Magnetically Aligned Phospholipid Bilayers with Positive Ordering: A New Model Membrane System

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ABSTRACT A stable smectic phospholipid bilayer phase aligned with the director parallel to the magnetic field can be generated by the addition of certain trivalent paramagnetic lanthanide ions to a bicellar solution of dimyristoylphosphatidylcholine (DMPC) and dihexanoylphosphatidylcholine (DHPC) in water. Suitable lanthanide ions are those with positive anisotropy of their magnetic susceptibility, namely Eu^{3+}, Er^{3+}, Tm^{3+}, and Yb^{3+}. For samples doped with Tm^{3+}, this phase extends over a wide range of Tm^{3+} concentrations (6–40 mM) and temperatures (35–90°C) and appears to undergo a transition from a fluid nematic discotic to a fluid, but highly ordered, smectic phase at a temperature that depends on the thulium concentration. As a membrane mimetic, these new, positively ordered phospholipid phases have high potential for structural studies using a variety of techniques such as magnetic resonance (EMR and NMR), small-angle x-ray and neutron diffraction, as well as optical and infrared spectroscopy.

INTRODUCTION

Magnetically oriented bilayered micelles, or "bicelles" (Ram and Prestegard, 1988; Sanders and Schwonek, 1992; Sanders et al., 1994) possess great potential as model membranes for in-depth structural and dynamic studies of integral and peripheral membrane polypeptides by magnetic resonance and other spectroscopic techniques. Bicelles are relatively small (diameter ~10–100 nm), planar, bilayered aggregates that form in aqueous solution from mixtures of long- and short-chain phosphatidylcholines such as dimyristoylphosphatidylcholine (DMPC) and dihexanoylphosphatidylcholine (DHPC). The function of the short-chain molecules is to coat the edges of the bilayered sections to protect the longer phospholipid chains from exposure to water, serving the same role as bile salts do upon digestion of phospholipid membranes in vivo. Bicellar size is a function of the molar ratio q = [DMPC]/[DHPC] (Sanders and Schwonek, 1992; Vold and Prosser, 1996), and when $2 < q < 6$, a magnetically aligned phase is readily formed. This phase exhibits nematic discotic order and is stable over a wide range of lipid concentration (typically 3–40% w/v), temperature (30–45°C), ionic strength, and pH (Sanders and Schwonek, 1992). Among the many virtues of phospholipid bicellar samples are 1) their chemical and dynamical stability under physiologically reasonable conditions; 2) their relative ease of preparation; 3) their rapid, spontaneous macroscopic alignment in magnetic fields stronger than ~1 T; 4) their rich phase diagram, which permits biophysical studies under morphologically different, but chemically similar conditions; 5) their stability upon reconstitution with a variety of membrane-associated peptides and proteins (Sanders and Landis, 1994, 1995); and last but not least, 6) their capability of preserving enzyme activity (Sanders and Landis, 1994, 1995).

For structural studies of membrane constituents, the pure phospholipid bicelles are preferable over several lyotropic nematic media proposed in the past: potassium laurate/decanol/NaCl/water) (Lawson and Flautt, 1967; Forrest and Reeves, 1981; Davis, 1988), detergent-phospholipid mixtures (Jansson et al., 1990), and even the more recently developed phosphatidylcholine/bile salt (Ram and Prestegard, 1988) and phosphatidylcholine/3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxyl-1-propane sulfonate (CHAPSO) (Sanders and Prestegard, 1990) bicelles. This is true not only because of their stability and capacity to incorporate membrane proteins, but because the short-chain DHPC is chemically identical to the lipids of biological membranes. Diacylglycerol kinase, a 122-residue integral membrane protein from *Escherichia coli* possessing three transmembrane segments, is one example of an enzyme that retains its activity when reconstituted into bicelles (Sanders and Landis, 1995), and is inactive in conventional detergent micelles. Moreover, if the peptide or protein interacts with or inserts into the DMPC-rich planar region of the bicelle (Sanders and Schwonek, 1992; Vold and Prosser, 1996), the polypeptide is not exposed to any strain associated with membrane curvature.

The phospholipid bicelles may be used as membrane mimetics for structural studies in both the anisotropic and the isotropic phase. Recent results for a short test peptide, the wasp venom mastoparan *Vespula lewisii* (INLKALAALA KKIL) (Vold et al., 1996, 1997), have illustrated the potential of isotropic DMPC/DHPC bicellar solutions in high-resolution NMR. Furthermore, Sanders, Vold, Opella,

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and their co-workers (Sanders and Landis, 1994, 1995; DiNatale et al., 1995; Howard and Opella, 1996) have demonstrated that ^{2}H, ^{13}C, and ^{15}N NMR spectra of peptides and small proteins are well resolved and informative in the magnetically oriented nematic bicellar phase, in which chemical shifts and resolved dipolar and quadrupolar splittings may be exploited to yield structural information. Use of the oriented phase also provides an opportunity for identification and assignment of spectral features that may be followed into the isotropic phase by simply adding more short-chain phospholipid.

One potential limitation of magnetically oriented bilayers as a medium for structural studies is that the bicelles (like all phospholipid bilayers) align with $\bar{\mathbf{n}}$, their director or average normal, perpendicular to the magnetic field, **H**. In this orientation, which is characterized by a negative orientational order parameter, $S_{l\bar{n}} = -1/2$, a well-resolved NMR spectrum with "single-crystal-like" lines will only be obtained if the bicelle or the molecule of interest undergoes fast axially symmetric motion; otherwise NMR spectra will exhibit cylindrical powder patterns. Furthermore, because the magnetic susceptibility anisotropy of phospholipids and α-helical protein segments have opposite signs, the addition of proteins containing membrane-spanning α-helices may cancel the tendency for the bilayers to align at all. For these reasons, positive magnetic alignment, i.e., alignment of phospholipid bilayers with their director parallel to the magnetic field, i.e., $S_{l\bar{n}} = 1$, has been the goal of several research efforts. For example, Sanders et al. (1993) explored changing the sign of the anisotropy of the magnetic susceptibility of DMPC/CHAPSO bicelles by adding aromatic molecules. 1-Naphthol had the desired effect, but the amount of 1-naphthol needed to flip the bicelles to positive alignment was 30 mol% or higher, which the authors (Sanders et al., 1993) concluded represented an unacceptable chemical perturbation of the phospholipid bilayers. Triton X-100, the partly aromatic nonionic detergent often used in biochemical studies, failed to "flip" the bicelles.

Recently it was observed (Prosser et al., 1996) that the addition of small amounts (1–10 mol% relative to DMPC) of one of the paramagnetic lanthanide ions Eu^{3+}, Er^{3+}, Tm^{3+}, or Yb^{3+} causes DMPC/DHPC bicelles to change their orientation from negative order, i.e., aligned with the director $\bar{\mathbf{n}}$ perpendicular to the magnetic field, to positive order, where the director is parallel to the magnetic field. The formation of a new positively ordered bilayered phase was inferred from the doubling of all of the quadrupolar splittings of the perdeuterated myristoyl chains. Small-angle neutron scattering experiments subsequently confirmed (Katsaras et al., 1997) that by combining DMPC, DHPC, Tm^{3+}, and H_2O in a molar ratio of 4.6:1:0.12:580, one obtains a highly ordered, smectic liquid crystalline mesophase at 40°C and 2.6 T, and in this paper we discuss those properties of bicelles and lanthanide ions that induce positive alignment. We present deuterium NMR data from headgroup- and chain-perdeuterated DMPC, which serve to characterize the Ln^{3+}-ion doped bilayered liquid crystalline phase of DMPC:DHPC:H_2O as a function of temperature, magnetic field strength, DMPC/DHPC ratio, NaCl concentration, and lanthanide ion concentration. We have focused on Tm^{3+} as the agent of realignment, because this ion has the largest positive magnetic anisotropy of the four candidates listed above (Bleaney, 1972).

We first present a brief discussion of the magnetic properties of phospholipid bilayers and of trivalent lanthanide ions. Subsequently, experimental details are presented, followed by the presentation and discussion of our ^{2}H NMR results for chain-perdeuterated bicellar DMPC. At the end of that section, we present (briefly) the effect of Ln^{3+} on headgroup deuterons. Our conclusions and predictions for use of these new systems are summarized in the last section.

MAGNETIC PROPERTIES OF PHOSPHOLIPID BICELLES

The currently accepted model of the DMPC/DHPC bicelle is illustrated in Fig. 1. The long-chain phospholipid DMPC is assumed to form a disk-shaped bilayer aggregate with DHPC, partitioning itself primarily on the rim of the disk, thereby minimizing the exposure of the longer fatty acid chains to water. This construct is supported by the observation (Sanders and Schwonek, 1992) of distinct ^{31}P NMR resonances for DHPC and DMPC in magnetically ordered bicelles. DMPC was found to resonate upfield from DHPC, which is exactly what one might predict from the properties of the ^{31}P shielding tensor (Duncan, 1990) in phospholipid molecules confined to undergo lateral diffusion over, respectively, a flat and a curved cylindrical surface whose

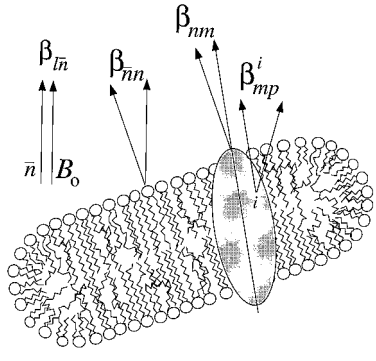

FIGURE 1 Cartoon of the Sanders bicelle formed in aqueous solutions of a mixture of long- and short-chain phospholipids at total lipid concentrations of 5–40% (w/v). The mesogenic unit is a disk-shaped, bilayered micelle (or stacks thereof), and the bicelle is shown to accommodate a test molecule—perhaps a membrane-spanning protein—with effective uniaxial symmetry. Also indicated are the angles that determine the orientational order parameters and the observed NMR spectral parameters: $\beta_{l\bar{n}}$ is the angle (here chosen to be 0°) between the average bicellar normal $\bar{\mathbf{n}}$ and $\mathbf{H}_0$ along the z axis in the laboratory frame (l); $\beta_{\bar{n}n}$ is the angle formed by $\bar{\mathbf{n}}$ and the instantaneous bicellar normal, $\mathbf{n}$, and β_{nm} represents the angle between $\mathbf{n}$ and an axis chosen to represent the molecule (m). Finally, β^{i}_{mp} represents the angle between this molecular axis and the principal axis (p) of the interaction tensor of interest, in this case, that of the electric field gradient at the site of deuteron i.

major symmetry axis is orthogonal to the magnetic field. A more quantitative assessment of bicellar morphology was obtained from the measurement of the order parameters for chain perdeuterated DMPC and DHPC (Vold and Prosser, 1996) in aligned samples that differed only in isotopic composition. The results clearly indicated that DMPC and DHPC partitioned exclusively to the planar center and to the surrounding rim of the bicelle, respectively.

