures of cationic plus anionic plus zwitterionic amphiphiles, where the cationic and anionic charges are balanced to produce

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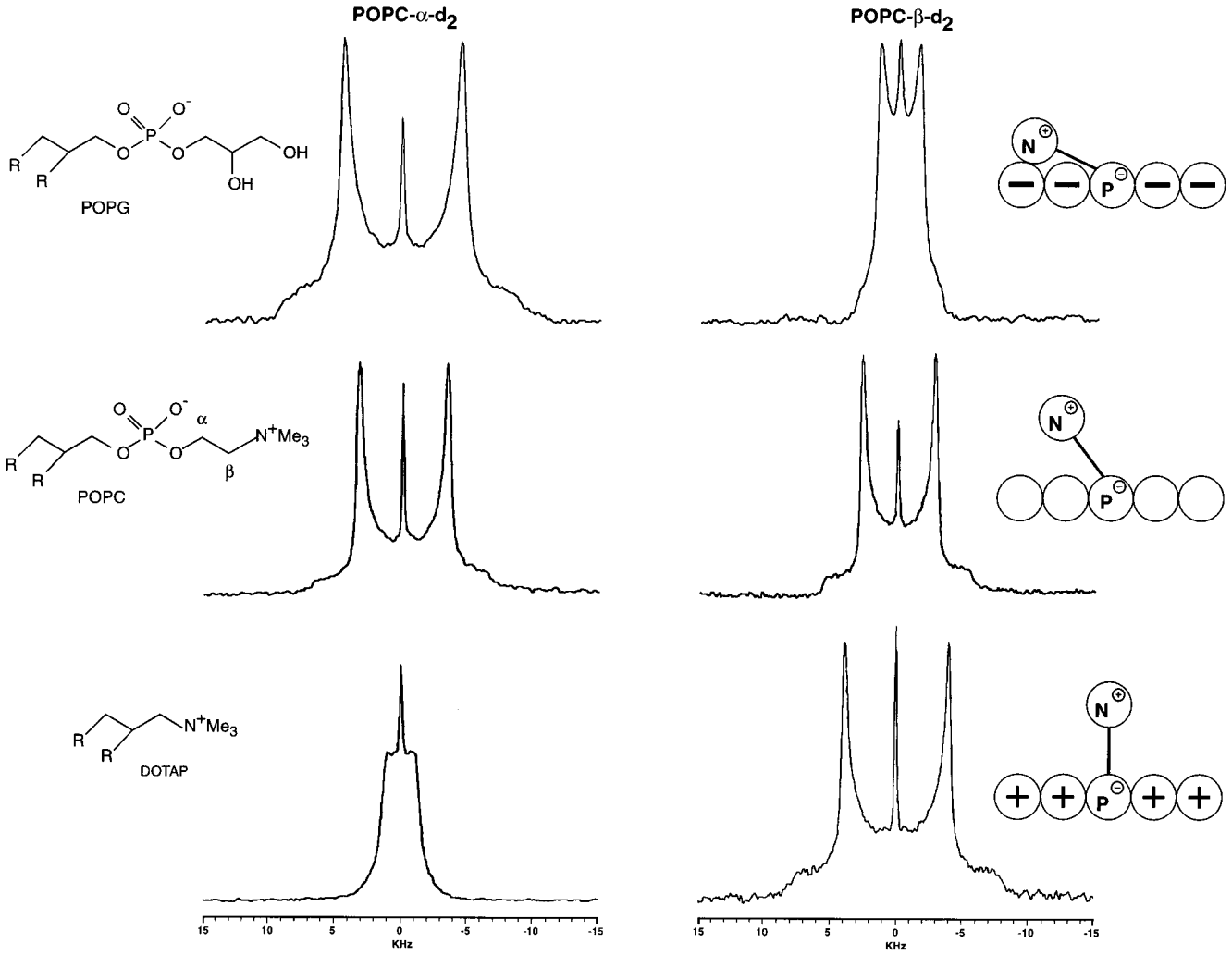

FIGURE 2. ^{2}H NMR spectra of choline-deuterated POPC in the presence of anionic (top), neutral (middle), and cationic (bottom) surface charges. Deuteron labels were located at either the α- or the *β*-position of the choline group of POPC, as defined in the chemical diagram. Anionic surface charges were introduced by mixing POPC with 20 mol % POPG. Cationic surface charges were introduced by mixing POPC with 20 mol % DOTAP. Anionic surface charges always produce changes in $\Delta\nu$ which are the inverse of those produced by cationic surface charges. The $\Delta\nu$ from POPC-α-d_2 always behaves opposite that from POPC-*β*-d_2. The phosphocholine group appears to undergo a conformation change consistent with a tilting of the entire phosphocholine group with respect to the plane of the membrane.

