Magnetically Aligned Phospholipid Bilayers at the Parallel and Perpendicular Orientations for X-Band Spin-Label EPR Studies

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Received September 5, 2000 Revised Manuscript Received January 22, 2001

Magnetically aligned phospholipid bilayers (bicelles) have been successfully used in a range of solid-state and solution NMR studies to macroscopically order both membrane-bound[1−5] and water-soluble macromolecules.[6−9] Sample orientation enables the efficient high-resolution measurement of anisotropic spectral parameters that provide valuable structural and dynamic information for both NMR and EPR studies.[6,10−13] A standard bicelle consists of a combination of long chain bilayer forming 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) phospholipids and short chain 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC) phospholipids that when mixed together under the correct conditions magnetically align. The negative sign of the magnetic susceptibility anisotropy tensor for the phospholipid bilayers dictates that the bicelles align with their bilayer normal oriented perpendicular to the direction of the static magnetic field.[4] Unfortunately, the standard DMPC/DHPC bicelle disks used in NMR studies do not completely align at the perpendicular orientation at low magnetic fields used for X-band EPR studies presumably due to the lack of sufficient negative magnetic susceptibility anisotropy. The inability to study magnetically aligned phospholipid bilayers at this orientation at low magnetic fields dramatically limits the usefulness of this technique for spin-label EPR studies. In this communication, we successfully demonstrate with spin-label EPR spectroscopy that magnetically aligned phospholipid bilayers can be investigated with their bilayer normals perpendicular with the magnetic field in a conventional X-band EPR spectrometer.

The addition of paramagnetic lanthanide alignment reagents with large positive magnetic susceptibilities (Eu^{3+}, Er^{3+}, Tm^{3+}, or Yb^{3+}) causes the phospholipid bilayer disks to align with their bilayer normals parallel with B_0.[14,15] Previously, we demonstrated that phospholipid bilayers doped with either Tm^{3+} or Yb^{3+} magnetically align at this orientation using spin-label EPR spectroscopy at X-band and discussed conditions for optimal alignment.[16,17] Here, with the aim of increasing the magnitude of the overall negative $\Delta\chi$ of the disks to induce a perpendicular bicelle alignment, we have added Dy^{3+} to the DMPC/DHPC bicelle matrix which alternatively possess a large negative $\Delta\chi$.[14,18] The ability to align lipid bilayer systems in the perpendicular as well as parallel orientation is valuable because the combination of both types of anisotropic spectra provides a more detailed and complete description of the structural and motional properties of the membrane-associated spin label than either type alone.[19,20]