When a sample containing deuterated phospholipids is macroscopically oriented, the deuterium spectrum normally consists of a collection of symmetrical doublets, characteristic of molecules undergoing fast, axially symmetrical motion. Coordinate systems and angles of orientation suitable for a proper description of uniaxial molecular and bicellar order, as well as the quadrupolar splittings observed in ^{2}H NMR spectra, are depicted in Fig. 1. The quadrupolar splitting, Δ^i_l, observed for the ith CD bond on the chain, may be accounted for in terms of 1) the average orientation, $\beta_{l\bar{n}}$, of the bicelle normal with respect to the magnetic field in the laboratory frame, l; 2) the angle, $\beta_{\bar{n}n}$, between the instantaneous, or local, bilayer normal $\mathbf{n}$ and the average bilayer normal, $\bar{\mathbf{n}}$; 3) the orientation, β_{nm}, of a molecular axis with respect to $\mathbf{n}$; and 4) the local angle, β^i_{mp}, between the molecular axis and the principal axis of the interaction tensor along the ith CD bond in a bicellar constituent. When the quadrupole coupling tensor is symmetrical, as is very nearly true for aliphatic deuterons, and when effective uniaxial symmetry pertains for an arbitrary probe molecule (represented in Fig. 1 by the prolate ellipsoidal object embedded in the bicelle), the observed splitting for deuteron i is given by

$$\Delta^i_l = \Delta^i_p \frac{1}{2}(3\cos^2\beta_{l\bar{n}} - 1)\frac{1}{2}\langle 3\cos^2\beta_{\bar{n}n} - 1\rangle \frac{1}{2} \cdot \langle 3\cos^2\beta_{nm} - 1\rangle \frac{1}{2}(3\cos^2\beta^i_{mp} - 1). \quad (1)$$

Parentheses are used in Eq. 1 to indicate time-independent quantities, and brackets refer to motionally averaged geometry. $\Delta^i_p = \frac{3}{2} e^2qQ/h$ is the splitting that would be observed for a stationary deuteron in a CD bond pointing along the external magnetic field. For deuterons bound to an sp^3-hydridized carbon, a value of 168 kHz is commonly used for the quadrupole coupling constant e^2qQ/h, and this value actually represents a time average over very fast vibrational motion. Equation 1 may be interpreted in terms of a product of uniaxial order parameters (Davis, 1981),

$$S^i_{lp} = S_{l\bar{n}}\, S_{\bar{n}n}\, S_{nm}\, S_{mp} = \frac{\Delta^i_l}{\Delta^i_p}. \quad (2)$$

Because $S_{l\bar{n}}$ specifies the ordering of the perfectly aligned bilayer relative to the magnetic field, "negative order" is represented by $S_{l\bar{n}} = -1/2$ for $\beta_{l\bar{n}} = 90°$ and "positive order" by $S_{l\bar{n}} = +1$ for $\beta_{l\bar{n}} = 0°$. At the more local level, we have chosen as the "molecular axis" the normal to the plane containing the three atoms in a methylene group, such that $\beta_{mp} = 90°$ and $S_{mp} = -1/2$. With these assignments, the remaining product of two order parameters, $S_{\bar{n}n}S_{nm}$, reflects the overall and internal mobility present in fluid bilayers (Marcelja, 1974; Jahnig, 1979; Dill and Flory, 1980).

In water, phospholipid membranes may spontaneously organize into uni- or multilamellar vesicles, the average radius of which depends on both the spontaneous radius of curvature associated with the particular lipid, and on the sample preparation history. As Helfrich and co-workers demonstrated (Boroske and Helfrich, 1978; Scholz et al., 1984), spherical vesicles may be deformed by magnetic fields into ellipsoids of revolution, so that a majority of the lipids are aligned with their long axes perpendicular to the field. The degree of macroscopic alignment depends on the relative magnitudes of the free energies of magnetic alignment and the total membrane curvature. The rate of realignment will be determined by the energy barrier associated with reorientation, which is a function of solution viscosity and concentration, as well as the size of the bilayered aggregates. The propensity of phospholipid bilayers to align in a magnetic field $\mathbf{H}$ is a consequence of the difference $\Delta\chi = \chi_\parallel - \chi_\perp$ between their volume magnetic susceptibilities parallel ($\chi_\parallel$) and perpendicular ($\chi_\perp$) to the long axes of the lipid molecules. The associated orientation-dependent component of the average Helmholtz free energy density of the membrane is given by (Scholz et al., 1984)

$$F(\beta_{ln}) = -\frac{1}{2}N\Delta\chi(\mathbf{n}\cdot\mathbf{H})^2 = -\frac{1}{2}N\Delta\chi H^2\cos^2\beta_{ln}, \quad (3)$$

where N is the number of molecules per unit volume. In their work on the microscopic observation of the realignment of cylindrical egg lecithin vesicles in a magnetic field, Boroske and Helfrich (1978) found $\Delta\chi = -(0.28 \pm 0.02) \times 10^{-8}$ erg cm^{-3} G^{-2} at 23°C, and subsequent work (Sakurai et al., 1980; Kawamura et al., 1981; Scholz et al., 1984) showed that the anisotropy arises mainly from the alkyl chains. It is readily seen from Eq. 3 that planar bilayers with $\Delta\chi < 0$ minimize the magnetic alignment free energy when, on average, the lipid long axes are perpendicular to the magnetic field ($\beta_{l\bar{n}} = 90°$).

The tendency of phospholipid bilayers to align in magnetic fields can actually be observed in some of the deuterium NMR phospholipid powder spectra published over the years (Brumm et al., 1992). It has also been deliberately exploited (Seelig et al., 1985; Speyer et al., 1987) in multilamellar phospholipid samples, but the alignment process is slow and unreliable in these highly viscous materials. The discovery of essentially spontaneous macroscopic magnetic alignment in 15–20% (w/v) aqueous samples of planar, mixed, bilayered micelles formed by phosphatidylcholine with bile salt (Ram and Prestegard, 1988), CHAPSO (Sanders and Prestegard, 1990), or DHPC (Sanders and Schwonek, 1992) was consequently greeted with enthusiasm by the NMR community. For such bicelles (Sanders and Landis, 1995), the alignment process is rapid, taking a few seconds, minutes, or at most an hour, depending on

sample composition (Prosser and Vold, unpublished observations). The combination of the two amphiphiles apparently results in the formation of a disk-shaped mesogenic unit that is small enough to lower the energy barrier associated with bilayer reorientation, and large enough to align when exposed to a magnetic torque. In addition, the bicellar systems are distinct from the vesicular phospholipid systems in which the vesicle undergoes deformation in the presence of a magnetic field (Scholz et al., 1984). The bicelles are presumably planar in the absence of the field, so there is no curvature free energy cost from bilayer deformation in the presence of the field.

The negative alignment of bicelles in a magnetic field is illustrated in Fig. 2 *A*. Symmetry arguments can be used to show that the bicellar DMPC/DHPC/H_2O solution forms a discotic nematic liquid crystal, i.e., a phase with no positional correlations between the mesogenic units, except perhaps locally. This phase may be envisioned as a family of disks suspended by strings of different length parallel to $\mathbf{H}_0$ and, as indicated in Fig. 2 *A*, the bicellar normals are randomly and uniformly distributed about the magnetic field direction. However, as demonstrated recently (Prosser et al., 1996), trivalent lanthanide ions with suitable magnetic properties can be used to realign the bilayers, so that the average orientation of their normals switches from $\beta_{l\bar{n}} = 90°$ to $\beta_{l\bar{n}} = 0°$. As illustrated in Fig. 2 *B* and discussed below, at high enough Ln^{3+} concentration, this new phase is smectic.

The trivalent lanthanide ions capable of "flipping" the bicelles from $S_{l\bar{n}} = -1/2$ to $S_{l\bar{n}} = +1$ are Eu^{3+}, Er^{3+}, Tm^{3+}, and Yb^{3+}, the same four ions whose substituted tris(acetylacetonate) chelates are used as "downfield shift reagents." The shift reagents (Hinckley, 1969) were recognized (Kurland and McGarvey, 1970; Bleaney, 1972; Horrocks, 1973) to come in two flavors, those that shifted nuclear resonances upfield, and those that shifted them downfield. For the lanthanides, the origin of the paramagnetic shift in high-resolution NMR spectra is predominantly dipolar, and for an effectively uniaxially symmetrical ligand field, the relation between magnetic susceptibility anisotropy and direction of shift is given (in ppm) by Kurland and McGarvey as

$$\frac{\Delta\nu}{\nu_0} = -\frac{\Delta H}{H} = \frac{\Delta\chi}{3r^3}(3\cos^2\beta - 1). \quad (4)$$

Here β is the angle between the principal axis of χ and the vector $\mathbf{r}$ between the ion and the nuclear spin, and it is readily seen that a nuclear spin located on or near the symmetry axis of the complex will experience deshielding, i.e., a downfield shift, when $\Delta\chi > 0$. The sign of $\Delta\chi$ is given directly by the sign of certain matrix elements required for calculation of the crystal field splittings for lanthanide ion complexes (Bleaney, 1972), and whereas the magnitude of the crystal field perturbation depends on the chemical properties of a specific complex, the sign is determined by the orbital and spin angular momenta of the ion itself. For the reader's convenience, the relevant coefficients, a_2^J, are listed in Table 1 with other magnetic properties of the rare earth ions. Table 1 shows that for Er^{3+}, Tm^{3+}, and Yb^{3+}, a_2^J is positive and, apparently, large enough to overcome the negative magnetic anisotropy of the bicelle itself. For Eu^{3+}, whose electronic ground state is diamagnetic, the positive magnetic anisotropy is a property of the $J = 1$ and $J = 2$ excited states that are populated at room temperature.

The most authoritative description of the magnetic properties of 4*f* ions and the effects of ligand perturbations may be found in the 1970 EMR text by Abragam and Bleaney (1970), and the relation between the magnetic susceptibility tensor and the paramagnetic shift in NMR spectroscopy can be found in papers by McGarvey (Kurland and McGarvey, 1970; McGarvey, 1979).

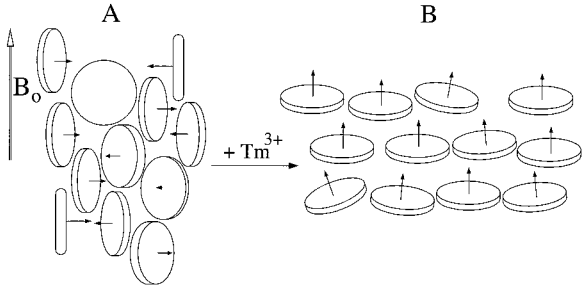

FIGURE 2 Long-range organization of bicelles with $\Delta\chi < 0$ (*A*) and $\Delta\chi > 0$ (*B*). In *A* the bicellar normals **n** form a cylindrical distribution, thereby forming a discotic nematic phase with order parameter $S_{l\bar{n}} = -1/2$, whereas those in *B* all align parallel to the field, i.e., with $S_{l\bar{n}} = +1$. The bicelles on the right are shown organized into layers, representing a smectic mesophase recently found by neutron diffraction measurements (Katsaras et al., 1997) on a sample with a high Tm^{3+} concentration. Note that the relative disk size, thickness, and spacing are not drawn to scale.

