

## Europium III Binding and the Reorientation of Magnetically Aligned Bicelles: Insights from Deuterium NMR Spectroscopy

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**ABSTRACT** Solid-state deuterium ( $^2\text{H}$ ) NMR spectroscopy was used to study the reorientation of magnetically ordered bicelles in the presence of the paramagnetic lanthanide  $\text{Eu}^{3+}$ . Bicelles were composed of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) plus 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine plus either the anionic lipid 1,2-dimyristoyl-*sn*-3-phosphoglycerol, or the cationic lipid 1,2-dimyristoyl-3-trimethyl ammonium propane. Alignment of the bicelles in the magnetic field produced  $^2\text{H}$  NMR spectra consisting of a pair of quadrupole doublets, one from the  $\alpha$ -deuterons and one from the  $\beta$ -deuterons of DMPC- $\alpha,\beta\text{-d}_4$ .  $\text{Eu}^{3+}$  addition induced the appearance of a second set of quadrupole doublets, having approximately twice the quadrupolar splittings of the originals, and growing progressively in intensity with increasing  $\text{Eu}^{3+}$ , at the expense of the intensity of the originals. The new resonances were attributed to bicelles having a parallel alignment with respect to the magnetic field, as opposed to the perpendicular alignment preferred in the absence of  $\text{Eu}^{3+}$ . Therefore, the equilibrium degree and kinetics of reorientation could be evaluated from the  $^2\text{H}$  NMR spectra. For more cationic initial surface charges, higher amounts of added  $\text{Eu}^{3+}$  were required to induce a given degree of reorientation. However, the equilibrium degree of bicellar reorientation was found to depend solely on the amount of bound  $\text{Eu}^{3+}$ , regardless of the bicelle composition. The kinetics of reorientation were a function of lipid concentration. At high lipid concentration, a single fast rate of reorientation (minutes) described the approach to the equilibrium degree of orientation. At lower lipid concentrations, two rates processes were discernible: one fast (minutes) and one slow (hours). The data indicate, therefore, that bicelle reorientation is a phase transition made critical by bicelle-bicelle interactions.

### INTRODUCTION

A novel phospholipid architecture, the bicelle (from bilayered micelle), has recently emerged as a model membrane system useful in a variety of high-resolution NMR experiments (Ram and Prestegard, 1988; Vold and Prosser, 1996; Vold et al., 1997; Sanders and Schwonek, 1992; Sanders et al., 1994). Bicelles differ from other phospholipid assemblies in that they lack an interior aqueous compartment and, being disc-shaped, are effectively planar. Bicelles form upon mixing long-chain lipid molecules, such as 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) with short-chain lipid molecules, such as 1,2 dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC). The molecules assemble such that the long-chain lipid molecules form the body of the discoidal bilayer, while the short-chain lipid molecules cover the edges of the disc.

A particularly useful characteristic of bicelles is that they spontaneously align upon being placed in a strong magnetic field. This occurs as a result of the interaction between the magnetic field and the magnetic susceptibility anisotropy of the bicelle, the latter being the sum of the small anisotropies of the individual molecules collected into the bicelle (Scholz et al., 1984). The alignment that spontaneously arises when bicelles contain phospholipids, which have a

negative magnetic susceptibility anisotropy, is such that the average bilayer normal is oriented perpendicular to the magnetic field direction. Bicelles can adopt an alternate orientation, in which the average bilayer normal is oriented parallel to the magnetic field, if their magnetic susceptibility anisotropy becomes sufficiently positive. Any species that has a positive magnetic susceptibility anisotropy and associates with lipid bilayers can induce a parallel alignment. These include species such as integral membrane proteins like gramicidin A, and lanthanide ions such as  $\text{Eu}^{3+}$  (Prosser et al., 1996, 1998a).

The spontaneous alignment of bicelles offers significant advantages for performing NMR conformational analysis. Solid state NMR spectra in oriented membrane systems contain narrow resonance lines sensitive to molecular orientation, as opposed to the broad powder pattern NMR spectra characteristic of non-oriented solids (Seelig et al., 1985). In solution state NMR, bicelles can induce a co-alignment of added molecules of interest, so that orientation-dependent interactions, normally absent due to isotropic molecular tumbling, become manifest and provide conformational information (Bax and Tjandra, 1997; Tjandra and Bax, 1997).

A particular advantage of the parallel-aligned bicelle state is that NMR spectral-narrowing results whether or not the bicelle-associated molecules undergo rapid long-axis reorientations. In a perpendicular-aligned bicelle state such a situation would produce powder pattern NMR line shapes, obscuring the desired orientation-dependent information. Another advantage of the parallel-aligned state is that the size of orientation-dependent

Received for publication 6 December 2000 and in final form 2 April 2001.

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0006-3495/01/07/255/11 \$2.00

NMR terms, such as the dipolar and quadrupolar interactions, are doubled relative to the perpendicular-aligned state, thereby enhancing resolution.

The parallel-aligned bicelle state is readily achieved by adding lanthanide ions (Prosser et al., 1996, 1998a,b). Ideally, one wishes to produce a homogenous parallel-aligned phase, while avoiding unwanted and potentially destabilizing interactions between the lanthanide and the molecule of interest, typically a protein or peptide. Schemes for dealing with these problems have been forthcoming (Prosser et al., 1998c). Multivalent cation binding to phospholipid membranes occurs at the level of the anionic phosphate group (Hauser et al., 1975, 1976), and is driven largely by membrane electrostatics. Nevertheless, little has been established regarding lanthanide ion binding specifically to bicelles, or the relationship between lanthanide binding, membrane electrostatics, and the transition from the perpendicular-aligned to the parallel-aligned state.

Recently, we demonstrated that solid-state deuterium ( $^2\text{H}$ ) NMR of choline-deuterated DMPC can be used to detect changes in bicelle surface charge (Crowell and Macdonald, 1999). In this report we apply the same  $^2\text{H}$  NMR technique to examine  $\text{Eu}^{3+}$  interactions with bicelles. We demonstrate that, by manipulating the initial membrane surface charge, it is possible to establish conditions under which both perpendicular-aligned and parallel-aligned bicelles coexist. This permits one to examine the relationship between the surface charge, the level of  $\text{Eu}^{3+}$  binding, and the transition between the two states, from both an equilibrium and a kinetics viewpoint.

## MATERIALS AND METHODS

### Materials

The lipids used in this work are as follows: DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine-1,1,2,2- $d_4$  (DMPC- $\alpha,\beta$ - $d_4$ ), DHPC, 1,2-dimyristoyl-*sn*-3-phosphoglycerol (DMPG), and 1,2-dimyristoyl-3-trimethylammonium propane (DMTAP). All were purchased from Avanti Polar Lipids (Alabaster, AL). All other materials, including  $\text{EuCl}_3 \cdot 9\text{H}_2\text{O}$ , were purchased from Aldrich (Milwaukee, WI).

### Preparation of bilayered micelles

The details of bicelle preparation have been described elsewhere (Prosser et al., 1998a). Identical methods were used here, with only slight modifications as reported previously (Crowell and Macdonald, 1999). Briefly, bicelles used in this report were prepared such that the ratio of long-chain: short-chain lipid molecules ( $q$ ) remained constant at 4.5. The ratio  $q$  is, strictly speaking, a ratio of the total cross-sectional surface area occupied by the head groups of the long-chain lipid molecules to that occupied by the head groups of the short-chain lipid molecules. Because the surface area per molecule is effectively the same for phosphatidylcholine and phosphatidylglycerol,  $q$  reduces to the molar concentration ratio (DMPC + DMPG or DMTAP)/DHPC. Because DHPC is extremely hygroscopic, it can be difficult to control  $q$ . Therefore, an aqueous DHPC solution was prepared immediately upon opening the container received from supplier, from which the necessary amount was added to the dry long-chain lipids.