an ostensibly neutral surface, the measured quadrupolar splittings diverge from the values expected for a neutral surface charge.[20] Instead, they suggest that the choline group engages in a series of one-on-one encounters with different charged species, each of which produces a distinct conformational change and each of which contributes to the average conformation reported by ^{2}H NMR. Langevin dynamics simulations indicate that changes in the internal torsional angles of the choline group occur in addition to any concerted choline tilt.[21] Moreover, surface charge is not the only physical variable capable of producing inverse changes in the quadrupolar splittings from the two choline deutero-labeling positions. Changes in membrane hydration[22−24] and hydrostatic pressure[25] produce effects qualitatively similar to those produced by surface charge. Evidently, a more precise determination of the conformation of the choline group in different surface charge environments is required. Recent NMR measurements of the ^{31}P−^{13}C dipole−dipole interactions within the choline head group[26,27] offer a promising means to achieve this goal.

Determining the pK_a of Membrane-Bound Ionizable Functional Groups

One of the foremost applications of the ^{2}H NMR sensitivity to surface charge is in the measurement of equilibrium binding isotherms for charged ligands interacting with membrane surfaces. Any chemical species may be examined, provided it is electrostatically charged and upon binding to lipid bilayers remains located at the membrane surface. One might regard this as the "zero-order" topographical distinction available via ^{2}H NMR, wherein one decides whether or not charges are present at the membrane surface. The variety of species which have been examined in this fashion is rather formidable, as may

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be judged from a recent catalogue of such ^{2}H NMR measurements.[28]

A more subtle topographical discrimination may be extracted from the ^{2}H NMR data, and this is with regard to the location of a bound species relative to the plane of the phosphocholine group. For instance, ligands with hydrophobic attachments, such as surfactants, would be expected to penetrate more deeply into the hydrophobic interior of the lipid bilayer than hydrophilic ligands such as aqueous ions. Moreover, cationic ligands should locate close to the POPC phosphate while anionic ligands might locate proximal to the POPC quaternary nitrogen.

To actually measure surface charge densities via ^{2}H NMR, one first quantifies the relationship between the quadrupolar splitting, from either POPC-α-d_2 or POPC-β-d_2, and the amount of bound surface ligand. At low levels of bound ligand such calibration curves are typically linear. Different ligands, however, produce different slopes. In general, cations produce greater effects than anions, while hydrophobic ligands, such as cationic surfactants, produce greater effects than hydrophilic ligands, such as calcium.[28,29] One concludes that the sensitivity of the ^{2}H NMR response provides information regarding the location of the ligand's charged group relative to the phosphocholine of PC.

All of these features of the ^{2}H NMR technique are illustrated in the example of *N*,*N*-dimethyl-D-sphingosine (DMS).[30] In addition, the case of DMS demonstrates that ^{2}H NMR provides a convenient means of determining the pK_a of membrane-bound ionizable groups *in situ*, as first shown by Watts and Poile.[31] DMS, shown in Figure 3, is a potent inhibitor of the membrane-bound regulatory enzyme protein kinase C (PKC).[32] The minimal structural prerequisites for any inhibitor of PKC are the presence of a positive charge and an 18-carbon chain. These are satisfied by both DMS and its non-aminomethylated parent compound sphingosine (SPG), but DMS is by far the more potent inhibitor.[32] One explanation for this difference is that the pK_a of membrane-bound DMS might differ from that of SPG. The pK_a of any ionizable functional group located at a membrane surface can shift markedly from its value in solution, due to differences in the local dielectric constant, ionic activities, or other factors. Consequently, *in situ* pK_a measurements are essential.

Figure 3 shows the pH dependence of the quadrupolar splittings from POPC-α-d_2 and POPC-β-d_2 lipid bilayer membranes to which 5 mol % DMS has been added. At pH 7.0 the added DMS causes the quadrupolar splitting from POPC-α-d_2 to decrease while that from POPC-β-d_2 increases, relative to their values at neutrality. This is consistent with an accumulation of cationic charges at the lipid bilayer surface. One immediately concludes that DMS associates with lipid bilayer membranes in such a fashion that its cationic dimethylamino group is located at the membrane surface. Separate calibration measurements show that at these levels DMS binds virtually quantitatively to the lipid bilayers and induces a large ^{2}H NMR response relative to other cationic species. One further concludes, therefore, that the cationic dimethylamino group of DMS locates close to the plane of the POPC phosphate group.