The orientational characteristics of the nitroxide label can be described by the molecular axes (*x*, *y*, and *z*) and are defined relative to the doxyl ring.[20] The *x*-axis is along the N−O bond, the *z*-axis is along the 2p π orbital of the nitrogen, and the *y*-axis is perpendicular to the *x*, *z* plane. Typical principal values for a nitroxide hyperfine tensor are $A_{xx} = 6$ G, $A_{yy} = 6$ G, and $A_{zz} = 32$ G.[20] In an aligned spectrum, the orientation that the spin label makes with respect to the magnetic field and the motion about its molecular axis will determine the observed hyperfine splitting. Figure 1 illustrates the effects of bicelle alignment (parallel and perpendicular) on the observed hyperfine splittings of two different spin labels (3β-doxyl-5α-cholestane (CLS) and 5-doxylstearic acid). EPR spectra A and D of Figure 1 display randomly dispersed bicelle disks and serve as an excellent basis for comparison between the subsequent perpendicular and parallel aligned bicelle EPR spectra.[17,21] The line shape and hyperfine splitting of the spectrum shown in Figure 1A is similar to previous data gathered at 318 K.[17] As illustrated in Figure 1, CLS aligns with its long axis (dotted line) parallel to the long axis of the phospholipids and undergoes rapid rotation ($R_{||}$) about this axis.[22] The nitroxide *y*-axis is approximately parallel to the long axis of the steroid derived spin probe.[22] Figure 1B shows the EPR spectrum of a DMPC/DHPC/Dy^{3+} bicelle sample doped with CLS. The observed increase in hyperfine splitting when compared to the randomly dispersed spectrum indicates that the bilayer normal of the Dy^{3+}-bicelle is aligned perpendicular to the magnetic field. At this orientation, the *y*-axis and axis of rotation are approximately perpendicular to the magnetic field; thus, the *x*- and *z*-tensoral components are averaged. The measured hyperfine splitting of 18.1 G is close to the theoretical value of $(A_{zz} + A_{xx})/2 = 19$ G. Conversely, Figure 1C shows an EPR spectrum of a Tm^{3+}-bicelle sample containing CLS. The observed 9.2 G hyperfine splitting is significantly reduced with respect to the unoriented and perpendicular aligned samples in spectra A and B of Figure 1 and is consistent with macroscopic orientation of the membrane bilayers such that their normals (and hence *y*-axis of associated CLS spin labels) are nearly parallel with B_0. The experimentally measured hyperfine splittings agree with values ranging from 17.5 to 19.0 G (perpendicular) and 8.0−10.0 G (parallel) in previously published mechanically oriented spectra with varying degrees of cholesterol.[21]

The alignment of the bicelle disks was further confirmed with a 5-doxylstearic acid spin label in which the long axis of the fatty

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10.1021/ja005574i CCC: $20.00
Published on Web 03/03/2001

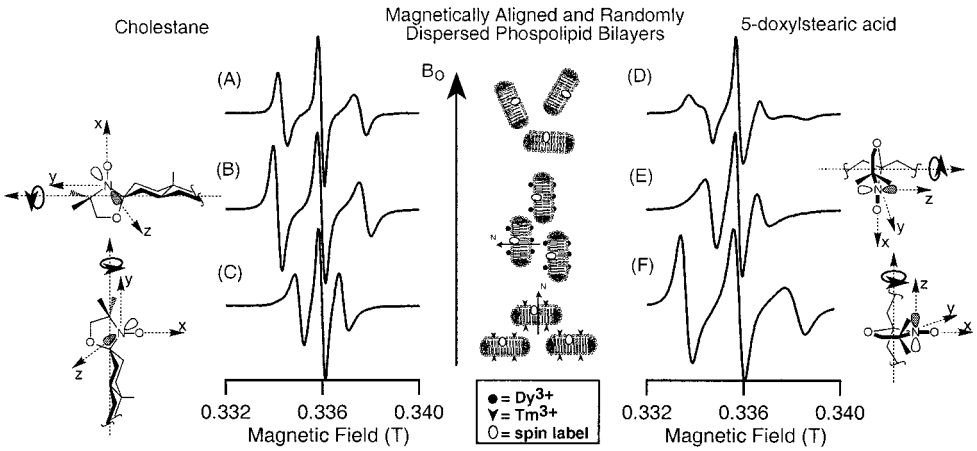

Figure 1. Comparison of magnetically aligned and randomly dispersed phospholipid bilayers with spin-label EPR spectroscopy. (A) EPR spectrum of randomly dispersed bicelle disks containing CLS. (B) EPR spectrum of magnetically aligned phospholipid bilayers (membrane normal is perpendicular with B_0) containing CLS and Dy^{3+}. (C) EPR spectrum of magnetically aligned bicelles (membrane normal is parallel with B_0) containing CLS and Tm^{3+}. (D) Same as spectrum A except 5-doxylstearic acid was used. (E) Same as spectrum B except 5-doxylstearic acid was used. (F) Same as spectrum C except 5-doxylstearic acid was used. The orientation and axis of rotation of the two spin labels with respect to the magnetic field and membrane normal of the bicelle are displayed on the edges of the figure. In the center, cartoons depicting the orientation of the disks are shown (bilayer normals shown as arrows). All samples consisted of DMPC/DHPC/cholesterol/Ln^{3+}/PEG2000-PE/spin label in molar ratios of 3.5/1.0/0.35/0.70/0.035/0.0056 in 100 mM HEPES buffer, pH 7.0. 10% molar cholesterol was added to all of our samples to decrease fluidity and stabilize the spin label within the bicelle. For the randomly dispersed spectra, the sample temperature was raised from 298 to 318K in the absence of B_0 prior to taking spectra at 308 K. All spectra were collected with a center field of 3350 G, sweep width of 100 G, $\nu = 9.39$ GHz, modulation amplitude of 1.0 G, and a microwave power of 2.5 mW. Conditions for magnetically aligning the phospholipid bilayer disks has been described previously.[17]

