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Pegylation of Magnetically Oriented Lipid Bilayers

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We report NMR data for magnetically oriented phospholipid bilayers which have been doped with a lipid derivatized with a polyethylene glycol polymer headgroup to stabilize samples against aggregation. ^{13}C, ^{31}P, and ^{2}H NMR data indicate that the incorporation of PEG2000-PE (1% molar to DMPC) does not interfere with the orientation properties of bicelles prepared at 25% w/v with or without the presence of lanthanide. Bicelles prepared at 10% w/v are also shown to orient when PEG2000-PE is added. The addition of PEG2000-PE to cholesterol-containing, lanthanide-flipped bicelles is shown to inhibit sample phase separation and improve spectral quality. Furthermore, the addition of PEG2000-PE to high w/v bicelles (40% w/v) is demonstrated to lead to an increase in overall sample order.

Key Words: **bicelles; magnetic orientation; pegylation; steric stabilization.**

INTRODUCTION

Aqueous solutions of a bilayer-forming phospholipid and a detergent that can break up the extended bilayers into disc-shaped micelles (bicelles) are now well-known to spontaneously orient in magnetic fields (*1*). Magnetically oriented phospholipid bilayers have been demonstrated to be useful platforms for studying membrane-bound molecules using solid-state NMR methods (*2–4*). In addition, there has been keen interest in using dilute solutions of bicelles (3–15% w/v) to weakly align soluble macromolecules and measure anisotropic NMR spectral parameters that can be used to refine high-resolution solution NMR structures (*5, 6*).

The use of bicelles to either incorporate membrane-bound molecules or weakly align soluble molecules requires that the medium be stable over the time required to perform a series of NMR experiments. Despite the enthusiasm and reported successes with bicelle systems, there have been several reports of problems with sample instability and precipitation (*7–10*). Instability of bicelle samples can be due to chemical decomposition of the lipids as well as phase separation resulting from interactions among neighboring bicelles. Ottiger and Bax have suggested strategies for minimizing chemical decomposition of bicelle samples, including maintaining the pH between 6 and 7 and replacing the diacyl phospholipids of regular bicelles with hydrolysis-resistant dialkyl analogs (*11*). Losonczi and Prestegard have demonstrated that the incorporation of charged amphiphiles into bicelles minimizes phase separation in dilute (5% w/v) bicelle solutions (*8*). The addition of lanthanides to bicelle systems to "flip" the bicelles by 90° (to an orientation where the bilayer normal is oriented parallel to the field) can lead to frustrating problems with sample precipitation. Prosser and co-workers have reported that the addition of negatively charged lipids and a lanthanide chelator can minimize these problems (*10*).

We were prompted to tackle problems of bicelle instability while developing a magnetically orienting phospholipid system for use in spin-label EPR spectroscopy (*12*). Crucial to the stability of the EPR samples and the success of these experiments was the addition of a small amount (1% molar to DMPC) of a phospholipid that had a soluble polyethylene glycol polymer tail attached to its headgroup, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol)2000] (PEG2000-PE, see Fig. 1). We added PEG-derivatized lipid to follow the lead from work on drug delivery liposomes which has shown that PEG-lipids prevent close contact of the membrane surfaces of liposomes and cells, prolonging liposome circulation time (*13*). Before we began adding PEG2000-PE to our EPR spin-labeled bicelle samples, our lanthanide-containing samples showed evidence of precipitation within a few hours of preparation and oriented poorly or not at all. Once we started adding PEG2000-PE to the bicelles, the samples showed high degrees of orientation and were stable over several days. Presumably, PEG2000-PE prevents neighboring bicelles surfaces from approaching each other and fusing together to produce insoluble aggregates.

Our success with PEG-derivatized lipids in our bicelles prepared for EPR spectroscopy led us to explore their use in NMR spectroscopy. Here we demonstrate that PEG-derivatized lipids can be reconstituted into bicelles over a range of w/v conditions (10–40% w/v) and that the pegylated bicelles still achieve high degrees of magnetic orientation in NMR samples. The ability of PEG-derivatized lipids to improve sample stability is demonstrated using data collected on lanthanide-flipped, cholesterol-containing bicelles. Finally, the added benefit that PEG2000-PE lipids can lead to an increase in sample order for high w/v bicelles is shown.