A sufficient volume of 150 mM NaCl was added to yield the desired lipid concentration. Typically, the samples consisted of 22% w/v of lipid in aqueous solution, with a total weight of lipid on the order of 40 to 50 mg. The lipids were hydrated by gentle mixing and centrifugation, followed by several cycles of heating to 40°C and cooling to 4°C. Bicelle formation was confirmed macroscopically by the presence of an optically clear solution. For samples requiring  $\text{Eu}^{3+}$ , a small aliquot of a concentrated aqueous stock solution of  $\text{EuCl}_3$  was added to a bicelle preparations. All bicelle preparations were stored at 4°C for 60 min prior to use, except for the kinetics experiments where samples were placed directly in the field after  $\text{Eu}^{3+}$  addition plus mixing.

### NMR spectroscopy

$^2\text{H}$  NMR spectra were recorded on a Chemagnetics CMX300 NMR spectrometer operating at 45.98 MHz, using a Chemagnetics wide line deuterium probe. The quadrupolar echo sequence (Davis et al., 1976) was employed using quadrature detection with complete phase cycling of the pulse pairs. Typical acquisition parameters are as follows: a 90° pulse length of 2.0  $\mu\text{s}$ , an interpulse delay of 30  $\mu\text{s}$ , a recycle delay of 200 ms, a spectral width of 100 kHz, and a 4K data size. Spectra were typically processed with exponential broadening of 50–100 Hz. Sample temperature was regulated during acquisition of the NMR spectra using a thermocouple linked to a feedback loop controlling the operation of a resistive heater through which the temperature-regulating air stream passed before reaching the NMR sample. The sample was held at the desired temperature for 30 min prior to signal acquisition, except for kinetics experiments in which signal acquisition commenced as soon as the sample was placed in the probe.

### Fluorescence assay of lanthanide binding

Binding of  $\text{Eu}^{3+}$  to bicelles was measured directly using a  $\text{Eu}^{3+}$  fluorescence assay. Briefly, dilute bicelle solutions (0.25 wt % lipid) of the same lipid composition and ionic strength used for the NMR experiments were titrated with  $\text{EuCl}_3$ .  $\text{Eu}^{3+}$  fluorescence was measured on a time-resolved Perkin-Elmer LS50B Spectrometer (Perkin-Elmer, Norwalk, CT) with excitation at 340.4 nm, and peak emission at 613.0 nm. Surface bound  $\text{Eu}^{3+}$  exhibits a quantum yield several orders of magnitude greater than aqueous  $\text{Eu}^{3+}$  due to reduced collisional quenching, but undergoes no shift in excitation or emission maxima (Saris, 1983). The measured fluorescence intensity is, therefore, largely due to surface bound  $\text{Eu}^{3+}$ , but contains a contribution from unbound  $\text{Eu}^{3+}$ . Background fluorescence and light scattering effects were eliminated by incorporating a 100- $\mu\text{s}$  time delay between excitation and fluorescence measurement, taking advantage, thereby, of the long  $\text{Eu}^{3+}$  fluorescence lifetime.

## RESULTS

### $^2\text{H}$ NMR spectra of DMPC- $\alpha,\beta$ - $d_4$ in bicelles and the effects of europium

Representative  $^2\text{H}$  NMR spectra of DMPC- $\alpha,\beta$ - $d_4$  incorporated into bicelles are shown in Fig. 1 for a bicelle sample with a long-chain lipid composition of 30/70 (M/M) DMTAP/DMPC. (All bicelle compositions ratios will be M/M.) Approximately 8 mol % of the total DMPC was deuterated. The series of spectra show the effects of increasing the  $\text{Eu}^{3+}$  concentration. Resonances are differentiated on the basis of whether they arise from  $\alpha$ -deuterons or  $\beta$ -deuterons, and whether they originate from the perpendicular-aligned ( $\perp$ ) or parallel-aligned ( $\parallel$ ) state.

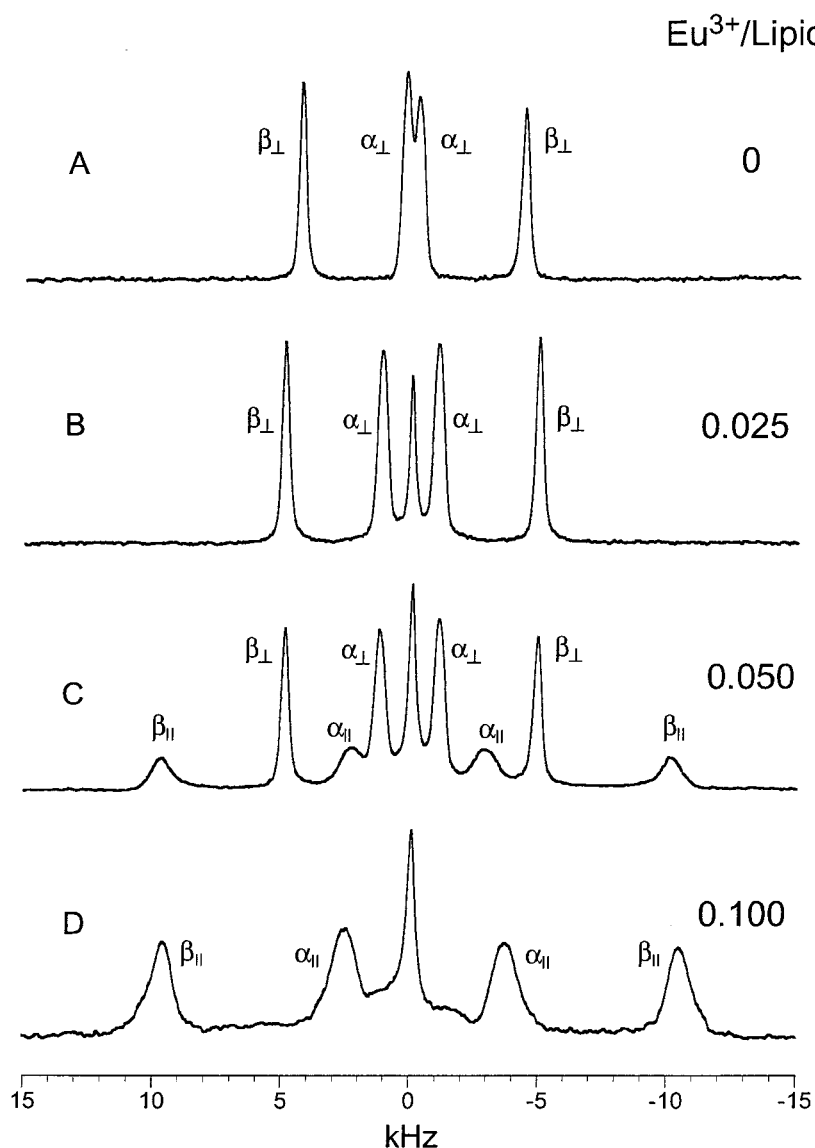


FIGURE 1 Representative <sup>2</sup>H NMR spectra of DMPC- $\alpha,\beta$ - $d_4$  present in 30/70 DMTAP/DMPC bicelles ( $q = 4.5$ , 21 wt % lipid in 150 mM NaCl, 40°C) as a function of added Eu<sup>3+</sup>, expressed as a molar ratio relative to the long-chain lipid content. The spectral assignments are discussed in the text.

Spectrum A was acquired from a sample with no Eu<sup>3+</sup> added. Consequently, the bicelles are in the perpendicular-aligned state with their bilayer normals oriented perpendicular to the direction of the magnetic field. In a liquid-crystalline bilayer, the lipids experience rapid anisotropic molecular diffusion about their long molecular axes. Consequently, for the case of a spin  $I = 1$  nucleus, like deuterium, attached to such a lipid, the quadrupolar Hamiltonian is not averaged to zero. Instead, there remains a residual quadrupolar interaction, the size of which is manifest in the so-called quadrupolar splitting,  $\Delta\nu$ , which obeys Eq. (1),

$$\frac{\Delta\nu}{\Delta\nu_0} = \frac{1}{2} * \langle 3 \cos^2 \alpha - 1 \rangle * \frac{1}{2} * \langle 3 \cos^2 \beta - 1 \rangle \quad (1)$$

where  $\Delta\nu_0$  refers to the static quadrupolar splitting (125 kHz),  $\alpha$  is the angle between the C-D bond direction and the

director axis of motional averaging (taken to be the lipid long axis parallel to the bilayer normal),  $\beta$  is the angle between the director axis and the magnetic field direction, and the angular brackets remind one that the measured quadrupolar splitting represents a time and ensemble average value.