MATERIALS AND METHODS

Sample preparation

Both the normal phospholipids, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphatidic acid (DMPA) and 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC), as well as those labeled with deuterium, chain-perdeuterated DMPC (DMPC-d_{54}), and DHPC (DHPC-d_{22}), single-chain labeled DMPC (DMPC-d_{27}) and choline-deuterated DMPC (DMPC-d_{13}) were purchased from Avanti Polar Lipids (Alabaster, AL). Typical NMR samples were prepared with mostly regular phospholipids, with each sample containing just a small fraction of the deuterated analog. It should be noted that chain-deuterated phospholipids received from Avanti often contain significant amounts of material in which the α-deuterons are exchanged with protons.

For NMR sample preparation, we initially simply weighed off the phospholipids in a reasonably dry environment. DHPC is extremely hygroscopic, however, and even with precautions, we found the DHPC content of early samples to be considerably below that planned. NMR samples are now routinely prepared from aqueous DHPC stock solutions prepared by immediately adding a known amount of deuterium-depleted H_2O to dry DHPC in the manufacturer's (preweighed) vial. This procedure allows for at least some consistency in sample preparation, although the ratio of DMPC to DHPC is still likely to be slightly higher than anticipated. It is consequently advisable to determine the relative amounts of the bicellar constituents by integration of ^{31}P NMR spectra of the samples.

TABLE 1 Magnetic properties of trivalent lanthanide ions*

Ion	State		g_L	$\mu_J = g_L\sqrt{J(J+1)}$[#] (calc)	μ_J[#] (obs)	CF coupling terms	
						a_2^J[§]	$a_2^J\langle J,M_J \mid \mathbf{O_2^0} \mid J,M_J\rangle$[¶]
La^{3+}		$^1S_0$	0/0	0.00	0.0		
Ce^{3+}	$4f$	$^2F_{5/2}$	6/7	2.54	2.51	−0.0571	−11.8
Pr^{3+}	$4f^2$	$^3H_4$	4/5	3.58	3.53	−0.0210	−20.7
Nd^{3+}	$4f^3$	$^4I_{9/2}$	8/11	3.62	3.55	−0.00643	−8.08
Pm^{3+}	$4f^4$	$^5I_4$	3/5	2.68	—	+0.00771	+4.28
Sm^{3+}	$4f^5$	$^6H_{5/2}$	2/7	0.84	1.46	+0.0413	+0.943
Eu^{3+}	$4f^6$	$^7F_0$	0/0	0.00	3.37	‖	
Gd^{3+}	$4f^7$	$^8S_{7/2}$	2	7.94	8.0	0	
Tb^{3+}	$4f^8$	$^7F_6$	3/2	9.72	9.3	−0.0101	−157.5
Dy^{3+}	$4f^9$	$^6H_{15/2}$	4/3	10.65	10.5	−0.00635	−181
Ho^{3+}	$4f^{10}$	$^5I_8$	5/4	10.61	10.4	−0.00222	−71.2
Er^{3+}	$4f^{11}$	$^4I_{15/2}$	6/5	9.60	9.5	+0.00254	+58.8
Tm^{3+}	$4f^{12}$	$^3H_6$	7/6	7.56	7.3	+0.0101	+95.3
Yb^{3+}	$4f^{13}$	$^2F_{7/2}$	8/7	4.53	4.5	+0.0318	+39.2
Lu^{3+}	$4f^{14}$	$^1S_0$	0/0	0.00	0.0		

*Data collected from tables presented by Herzberg (1944) and Bleaney (1972) for the lanthanide ions in their ground electronic state.
[#]The magnetic moments are given in units of the Bohr magneton, $\beta = 9.2741 \times 10^{-24}$ JT^{-1}.
[§]The coefficient a_2^J is referred to in the literature variously as a_2, α, and $\langle J\|\alpha\|J\rangle$. a_2^J and related coefficients are given for all values of J in table 20 in appendix B of Abragam and Bleaney (1970).
[¶] $\langle J, M_J \mid \mathbf{O_2^0} \mid J, M_J\rangle = g_L^2 J(J+1)(2J-1)(2J+3)$, and $\mathbf{O_2^0}$ is the equivalent of an NMR spin operator.
‖Paramagnetism originates in the $J = 1$ and $J = 2$ states, which are populated at room temperature and have positive values of a_2^J. Sm^{3+} also has a low-lying $J = 7/2$ state, which explains the higher experimental value of its μ_J.

Deuterium-depleted water (Cambridge Isotopes, Cambridge, MA) was used for all of the NMR samples, which contained 20% (w/v) total lipid. The lanthanide chloride hexahydrates (99.99–99.999%, Aldrich Chemicals, Milwaukee, WI) $[LnCl_2(H_2O)_6]Cl$ of yttrium, neodymium, holmium, europium, erbium, thulium, and ytterbium were dissolved in a small amount of deuterium-depleted water to yield 2.0 M solutions. When needed, aliquots of these solutions were added to the phospholipid solutions to give samples with the desired DMPC/Ln^{3+} ratio.

Unless otherwise mentioned, DMPC/DHPC ratios of $q = 3.2$ or 4.6 were used for samples discussed in this paper. Transparent phospholipid solutions (with or without lanthanide ions) were typically obtained in less than an hour by stirring or vortexing, heating to 40°C for 20 min, followed by cooling to 4°C for 20 min. The cooling part of the cycle is particularly useful, because the viscosity of the phospholipid solutions drops significantly below room temperature. In some instances, centrifugation and/or sonication was used with the aim of speeding up the sample clarification process, but this type of treatment had no discernible effect on the final sample. Finally, the solution was chilled to near 0°C, where the viscosity is ~1 cP (=1 mPa s) (Prosser et al., unpublished), 150 μl was pipetted into a short 5-mm NMR tube, and the sample was allowed to equilibrate at the desired temperature in the magnetic field.

Apparatus and NMR measurements

^{2}H quadrupole echo NMR spectra were acquired at either 55.3 MHz or 38.4 MHz. At 55.3 MHz, a General Electric GN500 spectrometer equipped with an 8.5-T Oxford Instruments wide-bore magnet, an ENI LPI-10 rf amplifier, and a Tecmag Libra spectrometer control system was employed. At 38.4 MHz, a Chemagnetics CMX-250/360 spectrometer was used, along with an ENI LPI-10 rf amplifier and a 5.9-T Oxford Instruments wide-bore magnet. The magnetic field homogeneity was not optimized regularly, so that the ^{2}H linewidth of D_2O was typically around 50–100 Hz, compared with the phospholipid linewidths of 300–400 Hz. Home-built probes delivering 2.0–2.5-μs 90° pulses to the sample via a 12-turn solenoid coil were used at either field. Both the normal $\pi/2$-τ-$\pi/2$-acq quadrupole echo sequence as well as the 4-pulse $\pi/2$-$\tau/2$-π-$\tau/2$-$\pi/2$-$\tau/2$-π-$\tau/2$-acq (Siminovitch et al., 1984) solid echo pulse sequence with $\tau = 50$ μs were used during these measurements. However, both paramagnetic shifts and quadrupolar splittings are modest for these samples, and comparisons showed that use of the 4-pulse sequence was unnecessary. A 0.5- or 1-s repetition time was used to acquire 500–5000 transients, which were processed with a 100- or 150-Hz exponential line broadening.

Tests for phospholipid exchange between isotropic and ordered components in DMPC/DHPC/Tm^{3+} samples were performed by selectively inverting the isotropic resonance and monitoring the spectral difference between π_{sel}-τ-$\pi/2$-τ_1-$\pi/2$ and 0-τ-$\pi/2$-τ_1-$\pi/2$ pulse sequences.

Determination of smoothed order parameter profiles

In some cases the orientational order of the phospholipid chains was determined by following the approach of Lafleur et al. (1989). This approach assumes a monotonic decrease of S^i_{mp} with increasing carbon number, i, and can be used to avoid the dilemmas presented by the presence of overlapping resonances from the alkyl chain CD_2 groups. The well-resolved innermost doublet, which arises from the terminal methyl groups, can be treated separately. The splitting of this doublet was multiplied by 3 before calculation of the order parameter, S^{14}_{lp}, to compensate for the rapid rotation of the methyl group about the ^{13}C–^{14}C bond. The remaining spectrum corresponds to 12 methylene groups per DMPC chain, and can consequently be divided into 12 doublet regions, each representing four CD_2 deuterons. To define the doublets, the spectrum was simultaneously integrated from the outermost peak on the left and the outermost peak on the right, proceeding inward. The area associated with each segment of the spectrum can be given the value 2, when the area under the methyl peaks is assigned the reference value 3. The order parameter S^i_{lp} for each segment of the chain(s) can then be defined as the splitting between the centroids of the first moment of each half of each spectral region. In this approximation, the differences in the $sn-1$ and $sn-2$ chains are neglected, as is the local oscillatory variation of S^i_{mp} for the first few carbon positions (Seelig and Seelig, 1980; Lafleur et al., 1989). The order parameters, $S^i_{lp} = \frac{2}{3}\Delta^i/e^2qQ/h$, were obtained from the splittings by using $e^2qQ/h = 168$ kHz.

