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Investigating magnetically aligned phospholipid bilayers with EPR spectroscopy at Q-band (35 GHz): optimization and comparison with X-band (9 GHz)

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Abstract

This paper presents the improvement and advantages of investigating magnetically aligned phospholipid bilayers (bicelles) utilizing electron paramagnetic resonance (EPR) spectroscopy at a microwave frequency of 35 GHz (Q-band) and at a high magnetic field strength of 1.25 T when compared to weaker magnetic fields for X-band EPR studies. The nitroxide spin label 3β-doxyl-5α-cholestane (cholestane or CLS) was inserted into the bicelles and utilized to demonstrate the effects of macroscopic bilayer alignment through the measurement of orientational dependent hyperfine splittings. The effects of different lanthanide ions with varying degree of magnetic susceptibility anisotropy were examined. The requirement of minimal amounts of the Tm^{3+} and Dy^{3+} lanthanide ions for well-aligned bicelles were examined for Q-band and compared with amounts required for X-band bicelle alignment studies. At a magnetic field of 1.25 T (when compared to 0.63 T at X-band), the perpendicular and parallel orientation were aligned with lower concentrations of Dy^{3+} and Tm^{3+}, respectively, and thereby eliminating/minimizing the unwanted effects associated with lanthanide-protein interactions. Thus, it is much easier to magnetically align phospholipid bilayers at Q-band when compared to X-band.

Keywords: Magnetic alignment; Q-band; X-band; EPR; Phospholipids; Cholestane

1. Introduction

A promising biophysical technique for studying uniaxially aligned phospholipid bilayers is based on the magnetic alignment of the membrane bilayers of a certain composition in a static magnetic field of high strength [1–6]. A recently developed model membrane system called magnetically aligned phospholipid bilayers (bicelles), [1,2,6,7] that spontaneously align in a magnetic field, has been demonstrated to be successful for nuclear magnetic resonance (NMR) spectroscopic studies of membrane and integral membrane proteins [2,3,7–14]. Generally, bicelles consist of a binary mixture of a long chain 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and short chain 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC) phospholipids [2,15]. Although bicelle model membrane systems were developed for NMR studies, one recent review article pointed out the enormous potential for examining oriented protein/phospholipids bicelle system with spin label electron paramagnetic resonance (EPR) spectroscopy [6]. EPR spectroscopy has been widely used by several researchers to study the orientational dependent behavior of nitroxide spin labels incorporated into aligned membrane systems [16–25]. In oriented membrane samples, the resulting EPR spectra reveal orientational dependent changes in the hyperfine splitting based upon the alignment of the spin label with respect to the magnetic field as well as a

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reduction in the corresponding linewidths. The anisotropic hyperfine coupling of aligned spin-labeled bicelles can provide a more detailed structural and orientational description of the spin label with respect to the membrane when compared to randomly dispersed bilayer samples.

The magnetic alignment of the bicelle system depends on several factors including the strength of the magnetic field (B_0), sample composition, the sign and the magnitude of the magnetic susceptibility anisotropy tensor ($\Delta\chi$), and the temperature of the system. Previous studies have shown that the overall magnetic susceptibility anisotropy tensor of the bicelle system plays a major role in the bicelle alignment [2]. Normally, the bicelles align with their bilayer normal perpendicular to the direction of the static magnetic field due to the negative sign of $\Delta\chi$. For low field X-band EPR experiments, the phospholipid bilayers do not fully align at this orientation without additional alignment reagents. The addition of Dy^{3+}, which posses a large negative $\Delta\chi$ [26], is required to induce a perpendicular bicelle alignment [27]. Conversely, the addition of paramagnetic lanthanide ions with a large positive magnetic susceptibility anisotropy tensor (Eu^{3+}, Er^{3+}, Tm^{3+}, and Yb^{3+}) changes $\Delta\chi$ to a positive value; thus, causing the bicelles to flip 90° such that the membrane normal is parallel with the direction of the static magnetic field [26,28]. Previous studies in this laboratory have 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 [27,29,30]. Thus, spectroscopic studies of magnetically aligned phospholipid bilayers can be conducted at two different orientations with respect to the direction of the static magnetic field. Recently, the minimum amount of lanthanide ions required for bicelle alignment in the presence of a magnetic field has been optimized for X-band EPR studies [31].