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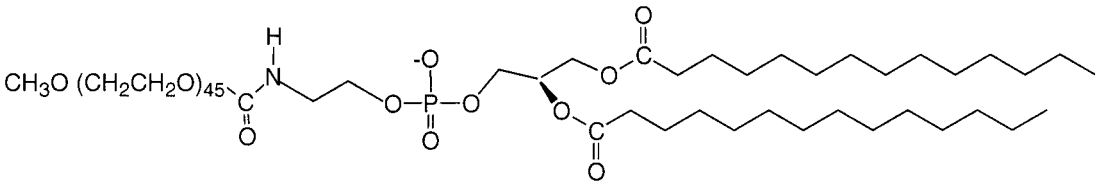

FIG. 1. Chemical Structure of PEG2000-PE lipid. (2000 refers to the approximate molecular weight for the polyethylene glycol polymer headgroup.)

RESULTS

For PEG-derivatized lipids to be useful in stabilizing bicelle systems, it first must be demonstrated that the presence of PEG-lipids does not interfere with the ability of the system to spontaneously orient in a magnetic field. Figure 2 shows ^{13}C and ^{31}P spectra of oriented 25% w/v bicelles both with and without the addition of PEG2000-PE (1% molar to DMPC). The positions of the two ^{31}P peaks (downfield DHPC, upfield DMPC) as well as the positions of the carbonyl peaks at ~180 ppm in the ^{13}C spectrum are particularly sensitive to orientation due to the effects of chemical shift anisotropy (*14*). As seen in Fig. 2, the addition of PEG-lipid does not significantly change the appearance of either the ^{13}C or the ^{31}P spectra. (The only difference in the ^{13}C spectrum of the sample with PEG2000-PE is the appearance of a sharp peak at 74 ppm which arises from the polyethylene glycol headgroup.)

Spectra of bicelles with a higher concentration of PEG2000-PE (3% molar to DMPC) were also collected at various w/v ratios (15–30% w/v) (data not shown). For w/v ratios below 25%, the spectra of 1% PEG2000-PE and 3% PEG2000-PE bicelles were nearly identical. However, at 25 and 30% w/v the spectra for 3% PEG2000-PE, although partially oriented, were broadened, consistent with possible "tangling" of neighboring discs and reduced motion.

After confirming that PEG2000-PE (at 1% molar to DMPC) did not interfere with bicelles which orient based on the diamagnetic susceptibility of the DMPC lipids, we tested the effect of PEG2000-PE on the orientation properties of lanthanide-containing bicelles. The lanthanide-induced flipping is evident in Fig. 3 by the changes in sign and magnitude of both ^{13}C and ^{31}P chemical shift anisotropy offsets with respect to those seen in Fig. 2 (*10*). As seen in Fig. 3, the addition of PEG2000-PE to lanthanide-doped bicelles does not significantly change the observed ^{31}P or ^{13}C carbonyl chemical shift anisotropy offsets. Consistent with the results shown in Fig. 2, the only change in the ^{13}C spectra upon the addition of PEG2000-PE is the appearance of a sharp peak at 74 ppm arising from the polyethylene glycol headgroup. Thus, PEG2000-PE does not interfere with the ability of lanthanide ions to flip the bicelles.

Next, we added PEG2000-PE to bicelles under the dilute conditions required to weakly orient soluble molecules and measure anisotropic parameters useful for refining high-reso-