RESULTS AND DISCUSSION

Effects of Ln^{3+} on DMPC chain deuteron spectra

^2H quadrupole echo NMR spectra of $q = 4.6$ DMPC/DHPC bicelles containing chain-deuterated DMPC-d_{54} and a variety of trivalent cations at different concentrations were obtained at 37°C for samples with $[DMPC]/[Tm^{3+}] = 10$. As illustrated in Fig. 3, the spectra are all characteristic of a well-aligned liquid crystalline bilayer in coexistence with a small amount of isotropic phase and, perhaps, some HOD. (We shall comment further on the isotropic component below.) Spectrum A of the undoped, negatively aligned bicelles is quite typical of DMPC-d_{54} containing bicelles and very similar to ^2H NMR spectra published previously (Sanders and Schwonek, 1992). Fig. 3 clearly reveals that the addition of Eu^{3+}, Er^{3+}, Tm^{3+}, and Yb^{3+} (spectra E–H) has the effect of increasing the quadrupolar splittings by a factor of ~2. In contrast, the addition of trivalent ions such as the diamagnetic ion Y^{3+} (B) and the paramagnetic ions Nd^{3+} and Ho^{3+} (C and D) leads to only a marginal increase in the splittings. To account for these observations, we

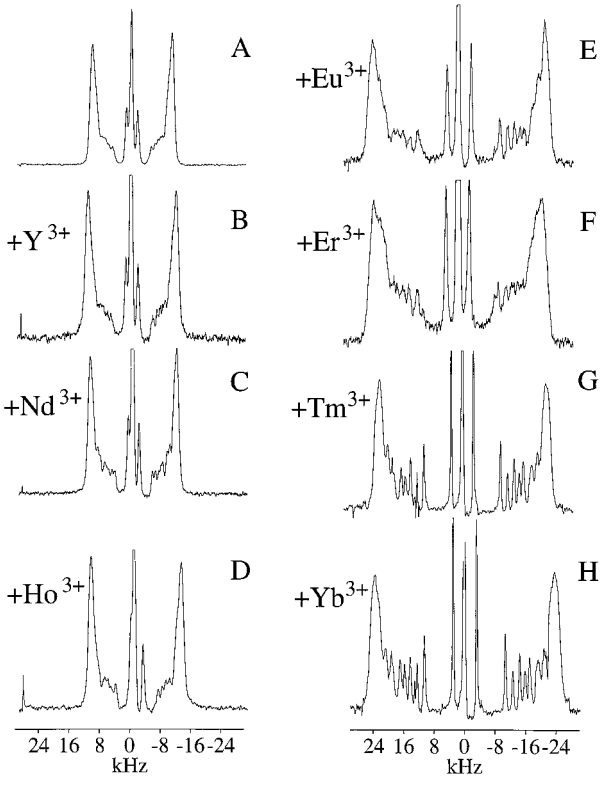

FIGURE 3 Deuterium quadrupole-echo spectra recorded at 37°C of DMPC-d_{54} in magnetically aligned DMPC/DHPC bicellar samples with 80% water (w/v) and $q = 4.6$. Spectrum A was obtained from an undoped sample; spectra B–H were from samples containing the indicated lanthanide ion in a ratio of Ln^{3+}/DMPC = 0.10. The magnetic field strength was 8.5 T (55.3 MHz) for all but G and H, which were obtained at 5.9 T (38.4 MHz). Note that spectrum F was obtained from a sample to which a small amount (DMPC/DMPA = 15:1) of dimyristoyl phosphatidic acid was added. The effects of including this minor amount of acidic phospholipid are discussed in the text.

define the observed quadrupolar splitting for deuterons on the ith carbon as

$$\Delta_l^i = \Delta_l^{i,0} + \delta\Delta_l^{i,1} + \delta\Delta_l^{i,2}, \quad (5)$$

with the three terms referring to the splitting observed in samples without trivalent ions ($\Delta_l^{i,0}$), to the incremental splitting observed in samples with trivalent ions that cause no realignment ($\delta\Delta_l^{i,1}$), and an additional increment observed in samples containing lanthanide ions that do ($\delta\Delta_l^{i,2}$).

The small increase (5–10%) in quadrupolar splitting observed in spectra B–D is of local origin and is assigned to $\delta\Delta_l^{i,1}$. Effects of di- and trivalent ions on the NMR spectra of phospholipids were first detected in 1973 (Levine et al., 1973) and subsequently discussed by several groups (e.g., Hauser et al., 1976, 1981; Brown and Seelig, 1977; Akutsu and Seelig, 1981). The increase was suggested to be due to tighter packing of the alkyl chains (Levine et al., 1973), induced by the ligation of neighboring phosphate groups to one ion, and the resulting reduction in surface area appears to be facilitated by a conformational change in the phospholipid headgroup (Brown and Seelig, 1977; Akutsu and Seelig, 1981). The strength of the trivalent-ion/lipid binding varies with the nature of the headgroup, with pH, and with the ionic strength of the solution, and for phosphatidylcholines and trivalent ions, effective binding constants in the range 100–1000 M^{-1} have been observed (Lin, 1980). On the basis of these earlier reports, we concluded (Prosser et al., 1996) that the small increase in Δ_l^i exhibited by the ^2H NMR spectra of DMPC/DHPC bicellar samples containing Y^{3+} ions arose (see Eq. 2) from an increase in the molecular order parameter S_{nm}, the local bilayer order parameter $S_{\bar{n}m}$, or both, i.e., in $S_{\bar{n}m}$. Similar effects can be observed in Fig. 3 for the DMPC spectra C and D of DMPC/DHPC bicelles doped with the paramagnetic ions Nd^{3+} and Ho^{3+}.

In contrast to these more subtle effects, the addition of sufficient Eu^{3+}, Er^{3+}, Tm^{3+}, or Yb^{3+} to a bicellar DMPC/DHPC solution causes the quadrupolar splittings to double. As shown in Fig. 3, for spectra E–H, the factor is 2.1–2.2, undoubtedly a combination of two effects: 1) a local increase in ordering, reflected in the splitting $\Delta_l^{i,1}$, in the phospholipid order parameter $S_{\bar{n}\bar{m}} = S_{\bar{n}n}S_{nm}$, just as in spectra B–D; and 2) a realignment of the average bilayer director such that it lies parallel to the field. The latter changes the average bilayer order parameter $S_{l\bar{n}}$ from $-1/2$ to $= +1$, with the result that $\delta\Delta_l^{i,2} = |\Delta_l^{i,0}|$. This 90° flip is induced by the binding of trivalent lanthanide ions with positive magnetic anisotropy to the phosphate groups of the phospholipid molecules, and in the process, the anisotropy of the bicellar magnetic susceptibility changes sign. As noted in the theoretical summary, the sign of $\Delta\chi$ for the lanthanide ions is determined by the sign of their crystal field coupling terms (Abragam and Bleaney, 1970), specifically, the sign of the coefficients a_2^J. For Er^{3+}, Tm^{3+}, and Yb^{3+} the electronic ground state suffices for a proper description of their magnetism, whereas for Eu^{3+} the positive anisotropy is a property of populated excited levels with $J = 1$ and $J = 2$.

Returning to the effect of the ions Nd^{3+} and Ho^{3+} upon the DMPC ^2H NMR spectra, it should be noted that the quadrupolar splittings Δ_l^i in spectra C and D within experimental error are no larger than in spectrum B of the sample containing Y^{3+}. In other words, $\Delta_l^{i,1}$ is identical for diamagnetic ions and paramagnetic ions. Ions with very large negative $\Delta\chi$ might be expected to reinforce the negative diamagnetic susceptibility of the phospholipids, thereby increasing the magnetic torque that drives the bicelles toward negative alignment. The fact that such an increase in Δ_l^i is not observed for either Nd^{3+} or, especially, Ho^{3+} simply means that the incremental energy of magnetic alignment for $q = 4.6$ discotic nematic bicelles is small compared with kT at 37°C.

The quadrupolar splittings for the plateau region, the two terminal CD_3 groups, and the ultimate ($i = 13$) CD_2 groups were measured at 37°C as a function of Ln^{3+} concentration for $q = 4.6$ DMPC/DHPC bicellar solutions containing Ho^{3+}, Y^{3+}, and Eu^{3+}. Normalized order parameters $S_{\bar{n}p}^i = S_{lp}^i/S_{l\bar{n}}$ were calculated from the splittings using Eq. 1 and $e^2qQ/h = 168$ kHz and plotted in Fig. 4. By dividing the observed order parameters S_{lp}^i for the ith carbon of the alkyl chains with $S_{l\bar{n}} = -1/2$ or $+1$, we remove from consideration whether samples have positive or negative order with respect to the magnetic field. In other words, $S_{\bar{n}p}$ reflects properties internal to the bicelle, including disorder of individual bicelle normals relative to the sample director $\bar{\mathbf{n}}$, motion of individual phospholipid molecules, and the orientation of particular CD bonds relative to a molecular axis. Inspection of Fig. 4 shows that the normalized order parameters $S_{\bar{n}p}^i$ are nearly identical for the three samples, regardless of the magnetic properties of the ion. The small differences observed are most likely due to inconsistencies in sample preparation, because the NMR samples were prepared before we appreciated the extreme hygroscopic nature of DHPC. Given the similar binding constants and other chemical properties of the rare earths, this is not particularly surprising, and we consider it highly likely that the individual order parameters, $S_{\bar{n}n}$ and S_{nm}^i, are also identical for the three kinds of ion at the same temperature and concentration. Note, however, the increase in resolution observed in spectra G and H in Fig. 3 for the samples doped with Tm^{3+} and Yb^{3+}, the ions with the largest positive magnetic anisotropy. (It should be noted that the spectrum in Fig. 3 *F* was obtained after adding not only Er^{3+}, but also a small amount of the anionic phospholipid, dimyristoyl phosphatidic acid, or DMPA, to DMPC/DHPC bicelles. It was hoped that the addition of a small amount of negatively charged lipid might improve the binding efficacy of the lanthanide to the surface. Instead, we observed that the sample turned white (opaque) and heterogeneous over long periods of time, presumably because of phase separation.) This change in appearance hints that subtle morphological changes occur for positively ordered samples with high Ln^{3+} ion concentration.

To investigate this phenomenon further, we carried out an NMR study of the effects of temperature and ion concentration on the ^2H DMPC spectra of Tm^{3+}-doped, magnetically aligned $q = 3.2$ DMPC/DHPC samples. Tm^{3+} was chosen because, according to Table 1, this ion has the largest positive value of a_2^J and may therefore be expected to have the greatest magnetic impact at the lowest concentration. Fig. 5 presents, in a 4×4 matrix, a subset of ^2H NMR spectra obtained at 5.9 T in one such study. In row 1 are shown two spectra of undoped bicellar samples; spectra 1,3 and 1,4 are missing, because the bicellar phase is unstable at temperatures above 45°C. The effects of adding Tm^{3+} ions are illustrated in rows 2–4: the quadrupolar splitting doubles, and between 35°C and 65°C, the spectra are all characteristic of a well-aligned liquid crystalline phase in equilibrium with varying amounts of an isotropic component. The spectral resolution is seen to increase dramatically with increasing temperature and increasing ion concentration, in concert with the growth and disappearance of the isotropic resonance. Ultimately, at high temperature and Tm^{3+} concentration, the isotropic peak vanishes, but at this point (see spectrum 4,4) the samples begin to deteriorate irreversibly.