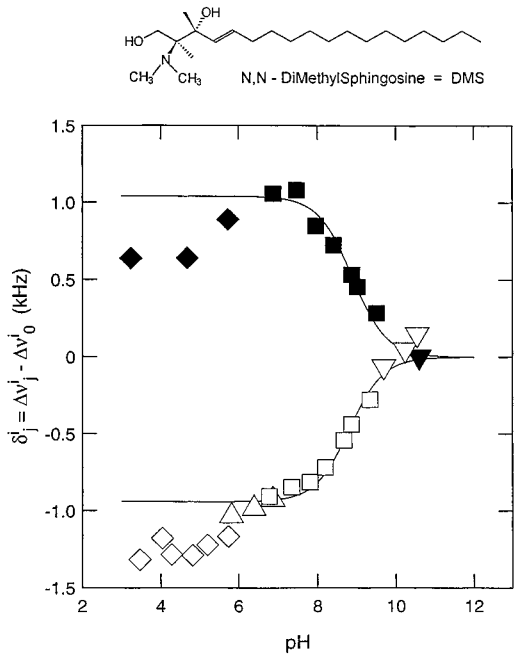

FIGURE 3. Experimental and simulated ^{2}H NMR DMS titration curves. Reprinted with permission from ref 30. Copyright 1995 Elsevier Science. The structure of DMS is shown at the top of the figure. The quadrupolar splittings from POPC-α-d_2 (open symbols) and POPC-β-d_2 (closed symbols) mixed with 5 mol % DMS are expressed in terms of their change relative to the absence of DMS, and plotted versus the solution pH. Different symbols denote different buffers used over different ranges of pH: citrate (diamonds), 2-(*N*-morpholino)ethanesulfonic acid (triangles), 1,3-bis[[tris(hydroxymethyl)methyl]amino]propane (squares), 3-(cyclohexylamino)-1-propanesulfonic acid (inverted triangles). The solid lines are simulations of the titration curve using eq 2 with values of k^α and k^β as reported elsewhere[30] and the pK_a of DMS equal to 8.8 for both deuteron-labeling positions.

When the pH of the aqueous bathing solution is rendered more basic, the quadrupolar splittings from both deutero-labeling positions revert to their values at neutrality, in a fashion consistent with a titration of a weak base from its protonated (cationic) form at lower pH to its deprotonated (neutral) form at higher pH. For the case of overall low surface charge, such that the surface pH does not differ greatly from the bulk pH, the pK_a of DMS may be approximated from the pH dependence of the quadrupolar splittings using the following equation:

$$\delta_j^i = \Delta\nu_j^i - \Delta\nu_o^i = \frac{k^i[T]_j}{1 + K_a/[H^+]} \qquad (2)$$

where the superscript i delineates the α- versus β-deutero-labeling position, the subscript j designates a particular total DMS concentration T, and k^i is the calibrated sensitivity of the quadrupolar splittings to DMS. The line of best fit, as judged by nonlinear least squares fitting of the predicted to the experimental POPC-α-d_2 and POPC-β-d_2 quadrupolar splittings, is shown as the solid line in Figure 3 and corresponds to the case pK_a = 8.8. The poor fit at low pH values is attributed to buffer specific, rather