For the case of non-oriented lipid bilayers, such as multilamellar vesicle preparations, all angles  $\beta$  are present simultaneously and the resulting spectrum reflects this powder distribution in the form of the familiar Pake-doublet <sup>2</sup>H NMR powder pattern spectral line shape. For the case of oriented lipid bilayers, such as bicelle preparations, one particular angle  $\beta$  is present across the entire sample. The resulting spectrum consists of a doublet of <sup>2</sup>H NMR resonances symmetrically displaced about the Larmor frequency. The quadrupolar splitting corresponds to the sepa-

ration between the resonances of the doublet, measured in Hz. Spectrum A shows two such quadrupolar splittings; the larger splitting originating with the  $\beta$ -deuterons and the smaller originating with the  $\alpha$ -deuterons of DMPC- $\alpha,\beta$ - $d_4$ .

One of the key reasons for employing choline-deuterated phosphatidylcholine is that the quadrupolar splittings may be used to monitor surface electrostatic charge (Seelig et al., 1987). For an ostensibly neutral bicellar surface in the perpendicular-aligned state, the values of the quadrupolar splitting from these two deuteron labeling positions are rather similar; 4.7 kHz for the  $\alpha$ -deuterons and 3.3 kHz for the  $\beta$ -deuterons (Crowell and Macdonald, 1999). In the presence of surface charges, the two quadrupolar splittings diverge in opposite directions from the control values. Moreover, for any one deutero-labeling position, the change in quadrupolar splitting for cationic surface charges is opposite to that for anionic surface charges. The direction of the change observed in spectrum A of Fig. 1 ( $\alpha$ -deuterons decrease to 1.2 kHz and  $\beta$ -deuterons increase to 8.5 kHz), relative to control bicelles lacking any surface charge, is that expected for bicelles bearing a cationic surface charge, consistent with the presence of the cationic amphiphile DMTAP.

Spectrum B shows the effects of adding  $\text{Eu}^{3+}$  to the bicelles at a concentration equivalent to 0.025  $\text{Eu}^{3+}$  per long chain lipid. The quadrupolar splitting from the  $\beta$ -deuterons increases, while the quadrupolar splitting from the  $\alpha$ -deuterons also increases, but only after having passed through a value of zero. Therefore, both quadrupolar splittings indicate a further accumulation of cationic charge at the 30/70 DMTAP/DMPC bicelle surface, consistent with  $\text{Eu}^{3+}$  surface binding.

In spectra B, C, and D there is a small isotropic component present. The same isotropic component is visible in corresponding  $^{31}\text{P}$  NMR spectra, indicating that it arises from small, non-oriented complexes. This isotropic component becomes problematic only at higher charged lipid contents.

Spectrum C shows the effects of adding  $\text{Eu}^{3+}$  to the bicelles at a concentration equivalent to 0.050  $\text{Eu}^{3+}$  per long chain lipid. The quadrupolar splitting from the  $\beta$ -deuterons increases again, as does that from the  $\alpha$ -deuterons, indicating, therefore, further  $\text{Eu}^{3+}$  surface binding. More significantly, two new pairs of quadrupolar splittings appear, having magnitudes approximately twice those of the original pair of splittings. This second set of quadrupolar splittings must arise from bicelles having a parallel alignment with respect to the magnetic field direction, the change in orientation having been induced by bound  $\text{Eu}^{3+}$ . Importantly, this spectrum demonstrates that under these conditions parallel and perpendicular bicelle orientations coexist.

Spectrum D shows that at a  $\text{Eu}^{3+}$  concentration equivalent to 0.10  $\text{Eu}^{3+}$  per long chain lipid, the bicelle population has converted entirely to the parallel alignment because the original quadrupolar splittings have disappeared com-

pletely. Relative to their magnitude in spectrum C, the quadrupolar splittings continue to increase, indicating yet further  $\text{Eu}^{3+}$  surface binding. Two other, more subtle effects of  $\text{Eu}^{3+}$  addition become evident in this spectrum. First, the quadrupolar doublets undergo a lanthanide shift (Kurland and McGarvey, 1970; Bleaney, 1972) corresponding, in this case, to an up-field shift of the center of the doublet relative to the isotropic shift in the absence of  $\text{Eu}^{3+}$ . This indicates close proximity of the deuterons to the bound  $\text{Eu}^{3+}$ . Second, the resonance lines become substantially broader in the presence of  $\text{Eu}^{3+}$ , a further indication of the proximity of the deuterons to bound  $\text{Eu}^{3+}$ .

Each of these effects — the changes in quadrupolar splitting, the lanthanide shifts, and the appearance of new quadrupolar splittings — is progressive with increasing  $\text{Eu}^{3+}$ . Each of these effects is consistent with binding of  $\text{Eu}^{3+}$  to the bicelle surface, accompanied by conversion of the bicelle from the perpendicular to the parallel alignment within the magnetic field. Each effect will be discussed now in greater detail.

### Europium III-induced changes in quadrupolar splitting

The effects of  $\text{Eu}^{3+}$  addition were investigated across a series of bicelle compositions ranging from 10 mole % DMPG to 30 mole % DMTAP. Fig. 2 illustrates the change in quadrupolar splitting of the  $\alpha$ - and  $\beta$ -deuterons of bicelle-incorporated DMPC- $\alpha,\beta$ - $d_4$  as a function of the concentration of added  $\text{Eu}^{3+}$  for the case of 10/90 DMPG/DMPC bicelles (A) and 30/70 DMTAP/DMPC bicelles (B).

In the presence of the anionic phospholipid DMPG (A), the quadrupolar splitting of the  $\alpha$ -deuterons increases, whereas that from the  $\beta$ -deuterons decreases, relative to their values in neutral bicelles; a response characteristic of the presence of anionic surface charges (Seelig et al., 1987; Crowell and Macdonald, 1999). Adding  $\text{Eu}^{3+}$  reverses this trend, an effect consistent with an accumulation of cationic charge at the bicelle surface due to  $\text{Eu}^{3+}$  binding. The same qualitative effect of  $\text{Eu}^{3+}$  is observed for both the perpendicular- and the parallel-aligned quadrupolar splittings. Where both sets of splittings can be measured simultaneously, the parallel-aligned splittings are virtually twice the magnitude of the perpendicular-aligned splittings. Note that neutralization of the initial DMPG anionic surface charge by  $\text{Eu}^{3+}$  binding, as roughly indicated by the return of the quadrupolar splittings to their values at neutrality, is only achieved at  $\text{Eu}^{3+}$  concentrations far exceeding those necessary to induce complete reorientation of the bicelles to the parallel-aligned state.