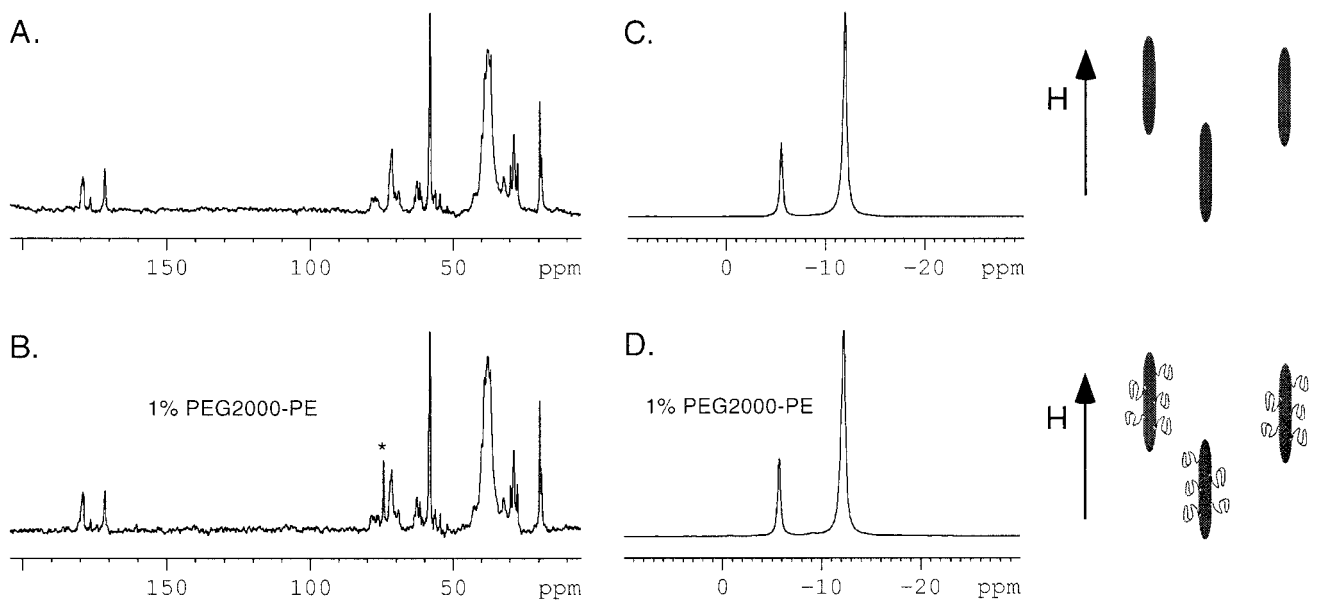

FIG. 2. ^{13}C–^{1}H decoupled spectra (A, B) and ^{31}P–^{1}H decoupled spectra (C, D) of 25% w/v DMPC/DHPC 3.5/1 bicelles in 50 mM HEPES, pH 7, at 313 K. (B, D) Included PEG2000-PE at 1% molar to DMPC. The cartoons at right illustrate the possible morphology of bicelles with and without the presence of PEG2000-PE. *In (B) indicates the peak from the PEG headgroup.

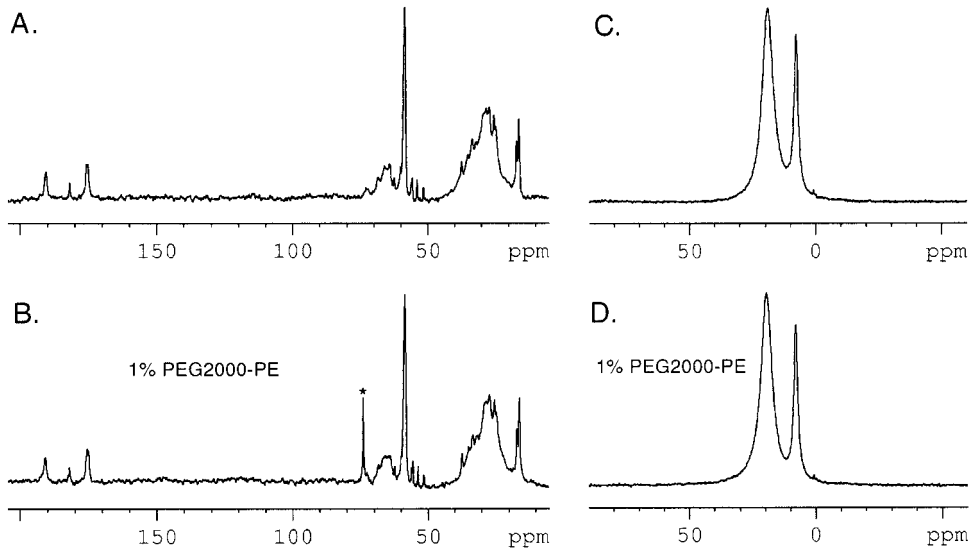

FIG. 3. ^{13}C–^{1}H decoupled spectra (A, B) and ^{31}P–^{1}H decoupled spectra (C, D) of 25% w/v DMPC/DHPC 3.5/1 bicelles with Yb^{3+} added (DMPC/Yb, 105:1 molar) in 50 mM Hepes, pH 7, at 313 K. (B, D) Included PEG2000-PE at 1% molar to DMPC. *In (B) indicates the peak from the PEG headgroup.