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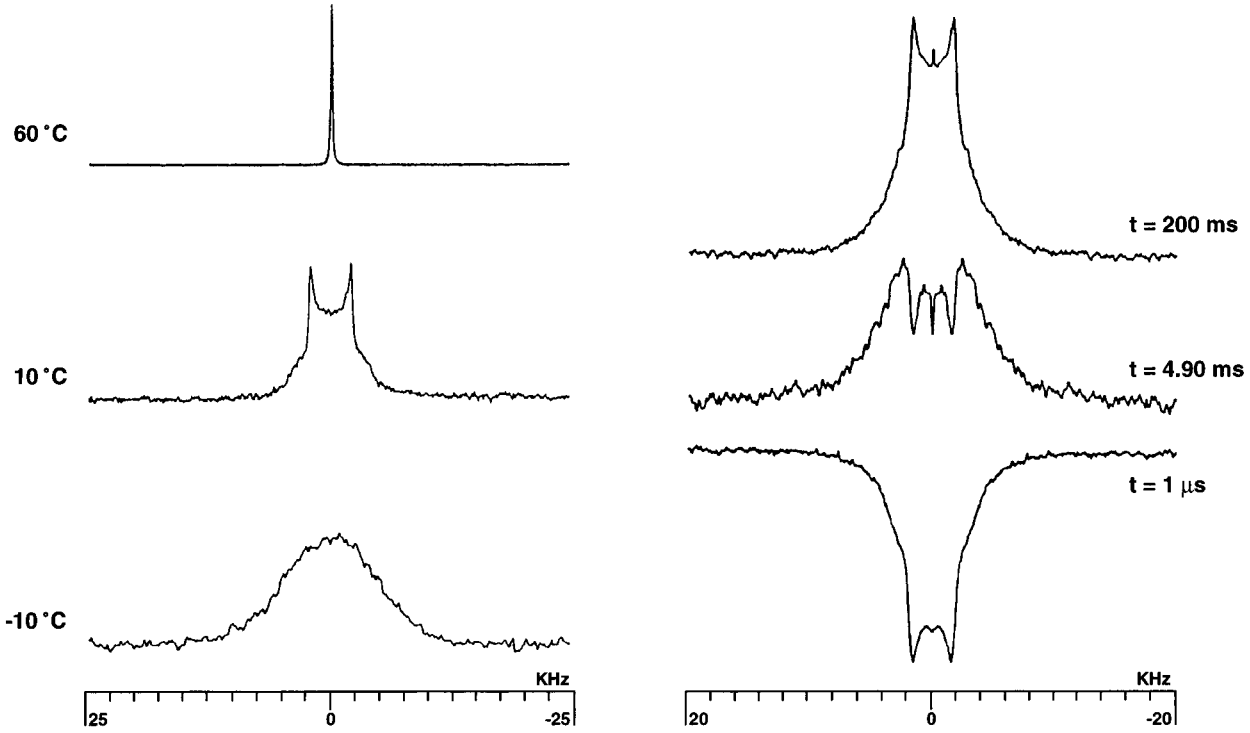

FIGURE 4. ^{2}H NMR detection of thermotropically-induced demixing of DMPA and POPC. Reprinted with permission from ref 33. Copyright 1993 Elsevier Science. On the left, ^{2}H NMR spectra are shown for POPC-β-d_2 in the presence of 50 mol % DMPA, as a function of temperature. Note the change in the $\Delta\nu$ from approximately 0 at 60 °C, above the T_g for both POPC and DMPA, to approximately 2.4 kHz at 10 °C, intermediate to the T_g values of POPC and DMPA. The ^{2}H NMR spectra on the right demonstrate unequivocally that at 15 °C the ^{2}H NMR spectrum from POPC-β-d_2 consists of a superposition of two spectral components with different T_1 relaxation times. Times t refer to the delay postinversion prior to acquisition of the ^{2}H NMR spectrum.

than pH specific, effects since anomalies are observed at low pH with or without DMS.

Given the value of the pK_a, it follows that at physiological pH over 90% of DMS exists in a cationic form. In comparison, SPG, with its membrane-bound pK_a of 8.5, also exists largely in a cationic form. Thus, the differences in their relative potencies as inhibitors of PKC do not originate with differences in their cationic versus neutral populations. Instead one must look to the relative affinities of DMS and SPG for the binding site on PKC.

Thermotropically-Induced Coexisting Lipid Domains of Distinct Composition

The case of DMS was one in which the charged species was evenly and homogeneously distributed throughout the membrane. In the fluid-mosaic biological membrane, however, there can exist long-lived domains having distinct composition and supporting distinct functions.[8] When the surface electrostatic charge within such a laterally-separated, inhomogeneous, membrane domain differs from that of its surroundings, ^{2}H NMR can provide information regarding the extent of domain separation and the domain composition and some indication regarding domain size.

One cause of domain separation is differences in the thermotropic properties of the constitutive lipids. All lipids undergo a temperature-induced transition from a fluidlike liquid-crystalline phase at high temperatures to a solidlike gel phase at temperatures below the transition temperature T_g characteristic for a given lipid. In mixtures of a high-T_g phospholipid, such as the anionic species 1,2-dimyristoylphosphatidic acid (DMPA) (T_g = 52 °C), with a low-T_g phospholipid, such as zwitterionic POPC (T_g = −5 °C), only at temperatures above T_g for both lipids are the two completely mixed at the molecular level. Figure 4 shows representative ^{2}H NMR spectra from a 50:50 mixture of DMPA + POPC-β-d_2 at different temperatures.[33] At 60 °C, a temperature greater than T_g for both DMPA and POPC, the ^{2}H NMR spectrum on the top left reflects a state of ideal fluid mixing in that the quadrupolar splitting falls close to 0 Hz, as expected for POPC-β-d_2 experiencing a highly anionically-charged membrane surface. At −10 °C, a temperature below the T_g of both DMPA and POPC, both phospholipids have entered the gel state. The corresponding ^{2}H NMR spectrum of POPC-β-d_2 consists of a broad ill-defined line shape, which cannot be interpreted in terms of the membrane surface charge.