In the presence of the cationic amphiphile DMTAP (B), the quadrupolar splitting from the  $\alpha$ -deuterons decreases while that from the  $\beta$ -deuterons increases, relative to their values in neutral bicelles, as expected in the presence of cationic surface charges. Adding  $\text{Eu}^{3+}$  heightens this re-



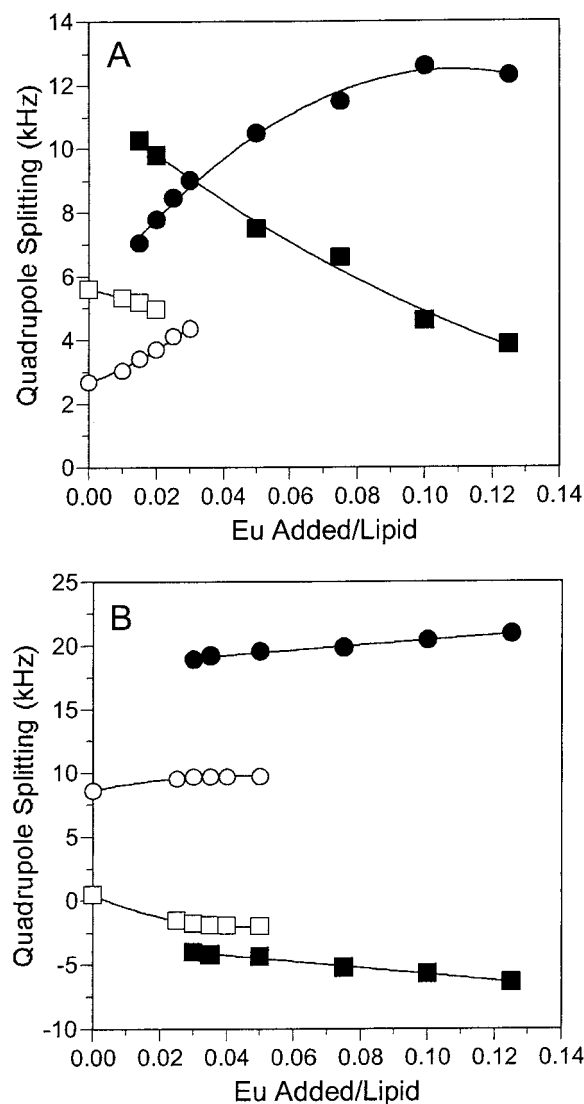


FIGURE 2  $^2\text{H}$  NMR quadrupolar splittings ( $\Delta\nu_Q$ ) of bicelle-incorporated DMPC- $\alpha,\beta\text{-d}_4$  as a function of added  $\text{Eu}^{3+}$ . (A) 10/90 DMPG/DMPC. (B) 30/70 DMTAP/DMPC. *squares*,  $\alpha$ -deuteron splitting; *circles*,  $\beta$ -deuteron splitting; *open symbols*, perpendicular-aligned bicelles; *closed symbols*, parallel-aligned bicelles.

sponse, indicating an additional accumulation of cationic charge at the bicelle surface due to  $\text{Eu}^{3+}$  binding, despite the initial cationic surface charge. Again, the same qualitative effect of  $\text{Eu}^{3+}$  is observed for both the perpendicular and the parallel-aligned quadrupolar splittings. Again, the splittings from the parallel-aligned state are virtually twice those from the perpendicular-aligned state. At higher  $\text{Eu}^{3+}$  concentrations there is little further change in the quadrupolar splittings, particularly in comparison to the case with 10/90 DMPG/DMPC bicelles.

Results similar to those described in detail above for the 10/90 DMPG/DMPC and 30/70 DMTAP/DMPC compositions were obtained for 100% DMPC bicelles and 10/90 and

20/80 DMTAP/DMPC bicelles. In the absence of  $\text{Eu}^{3+}$ , the quadrupolar splittings reflected the initial surface charge particular to the composition of the given charged amphiphile. Adding  $\text{Eu}^{3+}$  produced changes in the quadrupolar splittings indicative of the accumulation of cationic charge at the bicelle surface, even when the initial surface charge was cationic, and regardless of whether the perpendicular or the parallel bicelle orientation was being monitored.

### Europium III-induced lanthanide shifts

Lanthanide shifts can be decomposed into a combination of contact shifts, due to through-bond interactions, and pseudo-contact shifts, due to through-space interactions (Kurland and McGarvey, 1970; Bleaney, 1972). Contact shifts are generally much larger in magnitude than pseudo-contact shifts. Studies of lanthanide binding to phospholipids have shown that they produce mainly pseudo-contact shifts in the  $^1\text{H}$  NMR resonances from phospholipid polar head groups.  $^{31}\text{P}$  NMR resonances, on the other hand, show significant contact shifts. Hence, the lanthanides must coordinate directly to the phospholipid's phosphate group, thereby producing an indirect proximity effect on the head group protons (Hauser et al., 1975, 1976). The magnitude of the contact shifts observed with  $^{31}\text{P}$  NMR spectroscopy can be used to determine association constants for paramagnetic ions binding to phospholipid membranes (Grasdalen et al., 1977).

Fig. 3 illustrates the lanthanide shifts undergone by the  $^2\text{H}$  NMR resonances of DMPC- $\alpha,\beta\text{-d}_4$  in bicelles of various lipid compositions. Panel A compares lanthanide shifts undergone by the  $\alpha$ -deuterons versus the  $\beta$ -deuterons for the case of 10/90 DMPG/DMPC bicelles as a function of the added  $\text{Eu}^{3+}$  concentration. At low  $\text{Eu}^{3+}$  levels, when the perpendicular bicelle orientation predominates, the size of the shift is smaller than the  $^2\text{H}$  NMR line widths. At higher  $\text{Eu}^{3+}$  levels, when the parallel bicelle orientation predominates, the magnitude of the lanthanide shift is always greater for the  $\alpha$ -deuterons relative to the  $\beta$ -deuterons. This is in accord with the notion that lanthanide coordinates to the phosphate group of the phospholipids because the deuterons closest to the binding site should manifest the greatest lanthanide shift.

Panel B compares the magnitude of the lanthanide shift undergone by the  $\beta$ -deuterons for different bicelle lipid compositions. Clearly the shift is greater for the initially anionic bicelles, and decreases progressively with increasing initial cationic surface charge. This is in accord with the expectation that an anionic surface charge will attract greater levels of  $\text{Eu}^{3+}$  binding than a cationic surface charge.  $^{31}\text{P}$  NMR data are in agreement with these conclusions, but the breadth of the resonance lines preclude a meaningful determination of the magnitude of the shifts.

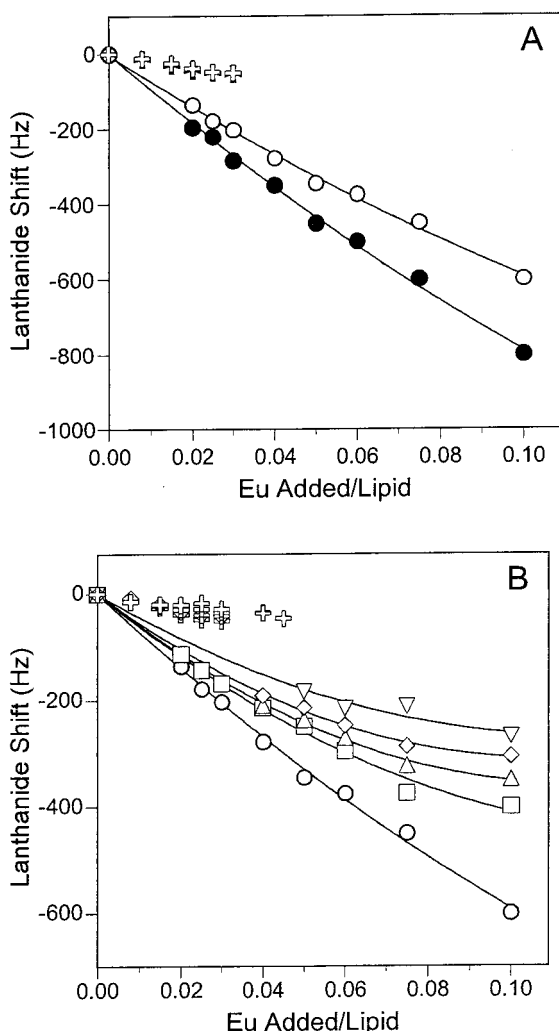


FIGURE 3  $\text{Eu}^{3+}$  lanthanide shift of bicelle-incorporated  $\text{DMPC-}\alpha,\beta\text{-d}_4$  as a function of added  $\text{Eu}^{3+}$  for different bicelle compositions. *A*)  $\alpha$ -deuteron shifts (closed symbols) versus  $\beta$ -deuteron shifts (open symbols) for 10/90 DMPG/DMPC bicelles in the parallel-aligned (circles) and perpendicular-aligned (crosses) states. *B*)  $\beta$ -deuteron shifts for different bicelle compositions in the parallel-aligned state. Circles, 10/90 DMPG/DMPC; square, 100% DMPC; triangles, 10/90 DMTAP/DMPC; diamonds, 20/80 DMTAP/DMPC; inverted triangles, 30/70 DMTAP/DMPC. The crosses show the corresponding lanthanide shifts for the perpendicular-aligned state, undifferentiated with respect to the bicelle composition.