acid is collinear with the long chains of the DMPC phospholipids. For the 5-doxylstearic acid in the parallel Tm^{3+}-bicelle alignment, the *z*-component and the axis of rotation are approximately parallel with the bilayer normal and the magnetic field (Figure 1F). The measured hyperfine splitting is equal to 22.0 G and matches a similar value of 22.5 G obtained at this orientation in the literature.[21,23] Conversely, for the perpendicular Dy^{3+}-bicelle alignment (Figure 1E) the *x*- and *y*-tensoral components are motionally averaged to reveal a measured hyperfine splitting of 11.4 G. This splitting agrees well with a literature value of 11.9 G.[21,23] The dynamics of the 5-doxylstearic acid are more complicated than CLS because rapid segmental motion occurs due to a large number of *gauche*−*trans* interconversions of the fatty acid chain resulting in a partial averaging of the hyperfine tensors.[21,24] Clearly, the orientational-dependent hyperfine splittings observed in spectra E and F in Figure 1 demonstrate the effects of macroscopic bilayer alignment when compared to the randomly dispersed spectrum in Figure 1D. All of the hyperfine splittings and the shape of the observed spectra displayed in Figure 1 closely resemble the EPR spectra in the literature obtained from mechanically aligned phospholipid bilayers on glass plates.[21−24] As a control, we prepared a DMPC/$DMPCd_{54}$/DHPC/Dy^{3+} bicelle sample and checked for alignment via NMR spectroscopy. The corresponding spectrum yielded a well-resolved ^{2}H NMR spectrum that matches the shape, breadth, and resolution of magnetically aligned bicelle spectra shown in the literature.[1]

Conformational changes within membranes have been shown to be small upon Ln^{3+} binding; however, minimizing these changes is important.[15] A phospholipid that chelates Ln^{3+} ions will be employed in future studies.[3]

The methodology described in this report enables high-resolution oriented EPR studies to be carried out at two different orientations with respect to the direction of the membrane normal and B_0. The anisotropic EPR data gleaned from these two distinct orientations will greatly enhance our ability to study membrane proteins with spin-label EPR spectroscopy and provide a more detailed structural and orientational picture of the probe with respect to the membrane when compared to randomly dispersed bilayers.[16,17] The bilayers in magnetically aligned bicelles are in the L_α phase and have the capacity to incorporate integral membrane proteins.[4,5] Thus, this alignment method will be useful for investigating site-specific spin-labeled membrane proteins. Finally, this magnetically aligned system can be used for both NMR and EPR studies and several published reports have already explicitly called for studies on phospholipid−protein interactions that combine well-resolved ^{2}H NMR results with EPR spin label studies.[25,26]

Acknowledgment. The EPR and NMR spectrometers were obtained from NSF Grants CHE9724192 and CHE9012532. G.A.L. acknowledges support from NIH Grant GM60259-01 and ACS-PRF 35352-G4. A.P. acknowledges support from Howard Hughes Grant No. 71199-520403. K.P.H. acknowledges support from Swarthmore College and NIH Grant GM57627-01.

JA005574I

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