At 10 °C, a temperature intermediate to the T_g of DMPA versus POPC, much of the DMPA phase separates into solidlike DMPA-enriched domains, leaving behind fluidlike POPC-enriched domains. The ^{2}H NMR spectrum at 10 °C reflects this state of separated coexisting lipid domains in that it consists of two overlapping spectral components. One component is broad and ill-defined, originating with those POPC-β-d_2 molecules trapped within the solidlike DPMA-enriched domains. The other component is a well-defined Pake pattern, originating with those POPC-β-d_2 molecules residing within the fluidlike POPC-enriched domains.

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The series of ^{2}H NMR spectra on the right in Figure 4 demonstrate unambiguously that, in mixtures of DMPA + POPC-β-d_2 at temperatures intermediate to the T_g values of the two constituents, the ^{2}H NMR spectrum consists of a superposition of two spectral components. The two, in fact, have different spin−lattice (T_1) relaxation times due to the different dynamic properties in the two phases from which they arise. If one inverts the populations of the nuclear spin states using an appropriate NMR pulse sequence, the populations will return to their equilibrium Boltzmann distribution with a characteristic time constant T_1. If one monitors their state shortly after the inversion (t = 1 μs), both spectral components have negative intensity. At a time postinversion which is much longer than either T_1 (t = 200 ms), both spectral components have returned to equilibrium and display positive intensities. However, if the T_1 relaxation times of the two spectral components differ sufficiently, then it is generally possible to find a postinversion delay time at which one spectral component displays a positive intensity while the other has a negative intensity. This is the case for t = 4.90 ms in Figure 4. Here, the broad spectral component corresponding to solidlike POPC-β-d_2 displays a positive intensity, while the Pake pattern spectral component corresponding to fluidlike POPC-β-d_2 is still negative.

The quadrupolar splitting measured for the liquid-crystalline spectral component at 15 °C is much larger than that measured at 60 °C, indicating that the POPC-β-d_2 experiences a much decreased anionic surface charge. This is consistent with a depletion of DMPA from the liquid-crystalline domains due to its phase separation into DMPA-enriched gel phase domains. Since the quadrupolar splitting is directly a measure of the local surface charge, an analysis of the quadrupolar splittings for both POPC-α-d_2 and POPC-β-d_2 over an entire temperature range and for a variety of mole fractions of DMPA/POPC yields a temperature−composition phase diagram, as illustrated in Figure 5.[33] The upper set of data points represent the *fluidus* curve, above which the DMPA and POPC are in a liquid-crystalline state and are ideally mixed. The lower set of data points represent the *solidus* curve, below which both DMPA and POPC are entirely in a gel state. Between the *solidus* and *fluidus* curves lies a region in which liquid-crystalline and gel phases coexist. From such a phase diagram the percent gel and percent liquid-crystalline phases, and the DMPA−POPC composition of either domain, may be determined using the classical tie-line method.

Electrostatically-Induced Coexisting Lipid Domains of Distinct Composition

Electrostatic attraction between anionic lipids and cationic proteins is known to produce a long-lived annulus or "boundary layer" of lipid surrounding certain membrane proteins.[34] However, attempts to quantify the electrostatics of such lipid−protein interactions via ^{2}H NMR have generally proved unfruitful, due either to an unfortunate time scale of lipid exchange between "bound" and "free" environments, or to some complication such as a change in the macroscopic lipid phase to a nonbilayer arrangement upon association with the protein.[35]

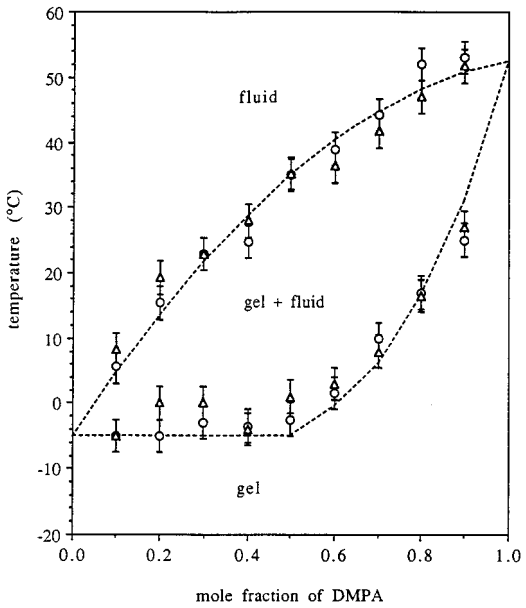

FIGURE 5. Temperature−composition phase diagram for POPC−DMPA mixtures obtained from analysis of the temperature dependence of the ^{2}H NMR quadrupolar splittings of POPC-α-d_2 (circles) and POPC-β-d_2 (triangles) mixed with various molar ratios of DMPA. Reprinted with permission from ref 33. Copyright 1993 Elsevier Science.