### Europium III-induced magnetic reorientation

As shown in Fig. 1,  $\text{Eu}^{3+}$  induces the appearance of a second set of quadrupole doublets in  $^2\text{H}$  NMR spectra of bicelles, having quadrupolar splittings twice those of the first. This is consistent with the established fact that  $\text{Eu}^{3+}$  induces a reorientation of bicelles from the perpendicular to the parallel alignment with respect to the magnetic field direction (Prosser et al., 1996; 1998a,b). Consequently, the normal to the bilayer, corresponding to the director axis of motional averaging, reorients from  $90^\circ$  to lie at  $0^\circ$  with respect to the magnetic field direction, producing a doubling

of the observed quadrupolar splitting as per Eq. 1. The spectra in Fig. 1 demonstrate that the two orientations can coexist and that they are in slow exchange with one another on the  $^2\text{H}$  NMR time scale. Consequently, the equilibrium degree of reorientation for a given  $\text{Eu}^{3+}$  concentration is obtained from the relative intensities of the two spectral components.

Fig. 4 *A* illustrates how the fraction of parallel-aligned bicelles varies with  $\text{Eu}^{3+}$  concentration for the five different bicelle lipid compositions investigated here. Clearly, the anionic DMPG-containing bicelles require the lowest  $\text{Eu}^{3+}$  concentration to achieve complete reorientation, while the cationic DMTAP-containing bicelles require the highest  $\text{Eu}^{3+}$  concentration. Qualitatively, this difference may be understood in terms of the Gouy-Chapman-Stern model of surface electrostatics, in which anionic surface charges from DMPG attract  $\text{Eu}^{3+}$  cations such that their concentration in the surface region exceeds their bulk concentration, producing enhanced binding levels (McLaughlin, 1989 and references cited herein). In contrast, cationic surface charges from DMTAP repel  $\text{Eu}^{3+}$  cations such that their concentration in the surface region is depleted relative to their bulk concentration, producing reduced binding. Therefore, differences in the amount of added  $\text{Eu}^{3+}$  required to induce a complete reorientation of bicelles from the perpendicular to the parallel orientation in anionic versus neutral versus cationic bicelles are likely to have arisen from differences in  $\text{Eu}^{3+}$  binding levels as influenced by surface electrostatics.

To confirm this interpretation,  $\text{Eu}^{3+}$  binding to bicelles of different charged amphiphile contents was assayed fluorescently as described in the methods section. Fig. 5 shows fluorescence intensity curves for the  $\text{Eu}^{3+}$  concentration range of interest for bicelle reorientation. The highest fluorescence intensity was found with DMPG-containing bicelles, in which the intensity was linearly related to the amount of added  $\text{Eu}^{3+}$ . The quantum yield of fluorescence for bicelle-bound  $\text{Eu}^{3+}$  is at least an order of magnitude higher than that of  $\text{Eu}^{3+}$  in solution (Saris, 1983), as the data in the figure make clear. Therefore, the fluorescence observed in the presence of bicelles is nearly entirely due to bound  $\text{Eu}^{3+}$ . Qualitatively, therefore, the fluorescence data indicate that increasing the cationic surface charge decreases  $\text{Eu}^{3+}$  binding, as expected from the Gouy-Chapman-Stern theory. Independent difference assays of  $\text{Al}^{3+}$  and  $\text{Tb}^{3+}$  binding to 10/90 DMPG/DMPC bicelles show that these trivalent cations bind quantitatively at these concentrations (data not shown). Consequently, the linear relationship between fluorescence intensity and  $\text{Eu}^{3+}$  concentration for the case of 10/90 DMPG/DMPC may be used as a calibration standard to extract the degree of  $\text{Eu}^{3+}$  binding from the  $\text{Eu}^{3+}$  fluorescence intensity for any other composition. Using polynomial fits such as shown in Fig. 5, the relationship between  $\text{Eu}^{3+}$  concentration and degree of

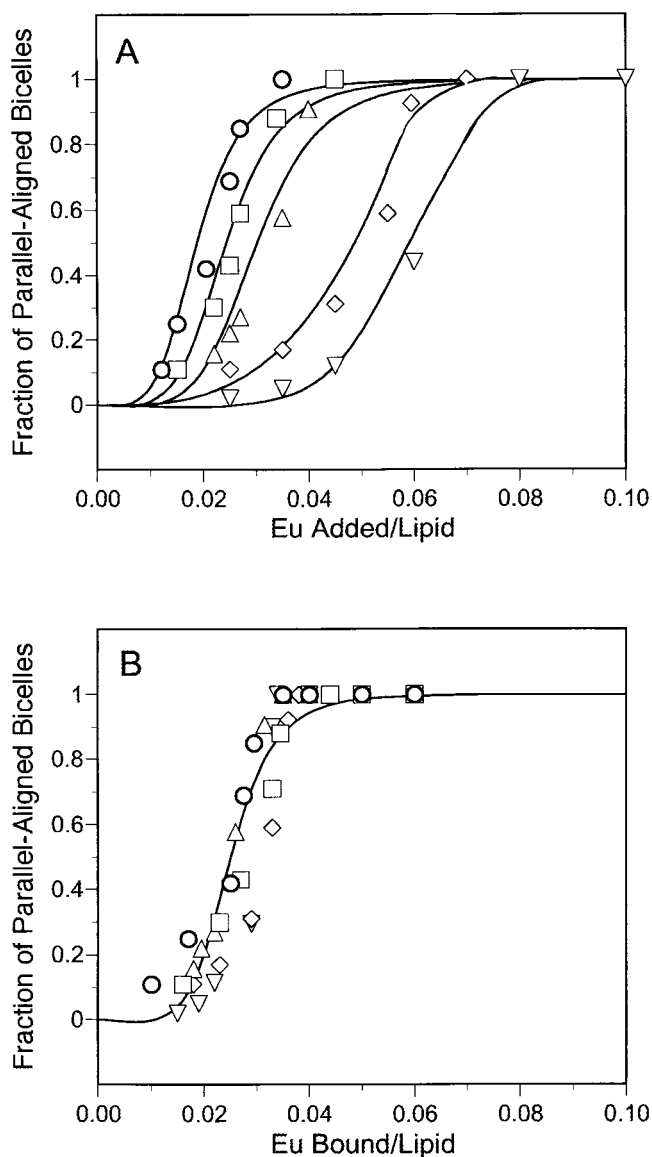


FIGURE 4 *A*) Fraction of parallel-aligned bicelles as a function of added  $\text{Eu}^{3+}$  for different bicelle compositions. Circles, 10/90 DMPG/DMPC; squares, 100% DMPC; triangles, 10/90 DMTAP/DMPC; diamonds, 20/80 DMTAP/DMPC; inverted triangles, 30/70 DMTAP/DMPC. The solid lines are meant only to guide the eye, although their sigmoidal shape clearly indicates cooperativity. *B*) Fraction of parallel-aligned bicelles as a function of bound  $\text{Eu}^{3+}$  for different bicelle compositions. Symbols as per *A*. The level of bound  $\text{Eu}^{3+}$  was assayed via fluorescence as described with respect to Fig. 5, and the curves for different bicelle compositions were shifted accordingly.

binding may be derived then for any  $\text{Eu}^{3+}$  concentration and any bicelle composition.