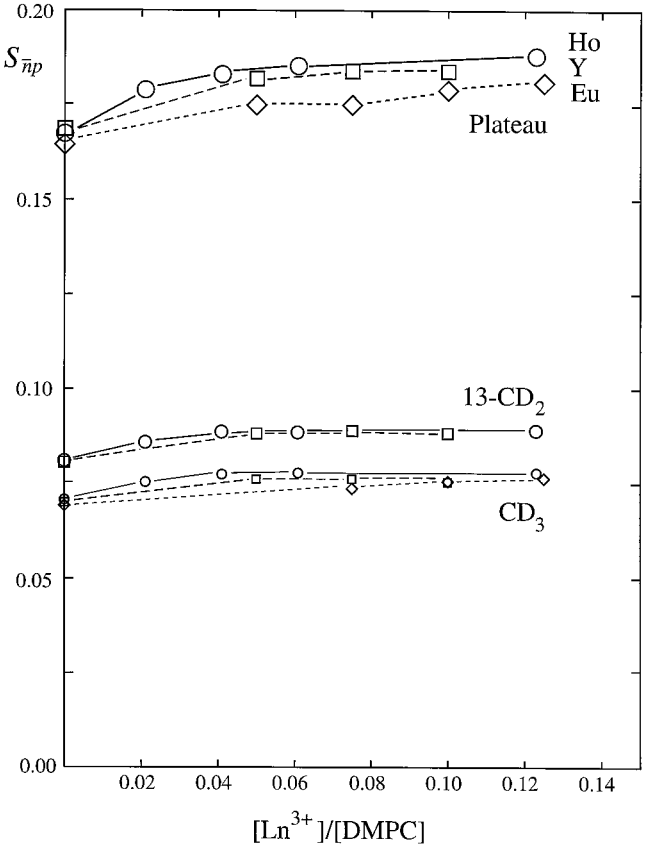

FIGURE 4 Orientational order parameters $S_{\bar{n}p} = S_{lp}/S_{l\bar{n}}$ for $q = 4.6$ DMPC/DHPC bicelles as a function of Ln^{3+} concentration. Depicted are order parameters for titration with the trichlorides of Ho^{3+} (*circles*), Y^{3+} (*squares*), and Eu^{3+} (*triangles*) at 37°C. The decreasing size of the symbols is associated with (in order) $S_{lp}/S_{l\bar{n}}$ for the plateau region, the terminal CD_2 group, and the CD_3 group. The order parameters were obtained from series of spectra, including B, D, and E in Fig. 3, and the quadrupolar splitting of the CD_3 group was multiplied by 3 before calculation of the order parameter to eliminate the (trivial) effect of methyl rotation.

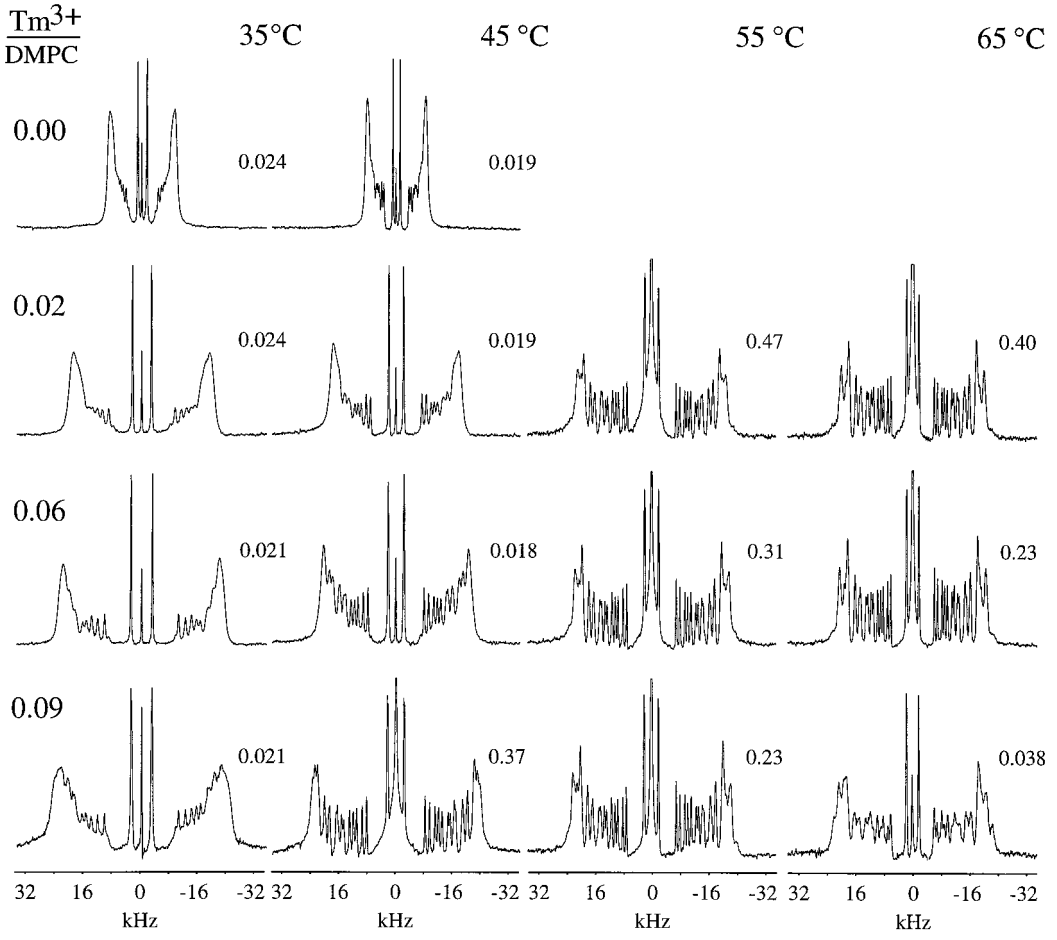

FIGURE 5 Deuterium solid echo spectra of DMPC-d_{54} recorded at 38.4 MHz as a function of temperature and Tm^{3+} concentration. The smaller numbers immediately to the right of each spectrum indicate the ratio of the intensities of the isotropic and oriented components.

It is important in this context to point out that the spectra presented in Fig. 5 were consistently recorded as a function of increasing temperature. This was done simply to avoid losing a valuable sample to overheating before the necessary measurements had been made. However, upon cooling the samples in the magnetic field, we later found that the smectic phase is stable at any temperature above the lower lying isotropic phase.

To ascertain that maximum alignment was reached at 5.9 T (38.4 MHz) and to test the reproducibility of the data presented in Fig. 5, deuterium quadrupole-echo spectra were recorded for two additional Tm^{3+}-doped DMPC/DHPC samples as a function of temperature and Tm^{3+} concentration at 8.5 T (55.3 MHz) and 12.9 T (84.4 MHz). The spectra (not shown) were found to be identical to those obtained at 5.9 T, showing that this new phase is stable over the temperature and concentration range investigated, and that 5.9 T is well above any threshold value of the magnetic field required for realignment. Furthermore, to determine whether exchange takes place between isotropic and aligned DMPC-d_{54}, a series of magnetization transfer experiments were conducted at 46°C on a DMPC-d_{54}/DHPC/Tm^{3+} sample with Tm^{3+}/DMPC = 0.13. The experiments involved inverting the isotropic peak with a 150-μs π-pulse and monitoring the change in intensity of the oriented component over a wide range of transfer times from 0.5 ms to 1 s. No evidence of exchange between the isotropic and oriented phases was found.

Fig. 6 depicts the temperature and thulium ion concentration dependence of the reduced orientational order parameter profiles for the myristoyl chains derived from the spectra in Fig. 5. Rather than plotting the normalized order parameter $S^{i}_{\bar{n}p}$ for selected resonances, we plot "smoothed" values (Lafleur et al., 1989) of $S^{i}_{\bar{n}p}$ as a function of carbon number i, as described in Materials and Methods. The addition of Tm^{3+} has the effect of scaling $S^{i}_{\bar{n}p}$ without significantly altering its shape, indicating that the L_α phase is preserved. However, for a given [Tm^{3+}]/[DMPC], the order parameter profiles are all indicative of a liquid crystalline bilayer phase whose classic plateau region gradually diminishes with increasing temperature (Lafleur et al., 1990a,b; Monck et al., 1992), whereas the mobility at the end of the chains increases. We also note that at the highest temperatures, increasing amounts of Tm^{3+} cease to affect the order parameter profile; in other words, a [Tm^{3+}] threshold value has been reached. At this point the samples

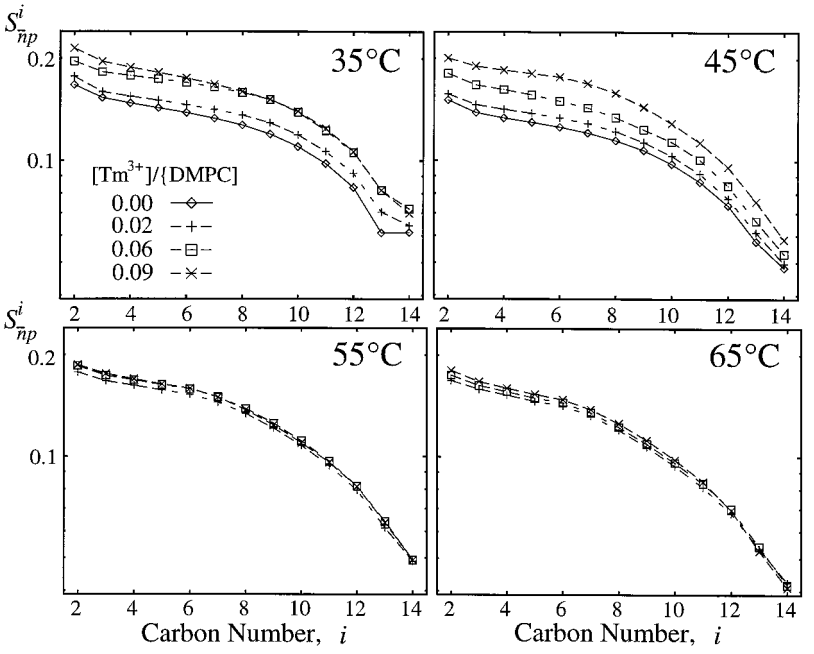

FIGURE 6 Reduced, smoothed chain-order parameter profiles corresponding to the DMPC-d_{54} spectra in Fig. 5. The order parameters were obtained as described in Materials and Methods, using e^2qQ/h = 168 kHz and η = 0.

become turbid, which together with the sudden growth of a significant isotropic peak, signals the onset of phase separation.

It is instructive to compare the temperature dependence of the order parameter profile as a function of Tm^{3+} concentration; results from the complete 5.9 T data set are presented in Fig. 7. For simplicity, Fig. 7 displays the temperature dependence of the normalized order parameter averaged over the 13 quadrupolar splittings characteristic of the myristoyl chain, in other words, $\langle S^i_{\bar{n}p} \rangle = (1/13)\Sigma_{i=2}^{14} S^i_{\bar{n}p}\rangle$ is plotted versus i for each of the four Tm^{3+} concentrations. As in Fig. 6, data obtained for the Tm^{3+}-free sample are indicated by diamonds; upon addition of 2 mol% Tm^{3+}, $\langle S^i_{\bar{n}p}\rangle$ increases by the 5–10% expected from the local ordering effects of trivalent ions. Below 50°C, $\langle S^i_{\bar{n}p}\rangle$ decreases in normal fashion with increasing temperature for this sample, but the decrease is interrupted just above 50°C by a prominent increase, followed again by a gradual decrease. The data for [Tm^{3+}]/[DMPC] = 0.06 (*squares*) show an overall large increase in order even at lower temperatures, and then exhibit a similar, if less prominent, reversal of the normal drop with temperature at 45°C. The very weak dip observed at 35°C for the [Tm^{3+}]/[DMPC] = 0.09 sample (*crosses*) may even be considered to indicate a morphological change, but only in the context of the resolution change observed in the fourth row of Fig. 5.