Recent studies of polyelectrolyte−membrane interactions have produced the first ^{2}H NMR characterization of long-lived, electrostatically-induced lipid domains of distinct composition.[36,37] The polyelectrolytes investigated to date include single-stranded DNA, poly(amino acids) such as polylysine and polyglutamate, and synthetic polyelectrolytes such as poly(sodium styrenesulfonate) (PSSS) and poly[(4-vinylbenzyl)trimethylammonium chloride] (PVTA). All exhibit similar propensities to induce lateral phase separation of lipid domains. Figure 6 shows the type of ^{2}H NMR spectrum which is obtained when charged lipid bilayer membranes are exposed to an oppositely-charged polyelectrolyte. In this particular case, the lipid bilayers are MLVs composed of a mixture of the cationic lipid DOTAP plus the zwitterionic lipid POPC-β-d_2, while the polyelectrolyte is the anionic species PSSS.

One observes two overlapping Pake patterns in the ^{2}H NMR spectrum. This proves that two distinct lipid domains coexist and that there is a slow exchange of individual POPC molecules between the two. The domains must be so large that POPC lateral diffusion within the plane of the membrane is too slow to permit individual POPC molecules to sample both environments. Since both spectral components are Pake patterns characteristic of fluid phospholipids, both domains are fluid. The quadrupolar splittings reveal the DOTAP compositions of the two domains, and one deduces that the PSSS-free domain is DOTAP-depleted, while the PSSS-bound domain is DOTAP-enriched, relative to the global bilayer-average DOTAP composition. Thus, PSSS preferentially sequesters cationic lipids from within the gemisch of the

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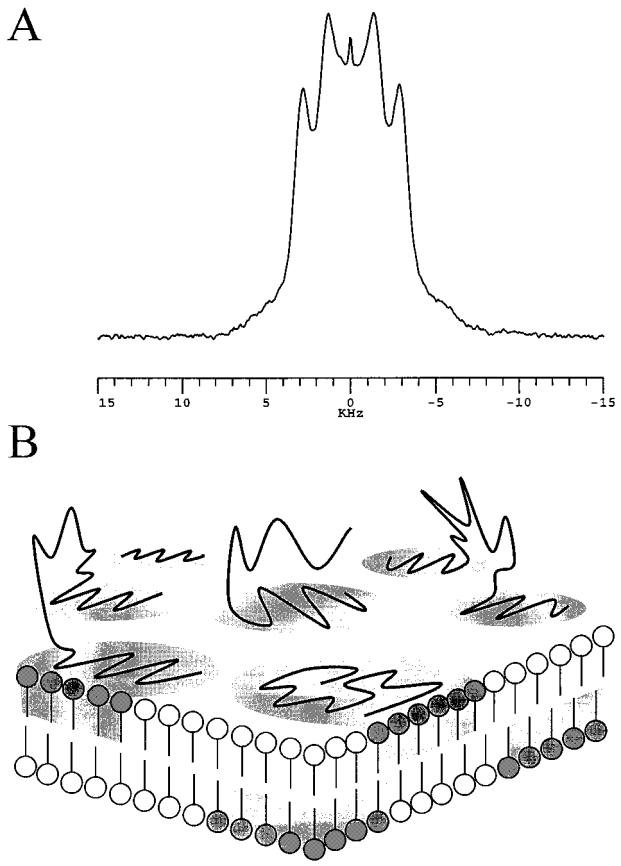

FIGURE 6. (A) ^{2}H NMR detection of electrostatically-induced demixing of DOTAP and POPC. Lipid bilayer MLVs composed of a 60:40 (mole: mole) mixture of POPC-β-d_2 plus DOTAP were exposed to a 20-mer of (anionic) poly(sodium styrenesulfonate). The two-component ^{2}H NMR spectrum indicates the presence of two separate POPC populations with different local surface charge environments. (B) A schematic of the polyelectrolyte-induced demixing of oppositely-charged-lipid-enriched versus -depleted domains.

lipid bilayer. Moreover, it can be shown that the PSSS-bound domains contain a 1:1 ratio of cationic DOTAP to anionic PSSS charges. From the relative areas of the two spectral components, one obtains the proportion of POPC contained within either domain. With the domain compositional information, this allows one to calculate the surface area occupied by the polyelectrolyte when bound to the membrane. For the case shown in Figure 6, the 4600 MW PSSS (number of monomers 22) produces a laterally-segregated domain composed of 22 DODAP monomers plus 22 POPC monomers, equivalent to a surface area of approximately 3000 Å^2.

