Magnetically Oriented Phospholipid Bilayers for Spin Label EPR Studies

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This paper reports the development of a model membrane system that spontaneously orients in the applied magnetic field of an EPR spectrometer. There has been a great deal of excitement recently over the use of magnetically oriented phospholipid bilayers (bicelles) for both solution and solid-state NMR spectroscopy.[1−4] Here we report nitroxide spin label data from a novel bicelle system which orients in the lower magnetic field of a conventional EPR spectrometer (<1 T for an X-band EPR spectrometer as opposed to ∼11.8 T for a 500 MHz NMR spectrometer). The system offers the opportunity to carry out EPR studies using a well-oriented highly hydrated model membrane system whose preparation is amenable to the reconstitution of labile membrane components such as integral membrane proteins. Furthermore, the magnetically orienting system reported here presents new opportunities to carry out both NMR and EPR studies on the same sample preparation providing complementary dynamic information in two different time scale regimes.

EPR studies of membrane systems utilizing nitroxide spin probes have greatly improved the view of the dynamic behavior of membrane components at a molecular level by providing information about the types and rates of motion, as well as the degree of organization of lipids.[5,6] Nitroxide spin labels are sensitive monitors of molecular organization and can provide detailed information for small quantities of biological systems. Nitroxide EPR studies have provided important insights into a wide range of membrane phenomena including information about lipid phase transitions[7] and information about how proteins interact with membrane lipids.[8]

It has long been known that the use of well-oriented multibilayers of lipids can greatly increase spectral resolution of EPR membrane studies as well as provide important data by allowing the collection of spectra obtained as a function of the angle between the magnetic field and the bilayers.[7,9,10] However, many of the existing alignment methods suffer from significant drawbacks such as labor intensive preparation, instability at high levels of hydration, and the requirement of procedures involving either organic solvents or elevated temperatures that can destroy labile membrane components such as proteins. Some procedures have been devised whose methods are more amenable to the study of membrane proteins. These methods include one study where whole cells were aligned in an external magnet (but not the magnetic field of the EPR spectrometer as reported here), followed by freezing of the sample before transferring the sample to an EPR spectrometer.[11] However, this method required freezing the samples and resulted only in partially aligned samples. Most recently Freed and co-workers reported the use of isopotential spin-dry ultracentrifugation to produce well-aligned films of both bacteriorhodopsin- and gramicidin-containing membranes.[12] This method enabled the collection of extensive oriented experimental data that, combined with quantitative theoretical analyses, provided a detailed description of protein/lipid interactions for membrane preparations containing 20 wt % water.

We think the method reported here offers significant advantages over previous alignment methods in terms of ease of preparation, stability at high degrees of hydration, and suitability for reconstitution of membrane proteins. The magnetically aligned samples described here are related to the systems initially introduced by Prestegard and co-workers[13] and subsequently adapted by Vold and co-workers[2] to align the bilayer normals of lipid bilayers so that they are parallel with the direction of the applied magnetic field. The oriented system we present here is composed of a mixture of a bilayer-forming phospholipid (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine, DMPC) and a short chain phospholipid (1,2 dicaproyl-*sn*-glycero-3 phosphocholine, DHPC) that breaks up the extended bilayers into what has been described as disc-shaped micelles (bilayered micelles, or bicelles) that are highly hydrated (in this case 75% aqueous). To characterize this system for EPR spectroscopy, we have utilized a steroid derivative nitroxide spin probe called 3β-DOXYL-5α-cholestane (cholestane). This rigid and elongated probe orients with its long axis parallel to the lipid acyl chains and reports on the general order of the lipids. The DMPC bilayers were enriched with 10% molar cholesterol to increase the local order in the membrane, restricting the movement of the spin probe and highlighting the effects of macroscopic bilayer orientation on the measured EPR spectra. The data reported here are from samples doped with Yb^{3+} ions (10% molar to DMPC).[14] This system was used because the large magnetic susceptibility induced on the lipid bilayers by the Yb^{3+} ions enabled it to be one of the easiest bicelle systems to orient in the magnetic field of an X-band EPR spectrometer.[15]

Crucial to the success of the experiment and stability of the sample was the addition of a small amount (1% molar to DMPC) of phospholipid that has a soluble poly(ethylene glycol) polymer tail attached to its headgroup, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol)2000] (PEG2000-

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(15) Oriented bicelles were prepared as follows. DMPC, cholesterol, and PEG2000-PE were co-solubilized in chloroform, rotovapped, and then placed under high vacuum for at least 8 h. In a separate flask, DHPC and cholestane spin label were also co-solubilized in chloroform, rotovapped, and then placed under high vacuum for at least 8 h. Half of the 100 mM HEPES buffer needed for the final solution was added to the flask containing DHPC/cholestane and the other half added to the flask containing DMPC, cholesterol, and PEG2000-PE. Both flasks then went through several cycles of vortexing and warming until all of the dried material was released from the sides of the flask. DHPC/cholestane solution was then added to the flask containing DMPC, cholesterol, and PEG2000-PE and vortexed until the sample was homogeneous. Finally, the solution went through two freeze/thaw cycles using liquid nitrogen. Ytterbium chloride hexahydrate was added as an aqueous solution. Samples were then transferred to a quartz flat cell for EPR spectroscopy. DMPC, DHPC, and PEG2000-PE were purchased from Avanti Polar lipids. All other materials were purchased from Sigma/Aldrich.