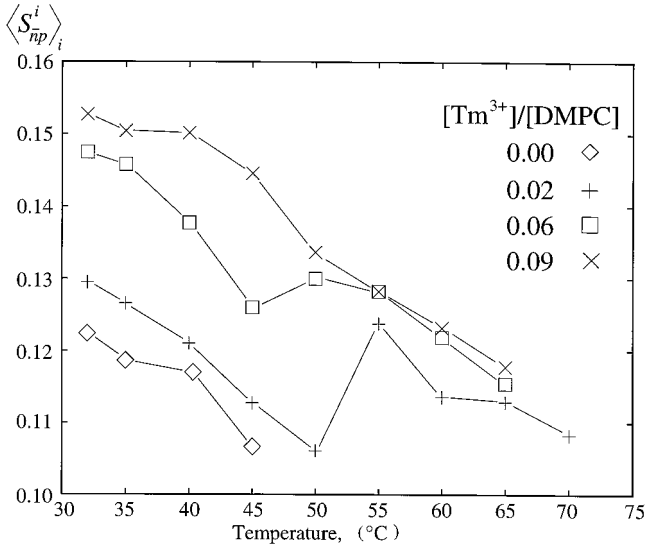

FIGURE 7 Temperature dependence of average, reduced order parameter $\langle S^i_{lp}\rangle = (1/13)\ \Sigma_{i=2}^{14}\ S^i_{lp}/S_{l\bar{n}}$ for the alkyl chains in q = 3.2 DMPC/DHPC bicelles with Tm^{3+}/DMPC ratios of 0.0, 0.02, 0.06, and 0.09.

We conclude that the onset of highly resolved DMPC-d_{54} spectra, the appearance of a significant isotropic peak, and the onset of turbidity signal a conversion from a positively aligned nematic to a positively aligned smectic phase in coexistence with an isotropic phase. The higher degree of order, as well as the higher resolution revealed in Fig. 5, should be a signature of well-ordered smectic layers expected to be better ordered than smaller, disk-shaped nematogenic units. One can imagine the bilayer morphology changing with increasing Tm^{3+} concentration from that of positively aligned bicelles to an intermediate form, with DMPC bilayers resembling slices of Swiss cheese that ultimately convert to well-ordered smectic layers in equilibrium with an isotropic solution of DMPC and DHPC, presumably in micellar form. At present we can only speculate about the composition of the isotropic phase, but we note that in more recent measurements (Prosser, manuscript to be published) on samples with only 60% water, the isotropic resonance is not observed. Fig. 5 shows that as the concen-

tration of Tm^{3+} is raised, the presumed nematic-to-smectic transition temperature, T_{NS}, is lowered. Based on additional ^2H spectra of DMPC-d_{54} measured at 5° intervals from 30°C to 65°C, we estimate that T_{NS} occurs at 50–55°C, 45–50°C, and 40–45°C for Tm^{3+}/DMPC ratios of 0.02, 0.06, and 0.09, respectively.

It should be noted that the NMR results for the smectic region were in fact confirmed by small-angle neutron scattering measurements (Katsaras et al., 1997). A DMPC/DHPC/Tm^{3+} sample with $q = 3.2$, 20% lipid, and [Tm^{3+}]/[DMPC] = 0.13 was found to be clearly smectic and very well aligned (mosaic spread < 1°) at 40°C and 2.6 T. On the other hand, our description of the lower temperature phase as nematic is based simply on the fact that the spectra (2,1), (2,2), (3,1), and (4,1) in Fig. 5, except for the factor of 2 in width, are very similar to those (A–D) presented in Fig. 3 and assumed to be discotic nematic. For Tm^{3+}-doped samples, this phase is only metastable in the magnetic field. All of the measurements illustrated in Fig. 5 were performed by stepwise increase of the temperature after a precooled sample had been inserted into the magnetic field. At 30–35°C, when first ordered, the phase with nematic morphology is kinetically trapped. However, after heating of the sample to 65°C, and then cooling it in the magnetic field, this phase is not recovered. Instead, the smectic phase remains to at least slightly below 28°C. The positively aligned nematic phase can be regenerated by removing the sample from the magnetic field, chilling it to 0°C, and reinserting it in the field.

A remaining question concerns the role of the short-chain lipid in the positively aligned smectic phase. Clearly, if the DMPC forms well-aligned smectic layers oriented perpendicular to the field, the DHPC may either participate in the layer formation, or it may be forced out and into mixed micelles in the isotropic phase. To address this question, we investigated $q = 3.2$ Tm^{3+}-doped samples as a function of temperature and thulium ion concentration, using chain-deuterated DHPC as a probe and [Tm^{3+}]/[DMPC] ratios of 0.028 and 0.083. The results of the measurements corroborate our conclusions based upon the DMPC-d_{54} ^2H spectra, and Fig. 8 illustrates the major points to be learned from the DHPC spectra. As was the case for the DMPC spectra, one can distinguish between the lower temperature nematic and the higher temperature smectic phase at both Tm^{3+} concentrations. The top spectrum in Fig. 8, which is similar to one reported previously (Vold and Prosser, 1996), was recorded at 39.4°C for a $q = 3.2$ DMPC/DHPC sample containing no thulium ions. The spectrum is narrow, and the few remaining α-methylene deuterons (see comments made in Materials and Methods) appears as just shoulders on the doublet from the other three methylene groups. In the spectrum immediately below, recorded at 33.3°C for a sample with [Tm^{3+}]/[DMPC] = 0.028, the quadrupolar splittings have doubled, indicating realignment of the bicelles to $S_{l\bar{n}} = 1$. As the temperature is raised into the region where the DMPC spectra in Fig. 5 show increased resolution, the DHPC splittings increase drastically and finally at 57°C, whereas most of the spectral intensity is found in an isotropic peak near the center of the bottom spectrum of Fig. 8, the remainder shows ordering more characteristic of an $S_{l\bar{n}} = 1$ bilayer. Thus it appears that the DHPC is partially absorbed into the DMPC bilayers, but the shorter chains do not fit in a planar layer, and eventually it is expelled into the isotropic phase.

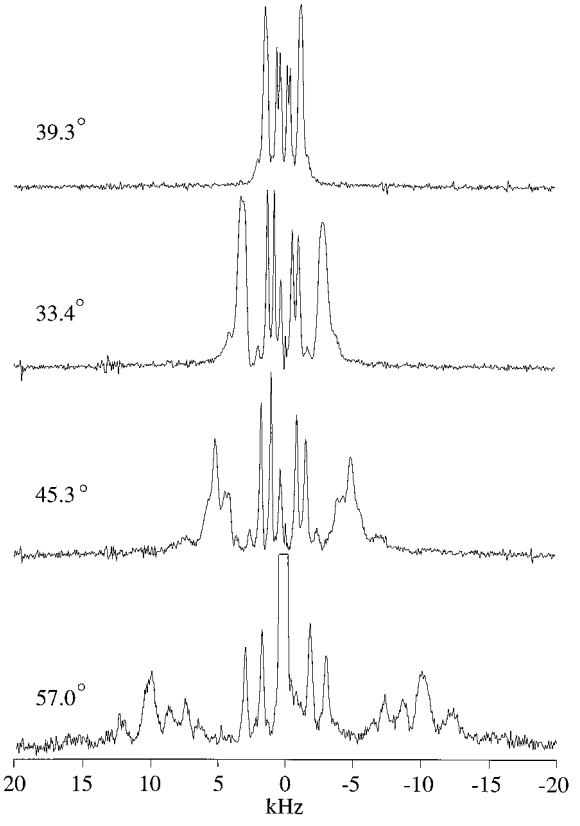

FIGURE 8 Representative DMPC-d_{22} spectra obtained at 5.9 T and the indicated temperatures of $q = 3.2$ DMPC/DHPC-d_{22} samples. The top spectrum contains no thulium ions, whereas in the three lower ones the Tm^{3+}/DMPC ratio is 0.028.

The DHPC spectra in Fig. 8 represent the change one would predict to take place as the bicellar structure is lost in an otherwise magnetically ordered phase. As discussed by Vold and Prosser (1996), the ratio Δ_S/Δ_L (where S stands for "short" and L stands for "long") of the α-deuteron quadrupolar splittings in spectra of chain-perdeuterated DHPC to that in spectra of DMPC is a critical indicator of the morphology of the mesogenic unit. For the ideal bicelle, the ratio at $q = 3.2$ is 0.236, and in Tm^{3+}-free bicelles the observed ratio was found to range from 0.20 at 29°C to 0.26 at 49°C. By comparison, for the thulium-doped sample with [Tm^{3+}]/[DMPC] = 0.028, Δ_S/Δ_L is observed to be 0.19, 0.21, and 0.22 ($\pm$0.01) at 35.3, 37.2, and 39.3°C, respectively, indicating that the bicellar morphology is preserved. In contrast, at higher temperatures for the thulium-doped samples, the DHPC-d_{22} splittings increase gradually with increasing temperature, whereas the DMPC splittings decrease. For example, Δ_S/Δ_L is observed to be 0.36 $\pm$ 0.02 at 45°C and 0.74 $\pm$ 0.05 at 57°C for Tm^{3+}/DMPC = 0.028,

suggesting that the small fraction of aligned DHPC remaining has been included in the DMPC bilayer.

Effects of Ln^{3+} ions on the DMPC headgroup deuteron spectra

The effects of trivalent lanthanide ions upon the ^2H NMR spectra of choline-deuterated DMPC (DMPC-d_{13}) in nematic solutions of $q = 4.6$ bicelles are illustrated in Fig. 9. Column A illustrates how the addition of yttrium chloride affects the quadrupolar splittings observed for the two choline methylene deuterons h_1 and h_2, increasing one splitting while decreasing the other. (To avoid confusion, we have refrained from using the traditional, but nonstandard, nomenclature, α and β, for the headgroup methylenes, because those labels are normally used for methylene groups in the fatty acids. Instead, we prefer the distinctly nonstandard, but more descriptive, labels h_1, h_2, and h_3.) This is clearly different from the uniform increase in the quadrupolar splittings observed in the fatty acid chain spectra in Fig. 3 after addition of the diamagnetic Y^{3+} ion, and reflects conformational changes within the phospholipid headgroup. As discussed by several authors (Hauser et al., 1976; Akutsu and Seelig, 1981), the conformational changes, although large enough to affect the packing of the phospholipid chains, are in fact minute, but the effects on the spectra are large, because certain bond angles critical for the magnitude of the quadrupolar splittings are close to the magic angle. The spectra shown in Fig. 9 *A* and the assignments indicated are consistent with those reported originally by Akutsu and Seelig (1981), who showed that the deuteron splitting associated with the methylene group h_1 closest to the phosphate group decreases with increasing trivalent ion concentration, whereas the splitting associated with the second methylene group h_2 increases.