In our laboratory, previous studies have extensively described the effect of lanthanide ions and experimental conditions necessary to macroscopically align phospholipid bilayers in the magnetic field of an X-band EPR spectrometer [27,30–32]. However, bicelle alignment studies using X-band EPR have certain limitations. Our X-band EPR studies indicate that the addition of large amount of lanthanide ions are necessary to magnetically align phospholipid bilayers at the low magnetic fields used in EPR spectroscopy. A potential drawback of adding lanthanide ions to perform alignment could cause paramagnetic line broadening due to protein–lanthanide interaction and complicate detailed analysis of spin-labeled EPR spectra [28]. So the complications caused by lanthanide–protein interactions may be possible especially at the higher concentration of lanthanide ions used for X-band alignment studies. Additionally, when bicelle samples are placed into the weak magnetic field typically used in a X-band EPR spectrometer, the bicelle is allowed to align in the presence of a high magnetic field (0.63 T) and then lowered to 3400 G to observe the nitroxide signal (at $g = 2$). A slight misalignment of the bicelles could be possible due to the sudden jump in magnetic field that may result in additional inhomogeneous broadening. Thus, it is important to study the bicelle alignment system at a higher magnetic field, which has several advantages over parallel experiments performed at X-band.

Almost all spin-labeling EPR studies of membranes are currently carried out at X-band (0.3 T) and only few studies have been reported at high magnetic field particularly with bilayer systems under physiologically relevant temperature [16,33–35]. Also few Q-band studies have been reported on the orientation of bilayers on glass plate using cholestane as a spin label [22,36]. Recently, a W-band EPR study has shown the improvement and advantages of using ultra high fields in the magnetic alignment of bicelles [37]. First of all, the bicelles are easier to align because the degree of bicelle ordering increases as square of magnetic field strength [2,6]. Obtaining well-aligned bicelles has never been a problem for NMR spectroscopy because the magnetic field strengths are much higher than those typically used in EPR spectroscopy. Second, by performing bicelle alignment at higher magnetic fields, we will be able to analyze the oriented spectra at the same magnetic field ($g = 2$; 1.25 T) at which the phospholipid bilayers align. Finally, the absolute point sensitivity is increased at a higher operating frequency; thus, membrane protein samples can be prepared on a much smaller scale, when compared to samples needed for X-band EPR spectroscopic experiments.

2. Materials and methods

1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC), and 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy-(polyethylene glycol)-2000] (PEG2000-PE) were purchased from Avanti Polar Lipids (Alabaster, AL). The cholesterol was obtained from Avocado Research chemicals (Ward Hill, MA). Thulium (III) chloride hexahydrate, dysprosium (III) chloride hexahydrate, 3β-doxyl-5α-cholestane (cholestane or CLS), and *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES) were obtained from Sigma–Aldrich (St. Louis, MO). Deuterium-depleted water was obtained from Isotec (Miamisburg, OH). All phospholipids were dissolved in chloroform and stored at −20 °C prior to use. Aqueous solutions of HEPES buffer and lanthanide ions in deuterium-depleted water were prepared fresh each day.