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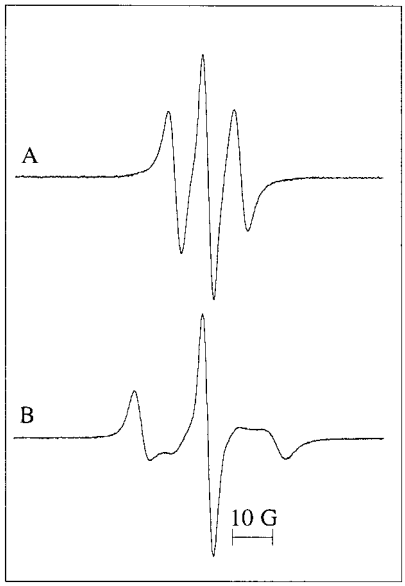

Figure 1. EPR spectra of cholestane incorporated into (A) macroscopically oriented bilayers composed of DMPC/DHPC/cholesterol/$YbCl_3$/PEG2000-PE/cholestane in the molar ratios 3.5/1.0/0.35/0.35/0.035/0.0056 in 100 mM HEPES buffer, pH 7.0. (B) multilamellar liposomes at 25% w/v with the same composition of (A) except DHPC was not included. Both spectra collected at 313 K on a X-band Bruker ER200D spectrometer (standard TE_{102} cavity) equipped with a variable temperature setup. Spectra were collected with a sweep width of 100 G, center field was 3355 G, 100 kHz field modulation frequency, scan time ~4 min and a microwave frequency of 9.5 GHz under nonsaturating conditions (signal intensity would still increase with increasing microwave power). Because the two spectra were calculated from two samples with slightly different volumes, an accurate comparison of spectral intensities is difficult. Peaks heights were thus normalized to give the same peak height of the center peak in both spectra. (However, our experience is that oriented samples gave higher relative spectral intensities than unoriented samples for the same amount of spin label.) The samples were equilibrated in the EPR magnet at 7400 G for 2 h prior to collection of spectra.[27,28]

PE). We added PEG-derivatized lipid to follow the lead from the literature on drug delivery liposomes which has demonstrated that PEG-PE provides a strong steric barrier that prevents close contact of membrane surfaces of liposomes and cells and prolongs liposome circulation time.[16] Before we began adding PEG2000-PE to our spin-labeled bicelle samples, our lanthanide-containing samples showed evidence of precipitation within a few hours of preparation and either 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 bicelle surfaces from approaching each other and fusing together to produce insoluble aggregates. We have also found that PEG2000-PE can be used for a range of different bicelle samples used for NMR studies and will offer advantages in other membrane bicelle preparations.[17]

Figure 1 clearly demonstrates the effects of macroscopic orientation on the EPR spectra of cholestane spin label in cholesterol-containing DMPC bilayers. The EPR spectra of nitroxide spin probes consist of a triplet due to the hyperfine interaction between the nuclear spin of the nitrogen (^{14}N) nucleus and that of the unpaired electron. At a microwave frequency of 9.5 GHz (~0.3 T magnetic field), the anisotropic contribution to the spectra is dominated by this hyperfine interaction. The standard system of coordinates for the N−O paramagnetic moiety is with the *x*-axis along the N−O bond and the *z*-axis along the nitrogen 2pπ orbital containing the unpaired electron density.[18] Typical principal values for the hyperfine tensor of the cholestane spin label in lipid bilayers are $A_{zz} = 33.8$ G, $A_{xx} = A_{yy} = 5.0$ G.[7,19] For cholestane, the nitroxide *y*-axis is approximately parallel to the long axis of the probe. Previous work has demonstrated that cholestane aligns with it long axis parallel to the long axis of the lipids and undergoes rapid rotation about this axis.[20] The reduction of the hyperfine splitting in Figure 1A with respect to the unoriented sample in Figure 1B is clearly indicative of macroscopic orientation of the membrane bilayers with their normals (and hence *y*-axis of associated cholestane spin labels) parallel to the applied magnetic field. The "apparent" splitting (measured splitting between low field and middle field spectral lines) in the oriented spectrum in Figure 1A is 8.7 G, which is in close agreement with values measured in previous published spectra with cholestane spin label incorporated into oriented ~10 mol % cholesterol containing phospholipid bilayers on glass plates.[20,21]

The magnetic ordering methodology described here provides a promising means of further extending high-resolution oriented EPR methods to a wider range of protein-containing membrane systems. As already demonstrated in several NMR studies, the lipid bilayers in magnetically oriented bicelles are in the liquid crystalline phase and have the capacity to incorporate membrane proteins.[22−24] Furthermore, this magnetically orientable 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 ^2H NMR results with EPR spin label studies.[6,12]

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(27) At 3400 G with 10% Yb^{3+}, the sample was partially oriented after 30 min. However, after the sample equilibrated in the magnet for 2 h with the field turned up to 7400 G, the spectral features characteristic of powder patterns disappeared. At higher concentrations of Yb^{3+}, orientation occurred much faster and at a lower magnetic field (3400 G); however, spectral broadening occurred presumably due to the interaction of Yb^{3+} with the nitroxide moiety of the cholestane spin labels (which has been shown to be at the level of the carbonyl groups of the lipids composing the bilayers[12]). Other spin labels that are buried deeper in the lipid bilayer should not be broadened to the same degree.
(28) Since the degree of orientation increases with the square of the magnetic field,[13] the use of high-field EPR spectroscopy holds promise. In addition to ordering effects, at higher magnetic fields the nitroxide Zeeman interaction exceeds that of the nitrogen hyperfine interaction, and since the *g*-tensor of nitroxide radicals is nonaxial, this opens the possibility of being able to study features of lateral and transverse ordering directly.[25, 26]