lution NMR structures. Although the primary purpose of doping bicelles prepared at ~25% w/v was to minimize the interaction between bicelles, PEG-lipids may also minimize undesirable interactions of soluble molecules with the lipid bilayer surfaces of the bicelles. Spectra from a 10% w/v bicelle sample with and without PEG2000-PE are shown in Fig. 4. The two ^{31}P spectra (Figs. 4A and 4B) are identical, consistent with the same degree of orientation of the two samples. The ^{2}H spectra (Figs. 4C and 4D) of the partially oriented D_2O solvent have very similar quadrupolar splittings (21 and 23 Hz, respectively), although the linewidths of the spectrum from the pegylated bicelle are broader. This linewidth difference could be related to the different hydration shell of pegylated bicelles since the headgroup of PEG2000-PE is known to bind a large number of water molecules (*15*).

The benefits of adding PEG2000-PE to bicelles are demonstrated in Figs. 5 and 6 using cholesterol-containing bicelles. Cholesterol constitutes about 30% of the mass of membrane lipids of many animal cell plasma membranes. There has been extensive work published on the role cholesterol plays in the

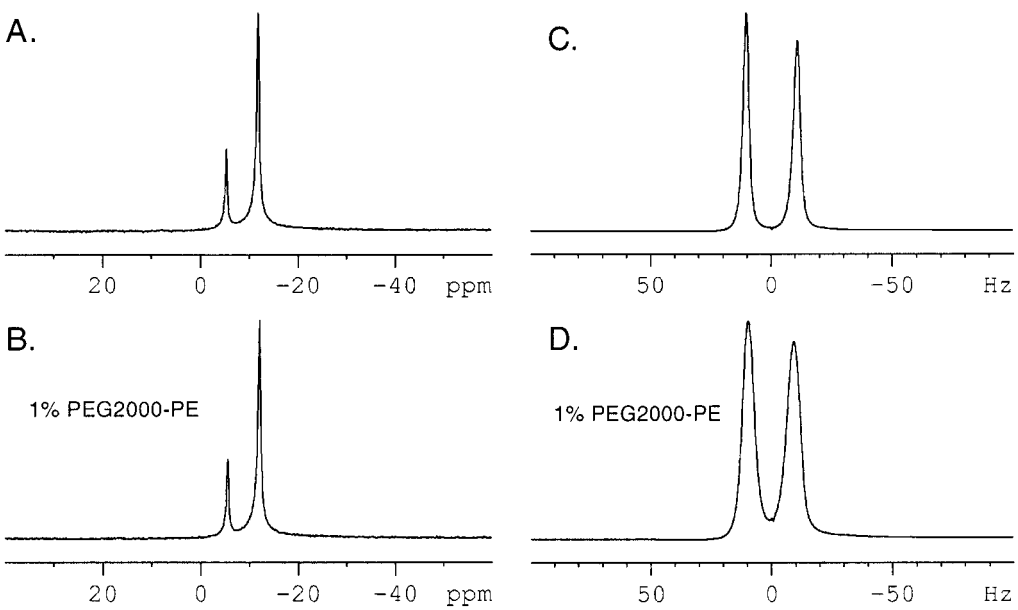

FIG. 4. ^{31}P–^{1}H decoupled spectra (A, B) and ^{2}H spectra (C, D) of 10% w/v DMPC/DHPC 3.5/1 bicelles in H_2O/D_2O at 313 K. (B, D) Included PEG2000-PE at 1% molar to DMPC.