A schematic summarizing the current picture of the polyelectrolyte-induced lipid domain structure is also shown in Figure 6. Binding of charged polyelectrolyte leads to the formation of oppositely-charged-lipid-enriched domains. Provided that excess charged lipid is available, the polyelectrolyte binds flat to the membrane surface, since a 1:1 cation:anion charge ratio is characteristic of the domains. When excess polyelectrolyte is present, "loops" and "trains" might well extend outward from the surface. The ^{2}H NMR data do not address directly the question of whether individual laterally-segregated domains aggregate into larger "patches" on the membrane surface. We note that the surface area occupied per polyelectrolyte, as calculated from ^{2}H NMR data, appears too small to account for the observation of slow exchange between bound and free domains,[37] implying that aggregation is likely. It is not yet known whether domain formation in one leaflet of the lipid bilayer correlates with domain formation in the opposite leaflet.

The greater significance of these ^{2}H NMR demonstrations is that it should now be possible to examine the role of variables such as polyelectrolyte molecular weight, hydrophilic−lipophilic balance, and membrane surface charge in determining the size and composition of electrostatically-induced domains, and to apply this understanding to biologically relevant situations.

Transbilayer Asymmetries of Surface Electrostatic Charge

In biological membranes the interior and the exterior surfaces are generally very different in composition, and biological organisms expend considerable effort in regulating and maintaining their distinct transbilayer asymmetries.[9] Until quite recently it has been rather difficult to distinguish the two faces of a lipid bilayer. Most ^{2}H NMR studies of lipid bilayers, for example, use MLVs as models of real membranes, for the very good reason that they are easy to prepare and yield high-quality spectra. However, the onionskin-like architecture of MLVs prevents their being used to generate transbilayer asymmetries. Instead one needs to employ unilamellar vesicles, such as that shown in Figure 1, wherein the aqueous medium is divided into two distinct compartments: that external to and that internal to the unilamellar vesicle. Likewise, the component phospholipids are divided into two, and only two, populations: those facing the vesicle interior and those facing the vesicle exterior. If giant unilamellar vesicles (GUVs) are employed, with diameters exceeding 500 nm, the ^{2}H NMR spectra are comparable to those obtained with MLVs.[38]

Using ^{2}H NMR, it is possible to resolve the interior and the exterior surfaces of such GUVs when the two differ with respect to their surface charge.[38] A simple means of creating a transbilayer surface charge asymmetry is to add an impermeant surface ligand, such as perchlorate anions or calcium cations, which binds to the lipid bilayer surface but does not permeate across the bilayer membrane.[29] Adding perchlorate or calcium to the exterior GUV solution produces a charged external membrane surface charge, leaving the interior surface electrostatically neutral. The charge at both surfaces may be monitored independently and simultaneously using ^{2}H NMR.[38]

Figure 7 shows the results of a more interesting transbilayer asymmetry of surface charge, in this case brought about by the transbilayer redistribution of a permeant surface ligand under the influence of a transmembrane potential $\Delta\Psi$.[39] The $\Delta\Psi$ alone produces no surface charge asymmetry, and therefore, one observes only a single quadrupolar splitting (top spectrum). When the potential sensitive surface ligand tetraphenylphosphonium (TPP^+) is added, it redistributes across the lipid bilayer in response to $\Delta\Psi$. For instance, if $\Delta\Psi$ is negative inside, then TPP^+ concentrates in the vesicle interior. TPP^+