The spectra in Fig. 9 *B* were obtained from a bicellar solution after addition of increasing amounts of europium chloride. At low concentration of Eu^{3+}, the h_1 deuterons show the expected decrease in quadrupolar splitting, whereas the h_2 deuteron splitting increases. However, the subsequent changes induced by increasing amounts of Eu^{3+} ion are more complex. The splittings double because the bicelles flip to the $S_{l\bar{n}} = 1$ orientation, and the Eu^{3+} ions induce shifts of the ^2H resonances. For the fatty acid chain deuterons (see Fig. 3), such shifts were barely detectable at 38.4 MHz. We have yet to pursue the analysis of the paramagnetic shifts observed in the headgroup spectra, but because the contact terms in the hyperfine interaction are likely to be negligible, it should be possible from such spectra to determine the location of the lanthanide ion relative to the phospholipid headgroups.

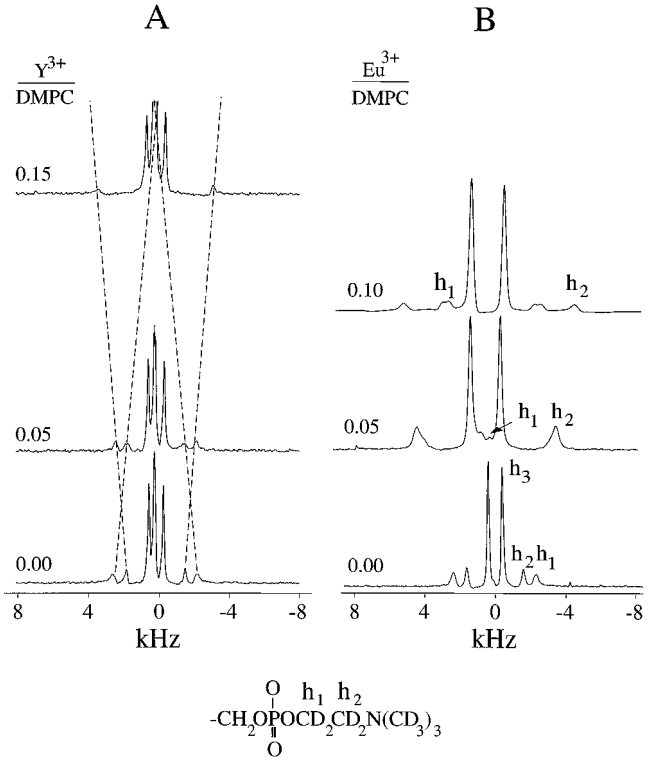

FIGURE 9 Deuterium solid-echo spectra obtained at 38.4 MHz and 45°C of headgroup deuterated DMPC (DMPC-d_{13}) in DMPC/DHPC $q = 3.2$ bicelles in the presence of diamagnetic, Y^{3+} (*A*), and paramagnetic, Eu^{3+} (*B*), trivalent ions. An exponential broadening of 50 Hz was applied to the data before Fourier transformation, and the magnet homogeneity was probably no better than 50–100 Hz.

CONCLUSIONS AND FUTURE PROSPECTS

In this report we have presented deuterium quadrupole echo NMR spectra that document the formation of a new, magnetically aligned, smectic phospholipid bilayered phase with positive ordering, i.e., with the bilayer normal $\bar{\mathbf{n}}$ parallel to the magnetic field, or $S_{l\bar{n}} = 1$. This is contrary to the normal negative alignment of phospholipid bilayers ($S_{l\bar{n}} = -1/2$), which arises because the anisotropy of the magnetic susceptibility $\Delta\chi$ of phospholipids is negative (Boroske and Helfrich, 1978). The new phase is obtained by the addition of any one of the four trivalent lanthanide ions, Eu^{3+}, Er^{3+}, Tm^{3+}, or Yb^{3+}, all of which have are characterized by $\Delta\chi > 0$. The sign of $\Delta\chi$ for the lanthanide ions is a property purely of the electronic angular momenta of the individual lanthanide ions and can be deduced from the usual symmetry based expansion of the crystal field coupling terms (Abragam and Bleaney, 1970; Bleaney, 1972). The ability to flip phospholipid bilayers follows the sign of the NMR chemical shifts induced in the presence of lanthanide shift reagents (Hinckley, 1969; Horrocks, 1973). In other words, those lanthanide ions that induce downfield shifts in isotropic solutions may also be used to realign phospholipid bilayers to $S_{l\bar{n}} = 1$.

The present study was conducted mostly with Tm^{3+}-doped DMPC/DHPC phospholipid samples with an overall lipid content of 20% by weight. Below certain temperatures, which depend on the thulium ion concentration, we have observed a positively aligned phase, which is bicellar and

probably discotic nematic. In this phase, the deuterium NMR spectra and their order profiles are quite similar to those observed for "normal" DMPC/DHPC bicellar samples and for samples doped with diamagnetic trivalent (e.g., Y^{3+}) and with $\Delta\chi < 0$ paramagnetic trivalent ions (e.g., Ho^{3+} and Nd^{3+}). Furthermore, the ratio between the quadrupolar splittings for DHPC and DMPC approaches that of the "ideal bicelle" (Vold and Prosser, 1996). In other words, the lower temperature phase may simply be a 90°-flipped version of the bicellar phase normally present in mixtures of DMPC, DHPC, and water.

As the sample temperature is raised in the magnetic field for the Tm^{3+}-doped samples, the quadrupolar splittings decrease normally, until a reversal sets in: the splittings suddenly increase, and the spectral resolution improves. Apparently, the sample undergoes a transition in which the bilayer order parameter $S_{\bar{n}m}$ increases, presumably because of a change in morphology. The bicelles may be aggregating to form structures similar to brickwork, or, alternatively, to bilayered Swiss-cheese-like sheets with DHPC lining the rim of the holes. The phase transition temperature is lower for higher concentrations of Tm^{3+} ion, but ultimately the phospholipids form a well-aligned smectic phase. Above the phase transition temperature, the bilayer order parameter is not strongly dependent on Tm^{3+} concentration. This is consistent with an extended bilayer domain, and the smectic nature of the macroscopically ordered high-temperature phase has in fact been independently established by small-angle neutron scattering experiments (Katsaras et al., 1997). For the samples studied here, the smectic phase was found to be in equilibrium with an isotropic phase. This phase contains both DMPC and DHPC, presumably present as mixed micelles of unknown morphology. Subsequent studies (Prosser, manuscript to be published) have shown this isotropic phase to be absent in samples containing 40% lipid. Our NMR studies have also revealed that the smectic phase can be obtained at any temperature above 28°C by simply heating the sample to well above the transition temperature, followed by slow cooling. Thus the low-temperature, positively aligned nematic phase is stable only for a freshly aligned sample.

For structural studies of membrane-associated peptides and proteins, the positively aligned phospholipid bilayers offer several advantages over those with negative order parameter. Larger dipolar and quadrupolar splittings and larger chemical shifts are observed than in the $\beta_{l\bar{n}} = 90°$ samples, although, at least for inhomogeneously broadened spectra, resonance linewidths may be increased as well. More importantly, in the $S_{l\bar{n}} = -1/2$ aligned bicelles, a well-resolved spectrum with "single-crystal-like" lines will only be obtained if the membrane-associated molecule of interest undergoes fast axially symmetrical motion. This confines solid-state NMR studies in such systems to small membrane peptides. In the $S_{l\bar{n}} = 1$, magnetically aligned DMPC/DHPC bilayers, there is no such restriction on the motional limit of the molecule of interest. Hence membrane-associated peptides or proteins of arbitrary size may be studied. There also is no conflict between the magnetic susceptibility anisotropies of the lanthanide-doped bilayer host and α-helical segments of membrane spanning proteins. This makes it feasible to incorporate transmembrane proteins/peptides into the model membranes at higher concentration without risk of losing alignment. In fact, since our first report (Prosser et al., 1996) of the positively aligned lipid/lanthanide systems, several groups (Czereski et al., 1996; Howard and Opella, 1996) have reported successfully orienting membrane peptides and proteins in Tm^{3+}-doped DMPC/DHPC bilayers. It should also be mentioned that magnetically aligned samples are simpler to work with than those mechanically aligned on glass plates. Not only does one avoid filling the NMR coil with a large amount of glass, which reduces the filling factor, but preparation of mechanically oriented samples is very tedious. Furthermore, the samples have a much shorter lifetime, because heating, cooling, and sample rotation all have a tendency to cause permanent misalignment. The higher water content and resulting larger interbilayer spacing of the magnetically oriented should be an advantage in studies of larger proteins, such as large receptor complexes. Mechanically aligned samples, on the other hand, have the advantage that they can be rotated in the field, so that transport properties such as translational and rotational diffusion and relaxation phenomena can be studied as a function of sample orientation.

The presence of free lanthanide ions in positively aligned bilayers is undesirable for structural studies of polypeptides. Even in the absence of specific lanthanide-binding sites, one can expect disturbing chemical influences of the ions on protein conformation. Furthermore, the chemical shifts induced by nonspecific coordination to the protein, mainly to free carboxyl groups (Perkins and Wüthrich, 1978), are likely to induce complications in spectral assignment. Such shifts were in fact observed in a study of Leu-enkephalin in DMPS/DHPC bicelles (DiNatale et al., 1995). The pentapeptide Tyr-Gly-Gly-Phe-Leu-OH, upon binding to the bicelles, exhibited significant chemical shifts upon the addition of thulium chloride to the sample, and whether those shifts were of pure paramagnetic origin, or whether conformational changes were involved was not clear. However, as recently shown (Prosser et al., manuscript to be published) by the use of a lanthanide complex containing a long alkyl chain that inserts into the bilayer, the paramagnetic shifts of the Leu-enkephalin deuterons can largely be avoided. This is a very promising result, because it demonstrates that one can use amphiphilic lanthanide chelates to avoid conformational perturbations of membrane-associated polypeptides.

In conclusion, we have shown that by combining paramagnetic dopants (Eu^{3+}, Er^{3+}, Yb^{3+}, or Tm^{3+}) with the DMPC/DHPC bilayered micelle system, one can generate a stable smectic bilayered phase in which the bilayer normal is parallel to the magnetic field. We anticipate that this new membrane mimetic may find many uses in magnetic resonance, in low-angle diffraction work, and in various types of optical and infrared spectroscopy to study structure and

dynamics of membranes and membrane proteins in a biologically mimetic milieu.