When the conversion from  $[\text{Eu}^{3+} \text{ added per lipid}]$  to  $[\text{Eu}^{3+} \text{ bound per lipid}]$  is performed in this fashion for the percent-reorientation curves in Fig. 4 *A*, the different curves for different bicelle compositions collapse onto a common curve, as shown in Fig. 4 *B*. Hence, the primary determinant

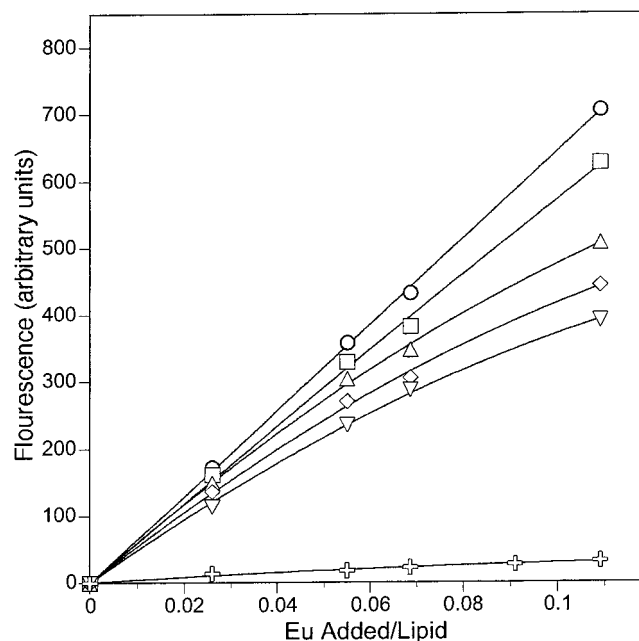


FIGURE 5 Evaluation of  $\text{Eu}^{3+}$  binding via fluorescence spectroscopy of bicelles plus  $\text{EuCl}_3$ . Free  $\text{Eu}^{3+}$  (crosses) has a very low quantum yield relative to bound  $\text{Eu}^{3+}$ . Consequently, the observed fluorescence is virtually entirely due to bound  $\text{Eu}^{3+}$ . Fluorescence intensities are shown for different bicelles compositions. Symbols as per Fig. 4 *A*. The solid lines are polynomial fits to the experimental data, and were used in the conversion of Fig. 4 *A* into 4 *B*, by relating  $\text{Eu}^{3+}$  Added/Lipid to  $\text{Eu}^{3+}$  Bound/Lipid for individual curves in 4 *A*.

of the equilibrium degree of bicelle reorientation is the  $\text{Eu}^{3+}$  binding level, other factors being constant. Furthermore, the charged amphiphile composition of the bicelle exerts an indirect influence on the degree of bicelle reorientation via the influence of surface electrostatics on the level of  $\text{Eu}^{3+}$  binding.

### Time course of bicelle reorientation

The  $^2\text{H}$  NMR spectra shown in Fig. 1 demonstrate that perpendicular- and parallel-aligned bicelles coexist within the same sample under particular conditions, and that there is only slow exchange of bicelles between the two orientations. In order to investigate more closely the stability of the distribution of bicelles between the two orientations, bicelles were prepared with concentrations of  $\text{Eu}^{3+}$  known to produce a mixture of the two orientations, and their  $^2\text{H}$  NMR spectra were monitored as a function of time.

Fig. 6 *A* shows the results of one such series of measurements, this for the case of 25/75 (M/M) DMTAP/DMPC bicelles, at 22% (w/v) lipid/water content, and with 0.035  $\text{Eu}^{3+}$  added per lipid. Each spectrum here required  $\sim 12.5$  min to acquire, so the series as shown represents a time course of  $\sim 6.7$  h. The time course of reorientation is most readily monitored by focussing on the two largest quadru-

polar splittings, which arise from the  $\beta$ -deuterons in the perpendicular- and parallel-aligned states. Immediately after placing the bicelles inside the magnetic field and equilibrating to 40°C, one observes coexisting perpendicular- and parallel-aligned bicelles, although the former predominate. With the passage of time, there is a slow conversion toward the parallel-aligned state over the course of several hours. Evidently the equilibrium distribution between the two bicelle orientations is not achieved rapidly under these conditions.

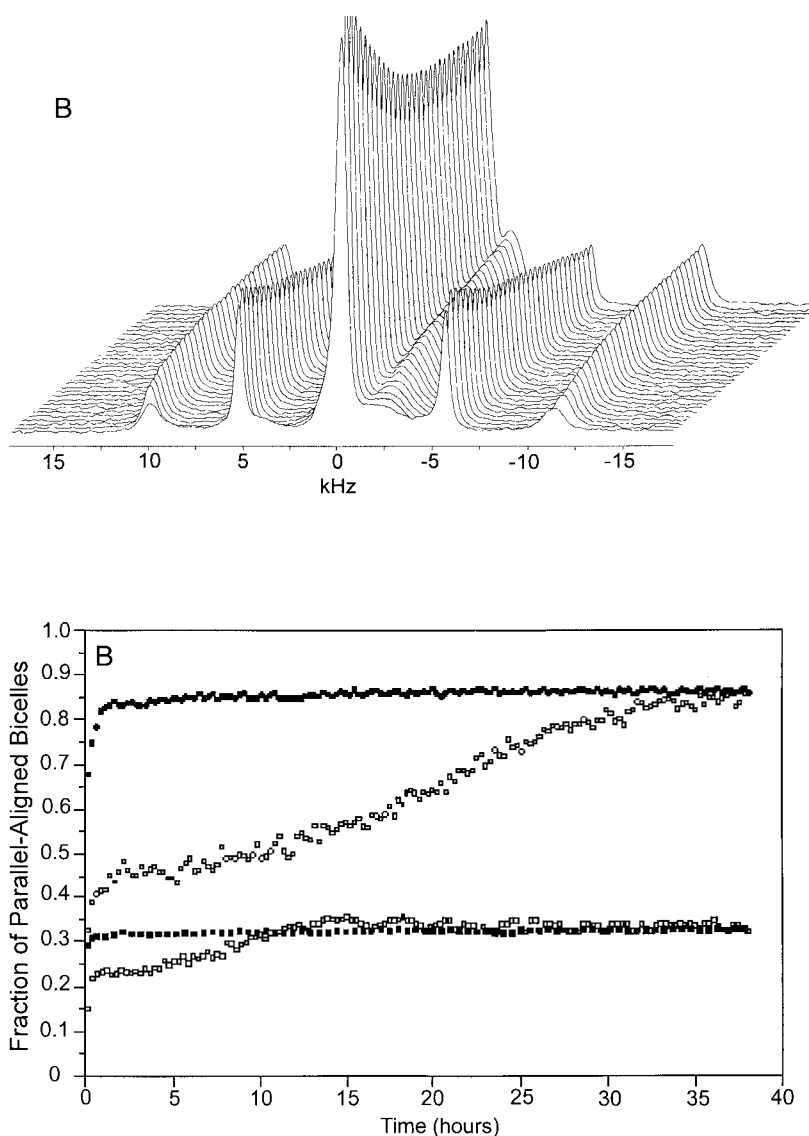
Fig. 6 *B* demonstrates that the time course of bicelle reorientation depends on the lipid/water content and may involve more than one rate process. At any one lipid/water composition, higher  $\text{Eu}^{3+}$  levels translate into greater equilibrium fractions of parallel-aligned bicelles, as expected. The equilibrium fraction of parallel-aligned bicelles is not dependent on the lipid/water content in this range, but the rate of approach to

equilibrium is. At the higher lipid/water content (31% w/v) the final state is achieved rapidly and immediately. At the lower lipid/water content (22% w/v) the equilibrium fraction of parallel-aligned bicelles is only attained after many hours in the magnetic field. There appear, in fact, to be two time scales involved at lower lipid contents. The first is an initial rapid development of parallel-aligned bicelles, with a rate similar to that observed at higher lipid contents. This is followed by a much slower phase of reorientation taking place over the course of many hours.