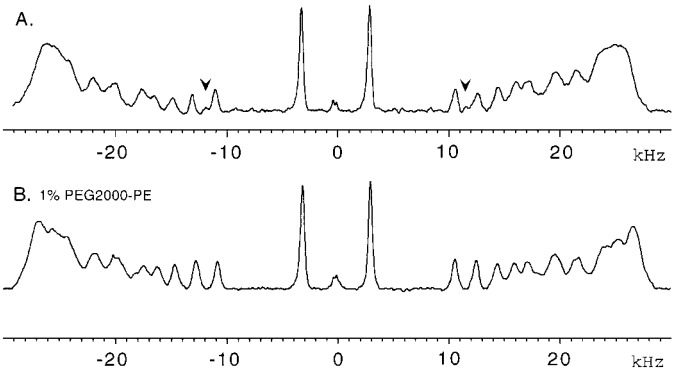

FIG. 5. ^{2}H spectra of 40% w/v DMPC/DHPC/cholesterol/Yb^{3+}/DMPC-d54 in the molar ratios 3.5/1/0.525/0.7/0.02 in deuterium-depleted water at 313 K. (B) Included PEG2000-PE at 1% molar to DMPC. Samples were equilibrated in magnet for 1.5 h prior to acquisition. Arrows in (A) indicate peaks resulting from phase instability.

phase properties and fluidity of membranes (*16*). We have made bicelles containing 5, 10, and 15% cholesterol (% molar to DMPC) and have found that these bicelles still spontaneously orient in an applied magnetic field (data not shown). Although we have not had significant problems with the 5 and 10% cholesterol samples, our bicelles containing 15% cholesterol have had repeated stability problems. The addition of cholesterol at 15% molar to DMPC changes the appearance of the sample, which is normally clear a few degrees below room temperature, to milky white. The addition of 1% PEG 2000-PE to these 15% cholesterol bicelles leads to a marked difference in appearance from being milky white to being translucent (although not entirely clear like bicelles lacking cholesterol). We collected ^{2}H spectra of 15% cholesterol bicelles doped with a small amount of chain-perdeuterated DMPC (DMPC-d54) and flipped with the addition of Yb^{3+} both with and without PEG2000-PE. As shown in Fig. 5, the sample with PEG2000-PE (Fig. 5B) has improved spectral resolution compared to the bicelles without PEG2000-PE (Fig. 5A). PEG2000-PE eliminates the extra small peaks present in Fig. 5A that degrade sensitivity and resolution. When we removed the sample without PEG2000-PE from the magnet it was evident that it had separated into multiple phases, which lends to the heterogeneity seen in Fig. 5A.

In addition to improving behavior of samples prone to instability, PEG2000-PE lipids have the additional benefit of increasing sample order in some bicelle samples. Figure 6 shows ^{2}H quadrupole spectra of bicelles that have cholesterol, but no Yb^{3+}. (Note that the quadrupolar splittings in Fig. 6 are $\sim$1/2 of those seen in Fig. 5 due to the fact that without Yb^{3+} the disks orient with their bilayer normals perpendicular to the applied magnetic field rather than parallel as they do with lanthanide present.) Figure 6A was collected on bicelles without PEG2000-PE and Fig. 6B was for bicelles with 1% PEG2000-PE. There is an increase in each of the quadrupole doublets in Fig. 6B with respect to the corresponding doublet in Fig. 6A. The quadrupole splittings for the outermost doublets (indicated in the figure) increase upon the addition of PEG2000-PE by 1170 Hz. An increase in splittings upon addition of PEG2000-PE was consistently observed in samples prepared at high w/v ratios (greater than 35% w/v). Since PEG-lipids have been shown not to change the normal structure or thickness of the bilayer interior (*17*) and there was an increase in quadrupole splitting for all of the doublets, we