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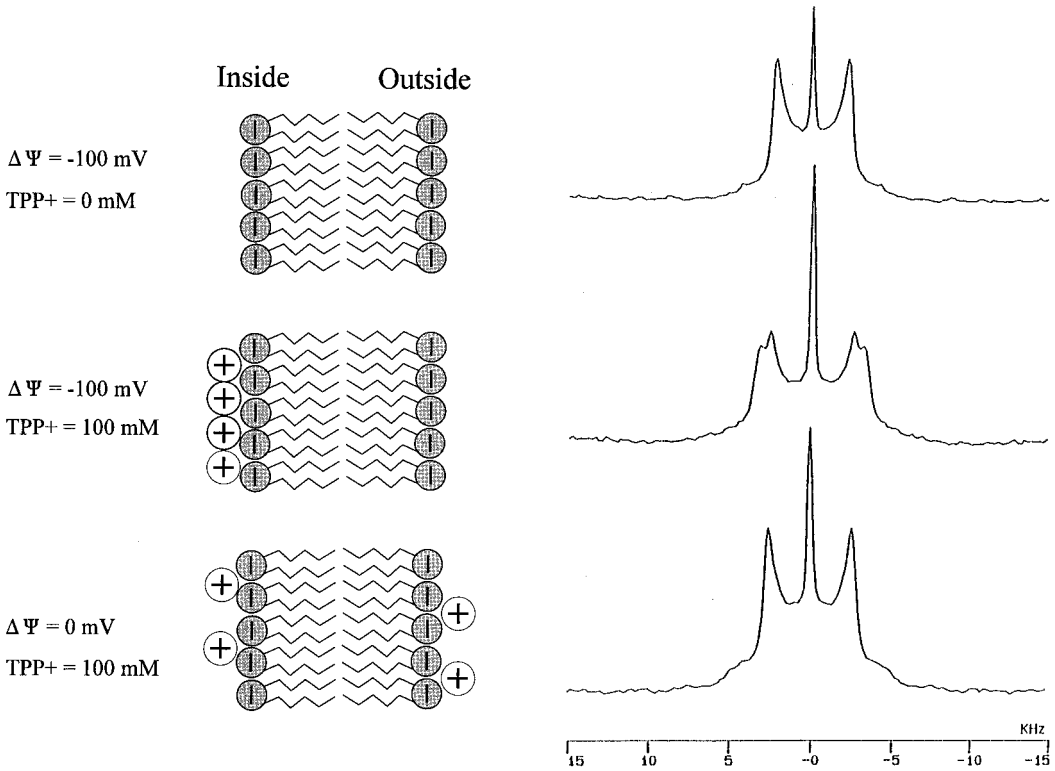

FIGURE 7. Resolving transbilayer asymmetries of surface charge in GUVs using ^{2}H NMR. The right-hand portion is reprinted with permission from ref 39. Copyright 1996 American Chemical Society. ^{2}H NMR spectra are shown for POPC-β-d_2 mixed 80:20 (mole:mole) with POPG (initially negative surface charge). $\Delta\Psi$ alone has little or no effect on the ^{2}H NMR spectrum. When TPP^+ is added, $\Delta\Psi$ (negative inside) causes it to redistribute to the vesicle interior, leading to greater TPP^+ binding to the interior vesicle surface, a different surface charge at the inner versus outer surface, and a ^{2}H NMR spectrum with two quadrupolar splittings. When $\Delta\Psi$ is dissipated, TPP^+ reequilibrates to equalize its concentration inside and outside the vesicle.

also binds to the available membrane surface, and the degree of binding depends on its concentration in the relevant aqueous compartment, among other factors. Consequently, the surface charge at the interior versus exterior face of the lipid vesicle differs, and one observes two quadrupolar splittings in the corresponding ^{2}H NMR spectrum (middle spectrum). The ratio of the integrated areas under the two Pake patterns producing the two quadrupolar splittings is virtually 1:1, since the phospholipid populations at the interior versus exterior face of the vesicle are equal. When $\Delta\Psi$ is entirely dissipated, TPP^+ reequilibrates so that its concentration is equal on either side of the vesicle, and one again observes a single quadrupolar splitting (bottom spectrum). One may construct a mathematical model relating the quadrupolar splittings at the interior versus the exterior vesicle surface with experimental variables such as $\Delta\Psi$, the vesicular trapped volume, the TPP^+ concentration, and the initial surface charge density. This allows a complete prediction of the expected resolution, permits optimization of conditions, and provides a benchmark against which to compare the behavior of other potential sensitive agents such as drugs, anaesthetics, and even peptides, proteins, and nucleic acids.[39]

Future Directions

The focus of this Account has been a description of "demonstration of concept" experiments, in which the current capabilities of the ^{2}H NMR method for measuring membrane surface electrostatic charge and the topography of surface charge distribution have been illustrated. Potential applications of these techniques are legion. The power to resolve transbilayer surface charge asymmetries, in particular, opens the door on an entirely new class of ^{2}H NMR experiment capable of monitoring protein insertion into membranes, or transmembrane "flip-flop" of amphiphiles, membrane fusion, DNA transmembrane transfer across the vesicle membrane, or pattern formation in vesicle templates. In fact, membrane fusion has already proved amenable to investigation via this ^{2}H NMR technique.[40]

The search for new capabilities continues. Space has not permitted a discussion of how the ^{2}H NMR molecular voltmeter technology has been transferred to the case of surface electrostatics in polymer colloids,[41] but one may anticipate successful transfers to other physical situations. New molecular voltmeters species should be possible, given that the most basic requirement of a large dipole moment is satisfied by a number of existing compounds, in particular the betaine and sulfobetaine surfactants in common use.

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