## DISCUSSION

The reorientation of magnetically aligned bicelles from their spontaneous perpendicular alignment to the lanthanide-induced parallel alignment is of interest because it represents

FIGURE 6 The time course of  $\text{Eu}^{3+}$  induced reorientation of magnetically aligned bicelles. (A) a series of  $^2\text{H}$  NMR spectra of DMPC- $\alpha,\beta\text{-d}_4$  in bicelles (22% w/v) composed of 25/75 DMTAP/DMPC as a function of time after addition of  $\text{Eu}^{3+}$  to a level of 0.032 per long-chain lipid. Each spectrum represents 12.3 min of signal acquisition time. The pair of resonances with a quadrupolar splittings of just over 10 kHz arise from  $\beta$ -deuterons in the perpendicular-aligned state. With the passage of time this intensity decreases, to be replaced by a new pair of resonances with a quadrupolar splitting just over 20 kHz, arising from  $\beta$ -deuterons in the parallel-aligned state. (B) The fraction of parallel-aligned bicelles as calculated using the relative intensities of the  $\beta$ -deuteron quadrupolar splittings from the differently aligned phases, showing the influence of different amounts of added  $\text{Eu}^{3+}$  and different total weight percent of lipid in the bicelle preparations. All bicelles were composed of 20/80 DMTAP/DMPC. Circles,  $\text{Eu}^{3+}/\text{Lipid} = 0.045$ ; squares,  $\text{Eu}^{3+}/\text{Lipid} = 0.032$ ; open symbols, 22% lipid (w/v); closed symbols, 32% lipid (w/v).





a unique example of a chemically-induced phase transition, and because the parallel-aligned state has special utility in studies of biomacromolecular structure. The NMR spectrum of a biomacromolecule associated with a magnetically-aligned bicelle contains orientation-dependent information expressed as residual dipolar and/or quadrupolar couplings. An analysis of such couplings can reveal otherwise inaccessible structural details. In the perpendicular alignment, in which the bilayer normal is oriented at 90° relative to the magnetic field direction, only molecules undergoing fast anisotropic rotation produce narrow resonance lines from which the desired couplings are readily extracted. In the parallel alignment, however, this stricture is removed, and even large transmembrane proteins should, in principle, be amenable to such NMR structural analysis. A better understanding of the nature of the lanthanide-induced reorientation of bicelles would aid in attaining such a goal. In the discussion that follows we highlight the novel insights provided by the  $^2\text{H}$  NMR studies reported here.

### Europium III is in fast exchange between free and bound populations

$\text{Eu}^{3+}$  binding to phospholipid bilayer surfaces is driven both by electrostatics and by chemical affinity considerations. Surface electrostatics influence the  $\text{Eu}^{3+}$  concentration available for binding in the solution immediately adjacent to the bilayer surface. This is plainly evident from the influence of anionic versus cationic surface charge on the level of  $\text{Eu}^{3+}$  binding. The chemical affinity of  $\text{Eu}^{3+}$  for the phosphate groups of the phospholipids is considerable, due to the fact that binding occurs even in the face of a countervailing cationic surface electrostatic charge.

Nevertheless, the  $^2\text{H}$  NMR spectra reveal that on the  $^2\text{H}$  NMR time scale  $\text{Eu}^{3+}$  ions are in fast exchange between free and bound states. Specifically, no separate resonances are observed for free versus  $\text{Eu}^{3+}$ -bound DMPC. Instead, the  $^2\text{H}$  NMR quadrupolar splittings reflect an average of the DMPC free and  $\text{Eu}^{3+}$ -bound populations. This also precludes the possibility of any lateral phase separation of any amphiphile induced by  $\text{Eu}^{3+}$  binding. The fact that the resonance lines are narrow indicates that any fluctuations from the average  $\text{Eu}^{3+}$  binding level per bicelle must be short-lived, because long-lived fluctuations would result in broadening of the  $^2\text{H}$  NMR resonances, reflecting the different surface charges amongst the bicelle population. The line broadening seen in such spectra is significant only at  $\text{Eu}^{3+}$  concentrations well above those which induce bicelle reorientation, and is attributable to dipolar interactions with bound  $\text{Eu}^{3+}$ .

Consequently, the  $^2\text{H}$  NMR spectra reveal that all DMPC on a given bicelle experience the same bicelle-average  $\text{Eu}^{3+}$  binding, and that all bicelles of a given orientation experience the same ensemble average level of  $\text{Eu}^{3+}$  binding.

The fact that the quadrupolar splittings for the parallel-aligned bicelles are virtually exactly twice those of the

perpendicular-aligned bicelles indicates, further, that the two populations experience virtually identical levels of  $\text{Eu}^{3+}$  binding and, therefore, are in equilibrium with one another in this respect.

The  $^2\text{H}$  NMR time scale of relevance here is the inverse of the quadrupolar splitting difference between the free and the bound states. Given that this could be on the order of 10,000 Hz, the maximum residence time of a  $\text{Eu}^{3+}$  ion at the bicelle surface would be on the order of 100  $\mu\text{s}$ .

Note that the state of fast exchange = equilibrium across the ensemble does not pertain necessarily in every situation. For example, when the DMPG composition of the bicelles is high, i.e., the initial surface charge density is highly anionic, adding  $\text{Eu}^{3+}$  produces flocculation, evident from the clouding of the sample along the  $\text{Eu}^{3+}$  diffusion trail from the point of addition. Often this inhomogeneity fails to dissipate over the course of several days. Moreover, other trivalent aqueous cations, such as  $\text{Al}^{3+}$ , produce separate free and bound resonances, indicating slow exchange (Crowell, Udit, and Macdonald, in preparation).

In summary, the  $^2\text{H}$  NMR spectra reveal that  $\text{Eu}^{3+}$  is in rapid equilibrium between free and bound states at a given bicelle surface and between perpendicular- and parallel-aligned bicelles. Consequently, the coexistence of perpendicular-aligned and parallel-aligned bicelle populations cannot be attributed to any long-lived inhomogeneity of  $\text{Eu}^{3+}$  concentration.

### Perpendicular-aligned and parallel-aligned bicelles coexist in slow exchange

The orientation of a bicelle in a magnetic field arises from the interaction between the field and the collective magnetic susceptibility anisotropy of the bicelle. The orientation dependence of the Helmholtz free energy is (Scholz et al., 1984)

$$F(\beta) = -\frac{1}{2} N \Delta\chi H^2 \cos^2 \beta \quad (2)$$

where  $N$  is the number of molecules per unit volume,  $\Delta\chi$  is the molecule's volume magnetic susceptibility anisotropy,  $H$  is the magnetic field strength, and  $\beta$  is the direction of the bicelle normal relative to the magnetic field. Phospholipids, having negative magnetic susceptibility anisotropies ( $\Delta\chi = -0.28 \pm 0.02 \times 10^{-8} \text{ erg cm}^{-3} \text{ G}^{-2}$ , Boroske and Helfrich, 1978), yield free energy minimums for the case,  $\beta = 90^\circ$ .

Prosser et al. (1996, 1998a) demonstrated that lanthanides, such as  $\text{Eu}^{3+}$ ,  $\text{Tb}^{3+}$ ,  $\text{Tm}^{3+}$ , and  $\text{Yb}^{3+}$  are capable of flipping bicelles from the perpendicular to the parallel orientation. The positive magnetic susceptibility anisotropy of the bound lanthanides overcomes the negative magnetic susceptibility anisotropy of the phospholipid bicelle, and the free energy minimum in Eq. 2 switches to  $\beta = 0^\circ$ .  $\text{Tb}^{3+}$  yielded the highest degree of reorientation for a given

concentration because it possesses the largest magnetic susceptibility anisotropy. From the perspective of examining the reorientation experimentally, this is somewhat of a disadvantage because the window of  $\text{Tb}^{3+}$  concentration yielding coexisting perpendicular and parallel alignments would be narrower than is the case for  $\text{Eu}^{3+}$ .