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REFERENCES

Abragam, A., and B. Bleaney. 1970. *In* Electron Paramagnetic Resonance of Transition Ions. Clarendon, Oxford.

Akutsu, H., and J. Seelig. 1981. Interaction of metal ions with phosphatidylcholine bilayer membranes. *Biochemistry.* 20:7366–7373.

Bleaney, B. 1972. Nuclear magnetic resonance shifts in solution due to lanthanide ions. *J. Magn. Reson.* 8:91–100.

Boroske, E., and W. Helfrich. 1978. Magnetic anisotropy of egg lecithin membranes. *Biophys. J.* 24:863–868.

Brown, M. F., and J. Seelig. 1977. Ion-induced changes in head group conformation of lecithin bilayers. *Nature.* 269:721–723.

Brumm, T., A. Möps, C. Dolansky, S. Brückner, and T. M. Bayerl. 1992. Macroscopic orientation effects in broadline NMR spectra of model membranes in high magnetic field strength—a method preventing such effects. *Biophys. J.* 61:1018–1024.

Czereski, L., T. Woolf, A. Grossfield, and C. Sanders. 1996. Solid State NMR Approaches to Membrane Protein Structure: Tools and Application to Diacylglycerol Kinase, 37th ENC, Asilomar, CA.

Davis, J. H. 1981. The description of membrane lipid conformation, order, and dynamics by ^{2}H NMR. *Biochim. Biophys. Acta.* 737:117–171.

Davis, J. H. 1988. ^{2}H nuclear magnetic resonance of exchange-labeled gramicidin in an oriented lyotropic nematic phase. *Biochemistry.* 27: 428–436.

DiNatale, J. S., S. A. Hunt, R. S. Prosser, and R. R. Vold. 1995. Deuterium Lineshape and Relaxation Studies of Magnetically Ordered Bilayers, International Chemical Congress of Pacific Basin Societies (Pacifichem 95), Honolulu, HI, December 17–22.

Dill, K. A., and P. J. Flory. 1980. Interphases of chain molecules: monolayers and lipid bilayer membranes. *Proc. Natl. Acad. Sci. USA.* 77: 3115–3119.

Duncan, T. M. 1990. A Compilation of Chemical Shift Anisotropies. Farragut Press, Madison, WI. P-20.

Forrest, B. J., and L. W. Reeves. 1981. New lyotropic liquid crystals composed of finite nonspherical micelles. *Chem. Rev.* 81:1–14.

Hauser, H., L. Pascher, R. H. Pearson, and S. Sundell. 1981. Preferred conformation and molecular packing of phosphatidylethanolamine and phosphatidylcholine. *Biochim. Biophys. Acta.* 650:21–51.

Hauser, H., M. C. Philips, B. A. Levine, and R. J. P. Williams. 1976. Conformation of the lecithin polar groups in charged vesicles. *Nature.* 261:390–394.

Herzberg, G. 1944. Atomic Spectra and Atomic Structure. Dover, New York.

Hinckley, C. C. 1969. Paramagnetic shifts in solutions of cholesterol and the dipyridine adduct of trisdipivalomethanatoeuropium(III). A shift reagent. *J. Am. Chem. Soc.* 91:5160–5162.

Horrocks, W. D. 1973. *In* NMR of Paramagnetic Molecules. G. N. La Mar, W. DeW. Horrocks, and R. H. Holm, editors. Lanthamide shift reagents and other analytical applications, Ch. 12. Academic Press, New York. 479.

Howard, K. P., and S. J. Opella. 1996. High resolution solid-state NMR spectra of integral membrane proteins reconstituted into magnetically oriented phospholipid bilayers. *J. Magn. Reson.* 112:91–94.

Jahnig, F. 1979. Molecular theory of lipid membrane order. *J. Chem. Phys.* 70:3279–3290.

Jansson, M., R. L. Thurmond, T. P. Trouard, and M. F. Brown. 1990. Magnetic alignment and orientational order of dipalmitoylphosphatidylcholine bilayers containing palmitoyllytsophosphatidylcholine. *Chem. Phys. Lipids.* 54:157–170.

Katsaras, J., R. L. Donaberger, J. P. Swainson, D. C. Tennant, Z. Tun, R. R. Vold, and R. S. Prosser. 1997. Rarely observed phase transition in a novel lyotropic liquid crystal system. *Phys. Rev. Lett.* 78:899–902.

Kawamura, Y., I. Sakurai, A. Ikegami, and S. Iwanayagi. 1981. Magnetoorientation of phospholipids. *Mol. Cryst. Liq. Cryst.* 67:77–88.

Kurland, R. J., and B. R. McGarvey. 1970. Isotropic NMR shifts in transition metal complexes: the calculation of the Fermi contact and the pseudocontact terms. *J. Magn. Reson.* 2:286.

Lafleur, M., M. Bloom, and P. R. Cullis. 1990a. Lipid polymorphism and hydrocarbon order. *Biochem. Cell Biol.* 68:1–8.

Lafleur, M., P. R. Cullis, B. Fine, and M. Bloom. 1990b. Comparison of the orientational order of lipid chains in the L_α and H_{II} phases. *Biochemistry.* 29:8325–8333.

Lafleur, M., B. Fine, E. Sternin, P. R. Cullis, and M. Bloom. 1989. Smoothed orientational order profile of lipid bilayers by ^{2}H NMR. *Biophys. J.* 56:1037–1041.

Lawson, K. D., and T. J. Flautt. 1967. Magnetically oriented lyotropic liquid crystalline phases. *J. Am. Chem. Soc.* 89:5489–5491.

Levine, Y. K., A. G. Lee, N. J. M. Birdsall, J. C. Metcalfe, and J. D. Robinson. 1973. The interaction of paramagnetic ions and spin labels with lecithin bilayers. *Biochim. Biophys. Acta.* 291:592–607.

Lin, G. S. B. 1980. Theory of cation-phospholipid-induced shape changes in a lipid bilayer couple. *Bull. Math. Biol.* 42:601–625.

Marcelja, S. 1974. Chain ordering in liquid crystals: structure of bilayer membranes. *Biochim. Biophys. Acta.* 367:165–176.

McGarvey, R. R. 1979. Nuclear magnetic resonance for rare earth complexes: theory. *In* Organiometallics of the *f*-Elements. T. J. Marks, and R. D. Fischer, editors. Reidel, Dordrecht, The Netherlands. 309–336.

Monck, M. A., M. Bloom, M. Lafleur, R. N. A. Lewis, R. N. McElhaney, and P. R. Cullis. 1992. Influence of lipid composition on the orientational order in *Acholeplasma laidlawii* strain B membranes: a deuterium NMR study. *Biochemistry.* 31:10037–10043.

Perkins, S. J., and K. Wüthrich. 1978. Structural interpretation of lanthanide binding to the basic pancreatic trypsin inhibitor by ^{1}H NMR at 360 MHz. *Biochim. Biophys. Acta.* 536:406–420.

Prosser, R. S., S. A. Hunt, J. A. DiNatale, and R. R. Vold. 1996. Magnetically aligned membrane model systems with positive order parameter—switching the sign of S_{zz} with paramagnetic ions. *J. Am. Chem. Soc.* 118:269–270.

Ram, P., and J. H. Prestegard. 1988. Magnetic field induced ordering of bile salt/phospholipid micelles: new media for NMR structural investigations. *Biochim. Biophys. Acta.* 940:289–294.

Sakurai, I., Y. Kawamura, A. Ikegami, and S. Iwayanagi. 1980. Magnetoorientation of lecithin crystals. *Proc. Natl. Acad. Sci. USA.* 77: 7232–7236.

Sanders, C. R., B. J. Hare, K. P. Howard, and J. H. Prestegard. 1994. Magnetically oriented phospholipid micelles as a tool for the study of membrane associated molecules. *Prog. NMR Spectrosc.* 26:421–444.

Sanders, C. R., and G. C. Landis. 1994. Facile acquisition and assignment of oriented sample NMR spectra of bilayer surface-associated proteins. *J. Am. Chem. Soc.* 116:6470–6471.

Sanders, C. R., and G. C. Landis. 1995. Reconstitution of membrane proteins into lipid-rich bilayered mixed micelles for NMR studies. *Biochemistry.* 43:4030–4040.

Sanders, C. R., and J. H. Prestegard. 1990. Magnetically orientable phospholipid bilayers containing small amounts of a bile salt analogue, CHAPSO. *Biophys. J.* 58:447–460.

Sanders, C. R., J. E. Schaff, and J. H. Prestegard. 1993. Orientational behavior of phosphatidylcholine bilayers in the presence of aromatic amphiphiles and a magnetic field. *Biophys. J.* 64:1069–1080.

Sanders, C. R., and J. P. Schwonek. 1992. Characterization of magnetically orientable bilayers in mixtures of dihexanoylphosphatidylcholine and dimyristoylphosphatidylcholine by solid-state NMR. *Biochemistry.* 31: 8898–8905.

Scholz, F., E. Boroske, and W. Helfrich. 1984. Magnetic anisotropy of lecithin membranes. A new anisotropy susceptometer. *Biophys. J.* 45: 589–592.

Seelig, J., F. Borle, and T. A. Cross. 1985. Magnetic ordering of phospholipid membranes. *Biochim. Biophys. Acta.* 814:195–198.

Seelig, J., and J. Seelig. 1980. Lipid conformation in model membranes and biological membranes. *Q. Rev. Biophys.* 13:19–61.

Siminovitch, D. J., M. Rance, K. R. Jeffrey, and M. F. Brown. 1984. The quadrupolar spectrum of a spin I-1 in a lipid bilayer in the presence of paramagnetic ions. *J. Magn. Reson.* 58:62–75.

Speyer, J. B., P. K. Sripada, S. K. Das Gupta, G. G. Shipley, and R. G. Griffin. 1987. Magnetic orientation of sphingomyelin-lecithin bilayers. *Biophys. J.* 51:687–691.

Vold, R. R., and R. S. Prosser. 1996. Magnetically oriented phospholipid bilayered micelles for structural studies of polypeptides: does the ideal bicelle exist? *J. Magn. Reson. B.* 113:267–271.

Vold, R. R., R. S. Prosser, and A. J. Deese. 1997. Isotropic solutions of phospholipid bicelles: a new membrane mimetic for high-resolution NMR studies of polypeptides. *J. Biomol. NMR.* 9:329–335.

Vold, R. R., R. S. Prosser, J. A. DiNatale, and J. S. Hwang. 1996. Towards Structural Elucidation of Membrane Bound Peptides and Proteins, 17th International Conference on Magnetic Resonance in Biological Systems, Keystone, Colorado, August 1996.