2.1. Sample preparation

The standard bicelle sample, consisting of 25% (w/w) phospholipids to solution with a q ratio (a molar ratio of long chain to short chain phospholipids) of 3.5, was made in 25 mL pear-shaped flasks. In the flask, DMPC, PEG2000-PE, DHPC, cholesterol, and cholestane were mixed in ratios of 3.5:0.035:1:0.35:0.0196, respectively. The chloroform in the flask was evaporated under a nitrogen gas stream and then the flask was placed under high vacuum overnight. The next day 100 mM HEPES buffer of pH 7.0 was added to the flask so that the amount of lipid in the sample was 25% (wt%). The sample was chilled in an ice bath and vortexed until all of the lipids were solubilized. The sample was sonicated with a Fisher Scientific FS30 bath sonicator (Florence, KY) for about 30 min with the heater turned off and ice added to the bath. The sample was subjected to three freeze (77 K)/thaw (room temperature) cycles to homogenize the sample and remove any air bubbles. Finally at 0 °C (ice bucket), an appropriate amount of a concentrated aqueous solution of lanthanide ion was added and mixed into the sample. For X-band EPR measurements, the bicelle samples (~50 μl) were drawn into 1 mm inner diameter capillary tube via a syringe. Both ends of the capillary tube were sealed with Critoseal (Fisher Scientific) and placed inside standard quartz EPR tubes (Wilmad, 707-SQ-250M) filled with mineral oil. For Q-band EPR measurements, the bicelle sample was placed in quartz capillaries, with an inner diameter of 0.3 mm (CV3040) from VitroCom (Mountain Lakes, NJ). The ends of the capillaries were sealed with Critoseal. The capillary with the sample was introduced into a VitroCom quartz tube with an i.d. 1.5 mm (CV1518Q) and sealed at one end. The typical sample volume inside the Q-band EPR tubes was about 3–5 μl.

2.2. EPR spectroscopy

For X-band studies, EPR experiments were carried out on a Bruker EMX CW-EPR spectrometer consisting of an ER041XG microwave bridge and a ER4119-HS cavity coupled with a BVT 3000 nitrogen gas temperature controller (temperature stability of ±0.2 K). Each spin-labeled EPR spectrum was acquired by taking a 42-s field scan with the center field of 3350 G, a sweep width of 100 G, a microwave frequency of 9.35 GHz, a modulation frequency of 100 kHz, a modulation amplitude of 1 G and the microwave power was set at 2 mW.

For Q-band studies, EPR spectra were recorded at a microwave frequency of 34.18 GHz on a Bruker EMX Q-band EPR spectrometer consisting of an ER051QG microwave bridge and a TE_{01x}—mode cylindrical Q-band cavity resonator (ER5106) coupled with a CF935 dynamic continuous flow cryostat. Each spin-labeled EPR spectrum was acquired by taking a 42-s field scan with a center field of 12,500 G, a sweep width of 100 G, a modulation frequency of 100 kHz, a modulation amplitude of 1 G and the microwave power was set at 2 mW.

2.3. Molecular order parameter (S_{mol}) calculations

The EPR spectra of a nitroxide spin label consists of three lines as a result of a system with an $S = 1/2$ unpaired electron coupled to $I = 1$ (^{14}N) nucleus. In a Cartesian coordinate system, the magnetic principal axes have the x-axis along the nitroxide N–O bond, the z-axis is along the 2pπ orbital of the nitrogen and the y-axis is perpendicular to the xz plane [24]. The order parameter S_{33} can be determined by measuring the resultant hyperfine splitting of the aligned spectra using the following equation:

$$S_{33} = [(A_{\parallel} - A_{\perp})/(A_{zz} - A_{xx})](a_N/a_{N'}), \quad (1)$$

where $A_{\parallel}$ and $A_{\perp}$ are the observed hyperfine splittings measured between the $m_I = +1$ and 0 spectral lines from the parallel and perpendicular oriented EPR spectra, respectively. The values $A_{xx} = 5.8$ G, $A_{yy} = 5.8$ G, and $A_{zz} = 30.8$ G were taken from a spectrum previously reported for the cholestane spin label [16]. $a_N = (A_{xx} + A_{yy} + A_{zz})/3$ represents the isotropic hyperfine splitting constants and is sensitive to solvent polarity. $a_{N'} = (A_{\parallel} + 2A_{\perp})/3$ is the solvent polarity correction factor for the hyperfine splitting. The S_{mol} molecular order parameter corresponding to the long molecular axis can be calculated from the following equation:

$$S_{mol} = S_{33}[(3\cos^2\theta - 1)/2]^{-1}, \quad (2)$$

where θ denotes the angle between the long molecular axis and 2pπ orbital of the nitrogen. In the case of the cholestane spin label, $\theta = 90°$ and $S_{mol} = -2S_{33}$.