One may exploit this relationship between net magnetic susceptibility anisotropy and net alignment to estimate the effective magnetic susceptibility anisotropy of a bound  $\text{Eu}^{3+}$  ion if it is assumed that the balance between negative and positive magnetic susceptibility anisotropies occurs when half the bicelles are perpendicular-aligned and half are parallel aligned. The number of  $\text{Eu}^{3+}$  ions bound per bicelle is estimated to be on the order of 250, for the situation that the average number of  $\text{Eu}^{3+}$  bound per lipid equals 0.030, i.e., when half the bicelles are parallel-aligned and half are perpendicular-aligned as per Fig. 4 B. This follows from the calculated bicelle radius of 295 Å for the case  $q = 4.5$ , using the relationship proposed by Vold and Prosser (1996) between the bicelle diameter,  $2R_{\text{bicelle}}$ , and the long-chain lipid/DHPC ratio,  $q$ :

$$2R_{\text{bicelle}} = rq[\pi + (\pi^2 + 8/q)^{1/2}] \quad (3)$$

where  $r$  is half the bilayer thickness ( $\sim 20$  Å). If DMPC occupies a cross-sectional area of  $\sim 65$  Å<sup>2</sup>, an individual bicelle will contain 8400 DMPC and bind 250  $\text{Eu}^{3+}$  at the half-parallel/half-perpendicular point. This suggests a magnetic susceptibility anisotropy of  $+9.4 \times 10^{-8}$  erg cm<sup>-3</sup> G<sup>-2</sup> per bound  $\text{Eu}^{3+}$  is needed to at least cancel the negative susceptibility anisotropy of the bicelle contributed by the phospholipids.

The up-field paramagnetic shifts undergone by the  $\alpha$ -deuterons and  $\beta$ -deuterons in the presence of  $\text{Eu}^{3+}$ , as shown in Fig. 3, agree with earlier reports by Hauser et al. (1975) on choline head group proton shifts caused by exposure to  $\text{Eu}^{3+}$ . Kurland and McGarvey (1970) relate the direction of the paramagnetic shift, the magnetic susceptibility anisotropy, and the geometry of the bound ion as

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

where  $\beta$  is the angle between the principle axis of  $\chi$  and the vector  $r$  between the ion and the nuclear spin. Both  $r$  and  $\beta$  remain undetermined. An up-field shift ( $\Delta\nu$  negative) and a positive  $\Delta\chi$  correspond to a negative value for  $(3 \cos^2 \beta - 1)$  for both the  $\alpha$ -deuterons and  $\beta$ -deuterons, suggesting  $0 \leq \beta \leq 54.7^\circ$ . Ignoring possible differences in  $\beta$ , the magnitude of the paramagnetic shifts shown in Fig. 3 indicate that  $r_\alpha < r_\beta$  as concluded by Hauser et al. (1975; 1976) and, as one expects, if  $\text{Eu}^{3+}$  binds to the phosphate group of the phospholipid.

Magnetic alignment of bicelles is a cooperative phenomenon, dependent on interactions between neighboring bicelles. At high water contents, for instance, only isotropic, and not oriented, phases result (Raffard et al., 2000). The

sigmoidal shape of the Europium III-induced transition from a perpendicular orientation to a parallel orientation observed here is another manifestation of cooperative interactions between bicelles. As pointed out by Struppe and Vold (1998), when the excluded volume per bicelle exceeds the volume available to each particle, a transition to an ordered phase occurs. For a case typical of that investigated here (20% w/v lipid,  $q = 4.5$ ,  $R = 295$  Å,  $3.2 \times 10^{16}$  bicelles per ml) the center-to-center bicelle separation is estimated to be 315 Å, i.e., on the order of the bicelle radius, and extensive bicelle-bicelle interactions are expected.

Likewise, the phase transition from the perpendicular-aligned to the parallel-aligned phase is of necessity a cooperative phenomenon. In the absence of  $\text{Eu}^{3+}$  Eq. 2 predicts  $\beta = 90^\circ$  for phospholipid-containing bicelles. Alignment is favored by interactions between bicelles, and opposed by the randomizing influence of thermal fluctuations. In the absence of  $\text{Eu}^{3+}$ , regions with parallel alignment are initially too energetically unfavorable to occur with significant probability. As  $\text{Eu}^{3+}$  is added, the energy difference between the two different alignments decreases, and the energy cost of producing a region of parallel orientation approaches zero. Either perpendicular alignment or parallel alignment occurs, rather than an isotropic phase, because of interactions between bicelles, in the spirit of a spin  $\frac{1}{2}$  Ising model. Therefore, regions of perpendicular alignment coexist with regions of parallel alignment. Near the critical  $\text{Eu}^{3+}$  level at which Eq. 2 indicates that the more energetically favored alignment changes from  $\beta = 90^\circ$  to  $\beta = 0^\circ$ , one expects that regions of perpendicular and parallel alignment occur on all length scales ranging from the inter-bicelle distance up to some maximum dictated by thermal fluctuations. As further  $\text{Eu}^{3+}$  is added the situation eventually reverses, such that the existence of regions with parallel alignment becomes so energetically unfavorable as to be improbable. A statistical thermodynamic model of this transition is currently being developed with a primary goal being to extract the cooperative unit size.

The kinetics of bicelle reorientation exhibit further manifestations of the importance of bicelle-bicelle interactions. At lower water contents, the rate of approach to the equilibrium degree of perpendicular versus parallel alignment is rapid, occurring within a matter of minutes, and involves a single apparent rate constant. The equilibrium alignment is dictated by the  $\text{Eu}^{3+}$  level. Close interactions between bicelles dictate that all bicelles approach the equilibrium condition with the same rate. At higher water contents, the same equilibrium degree of perpendicular versus parallel alignment is eventually achieved for a given  $\text{Eu}^{3+}$  level, but the overall rate of approach to equilibrium is much slower. This follows from the reduced cooperativity between bicelles expected at higher water content and greater bicelle-bicelle spacing. Two rate constants are involved at higher water content: one that is as rapid as that observed at lower water contents, and one that is much slower. We suggest that the slower rate process corresponds to

accretion of individual bicelles onto the surface of a region of parallel alignment from the surface of an adjacent region of perpendicular alignment.

### Some consequences for biomacromolecular conformation studies

The parallel-aligned state has particular advantages for structural studies of bicelle-associated macromolecules, as mentioned previously. When inducing the parallel alignment one desires to avoid excess lanthanide in order to minimize lanthanide interactions with the macromolecule of interest, without sacrificing the interactions between the macromolecule and the bicelles. Typically, both such interactions will contain electrostatic contributions. The trick is to bind sufficient lanthanide to the bicelle such that parallel alignment is achieved, but retaining sufficient net anionic bicelle surface charge such that the macromolecule (typically overall cationic) also associates with the bicelle. The studies reported here indicate that with 10/90 DMPG/DMPC bicelles  $\text{Eu}^{3+}$  binding is quantitative up to and beyond the level of added  $\text{Eu}^{3+}$  sufficient to produce 100% parallel alignment ( $\text{Eu}^{3+}$  added per lipid, 0.04). The  $^2\text{H}$  NMR quadrupolar splittings indicate that such bicelles are nearly neutral or possess a slight net cationic charge. To produce a net anionic bicelle surface charge in the parallel-aligned state therefore one need only alter the bicelle composition to include a greater percent of anionic amphiphile. This would serve to attract electrostatically a cationic macromolecule, but minimizing excess lanthanide, because lanthanide binding would remain quantitative at the higher anionic surface charge. A complication evident with  $\text{Eu}^{3+}$  is that its binding is readily reversible, as apparent from its fast exchange, and a binding equilibrium would be set up between bicelles and any macromolecule present. It might be preferable, therefore, to employ a lanthanide with the highest possible chemical affinity for phospholipids.

We thank Prof. M.A. Winnik of this department for the use of his Perkin-Ekmer time-resolved fluorescence spectrometer, and Mr. Xin Lu for expert instruction in its use. Supported by a grant from the Natural Science and Engineering Research Council of Canada (to P.M.M.). K.J.C. thanks the Sumner Foundation for Fellowship support.

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