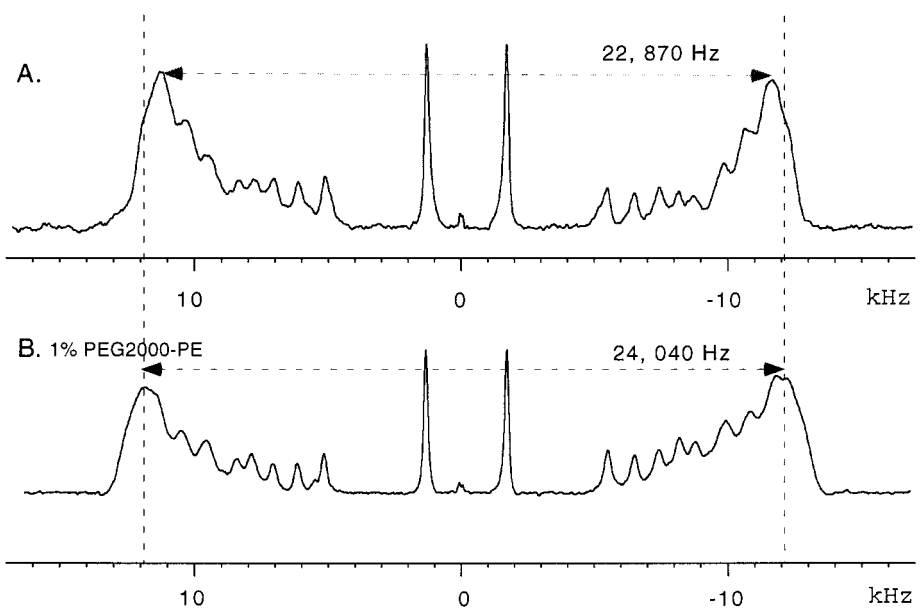

FIG. 6. ^{2}H spectra of 40% w/v DMPC/DHPC/cholesterol/DMPC-d54 in the molar ratios 3.5/1/0.525/0.02 in deuterium-depleted water at 313 K. (B) Included PEG2000-PE at 1% molar to DMPC.

propose that the source of this increase in quadrupolar splitting is a small overall increase in sample order. Even small increases in sample order can make a difference in significantly improving resolution of oriented samples with a high degree of spectral overlap. Note that the increase in sample order upon addition of pegylated lipids was not seen in Figs. 2–4. Figures 2–4 were for bicelles prepared at lower w/v ratios than the 40% w/v samples used in Fig. 6.

DISCUSSION

As illustrated in Figs. 2–6, bicelles can magnetically orient when doped with PEG2000-PE. The results in Fig. 5 and our recently published results with bicelles prepared for oriented EPR spin-label studies demonstrate that PEG2000-PE stabilizes bicelle samples against precipitation (*12*). What properties do PEG-lipids have that would promote stability in lipid-bilayer-based systems? This question is extensively addressed in the literature on drug delivery liposomes using methods such as X-ray diffraction, electron microscopy, and micropipet manipulation (*13*). PEG-lipids are commonly referred to as "stealth" lipids for their ability to shield surfaces from recognition and binding. The primary mechanism for PEG-lipid's protective properties has been reported to be steric stabilization, where the bulky water soluble polymer extends away from the surface and provides a barrier against the approach and adsorption of other particles. As seen in Fig. 1, the PEG-lipid headgroup also bears a negative charge. Thus the contribution of electrostatic repulsion to the interaction of pegylated surfaces should be considered. Studies on the repulsion of pegylated surfaces at various salt concentrations have indicated that for PEG2000-lipids the electrostatic contribution to the repulsion of neighboring surfaces is relatively small, particularly at the high ionic strengths relevant for drug delivery (*17*). However, they indicate that at low ionic strengths, electrostatic repulsion can play a role in the interaction of pegylated surfaces at low pressures and large separations. Another important finding for the application of PEG-lipids to bicelle studies of membrane-bound molecules is that the addition of PEG-lipids does not change the normal structure or thickness of the bilayer interior (*17*).

The repulsive barrier created by PEG-lipids inhibits the contact-destabilization that neutral lipid bilayers, in the absence of surface polymer, can experience due to interbilayer van der Waals forces. This suggests that the addition of PEG-lipids to bicelles could keep neighboring bilayer surfaces apart, limiting aggregation. In addition to limiting the interaction of lipid bilayer surfaces, PEG-lipids have potent protein-repelling activity (*18*). The addition of PEG-lipids to dilute bicelles prepared to weakly orient soluble molecules holds the promise of preventing unwanted binding of soluble macromolecules that have a hydrophobic patch or other affinity for bilayer surfaces. These dilute stealth bicelles could help solve the previously reported problems of sample instability reported with conventional unmodified bicelles (*7*).

How much PEG-lipid is needed to cover the bilayer surface and thus protect the bicelle from interactions that could lead to undesirable aggregation? The extent of coverage depends on the conformation of the polymer headgroup. The physical properties of the polymer headgroup of PEG-lipids depend on how concentrated they are in lipid bilayers (*18, 19*). When the density of PEG-lipid is low in the bilayer, the polymer chains do not interact laterally and the chains forms separate compact "mushrooms" (as shown in the cartoon in Fig. 2). When the PEG-lipid density is high, the chains overlap laterally and extend to form "brushes." The specific grafting levels that correspond to either mushroom or brush depend on the length of the PEG-lipid headgroup (*18*). At 1% molar to DMPC, PEG2000-PE is predicted to be in mushroom form (*18*). The extension of the mushroom away from the surface is on the order of the Flory radius ($R_f = aN^{3/5}$), where N is the degree of polymerization ($N = \sim 45$ for PEG2000) and a is the monomer size ($a = 3.5$ Å for PEG2000). Thus R_f and the extension of the headgroup of PEG2000-PE from the bilayer surface is ~35 Å (*20*). Calculations using the theoretical cross sections of the PEG polymer and the bilayer lipids indicate that ~1.5 mol% PEG2000-PE in the mushroom form is needed to completely cover the surface of a phosphatidylethanolamine bilayer (*18*).

The extension of the headgroup of PEG-lipids away from the lipid surface can help explain the observed increase in order parameter for high w/v bicelle samples. Since the PEG-lipids extend out from the bicelle surface and increase the effective volume taken up by each bicelle, the PEG-lipids reduce the free volume between neighboring bicelles, increasing order. By using different mole percentages of PEG-lipid as well as using some of the other commercially available PEG-lipids with different polymer headgroup lengths, one could potentially optimize a particular bicelle system in terms of both surface coverage and orientation properties.

CONCLUSIONS

We have demonstrated that PEG2000-PE can be reconstituted into bicelles and that pegylated bicelles still achieve high degrees of magnetic orientation. Furthermore, we have shown that pegylated lipids improve sample stability of cholesterol-containing lanthanide-flipped bicelles. For high w/v ratio bicelles, pegylation also leads to an overall increase in sample order. We think that pegylation holds promise for bicelles prepared under the dilute conditions required to weakly orient soluble molecules and measure anisotropic parameters useful for refining high-resolution NMR structures. The presence of PEG polymers at the surface of bicelles may minimize unwanted binding of soluble macromolecules. Finally, since PEG-lipids are commercially available and straightforward to reconstitute, they have the potential for solving instability problems across the range of different bicelle media currently being explored for a variety of different applications (*21*).

EXPERIMENTAL

1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine-d54 (DMPC-d54), 1,2-dicaproyl-*sn*-glycero-3-phosphocholine (DHPC), and 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol)2000] (PEG2000-PE) were purchased from Avanti Polar Lipids (DMPC and PEG2000-PE as lyophilized powders and DHPC as a chloroform solution.) All other materials were purchased from Sigma/Aldrich. Oriented bicelles were prepared as follows. DHPC in chloroform was rotovapped and then placed under high vacuum for at least 8 h. Half of the buffer needed for the final solution was added to the flask containing the dried-down DHPC and the other half added to a separate flask containing the DMPC and PEG2000-PE. Both flasks then went through several cycles of vortexing and warming until all the dried material was released from the sides of the flask. (If cholesterol was to be added, it was mixed with the flask containing DMPC and PEG2000-PE.) The DHPC solution was then added to the flask containing DMPC and PEG2000-PE and vortexed until the sample was homogeneous. Finally, the solution went through a freeze/thaw cycle using liquid nitrogen. Ytterbium chloride hexahydrate was added as an aqueous solution. Samples were then transferred to 5-mm tubes for NMR spectroscopy. All spectra were collected on a Bruker DRX 400 MHz spectrometer using a 5-mm tunable broadband probe (100.61 MHz for ^{13}C, 161.98 MHz for ^{31}P, and 61.42 MHz for ^{2}H). Samples were not spun and ^{31}P and ^{2}H spectra were acquired with the deuterium field frequency turned off. Samples equilibrated for at least 30 min at 313 K in the magnetic field prior to acquisition.

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