3. Results

3.1. Optimization of bicelle alignment at X- and Q-band: a comparison

3.1.1. Effect of lanthanide ions

The Q-band EPR spectra of the cholestane spin label incorporated into oriented (20% molar Dy^{3+} or Tm^{3+} with respect to DMPC) and randomly dispersed DMPC/DHPC bicelles at 318 K are shown in Fig. 1. At 0% Ln^{3+} (Fig. 1A), the spectral line shape and hyperfine splitting show that the bicelles are not aligned with respect to direction of magnetic field. This indicates that the bicelles do not completely align at the perpendicular orientation at a magnetic field of 1.25 T used for Q-band EPR studies. At the higher magnetic fields used in the NMR studies, phospholipid bilayers of this composition align such that the bilayer normal is perpendicular to the

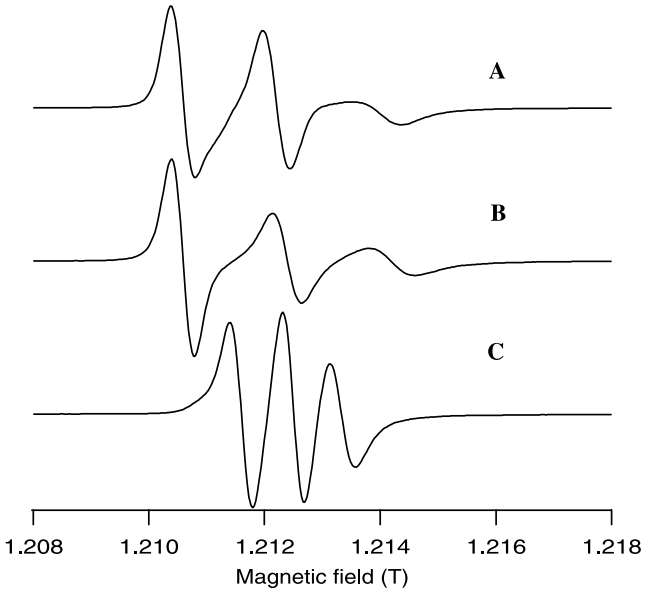

Fig. 1. EPR spectra of a cholestane spin label incorporated into oriented and randomly dispersed DMPC/DHPC/cholesterol phospholipid bicelles. Spectra were taken at 318 K and a static magnetic field strength of 1.25 T was used to align the bicelle samples. (A) EPR spectrum of randomly dispersed bicelles without adding lanthanide ions (B) 20% molar Dy^{3+} with respect to DMPC spectrum indicated that aligned phospholipid bicelles such that the bilayer normal was perpendicular to the direction of applied magnetic field (C) 20% molar Tm^{3+} with respect to DMPC spectrum revealed a aligned phospholipid bilayers such that the bilayer normal was collinear with the static magnetic field.

direction of magnetic field. A previous X-band study reported the requirement of lanthanide ions for the alignment of bicelles [27]. As shown in Figs. 1B and C, the spectral line shape and hyperfine splittings indicate that the lanthanide-doped bicelles are oriented in two different orientations with respect to the direction of static magnetic field. This observed spectral line shapes and hyperfine splittings are similar to previously published mechanically oriented Q-band EPR spectra of cholestane egg lecithin bilayers on glass plate [22] at 0° and 90° orientation with respect to the direction of the static magnetic field. At 20% molar Dy^{3+} with respect to DMPC, the hyperfine splitting observed in the EPR spectrum of Fig. 1B is increased when compared to the EPR spectrum of randomly dispersed bicelles in Fig. 1A. Previous studies have shown that cholestane aligns with its long molecular axis parallel to the long axis of the phospholipid bilayers and undergoes a rapid rotation about this long axis ($R_{\|}$) [21]. The nitroxide y-axis is approximately parallel to the long axis of the cholestane spin label [21]. At the perpendicular orientation, the y-axis (rotational axis) is perpendicular to the static magnetic field; thus, the x- and z-tensoral components are averaged ($A_{xx} + A_{zz}/2 = 19$ G). Therefore, the observed increase in hyperfine splitting (18.1 G) when compared to the randomly dispersed spectrum (16.1 G) indicates that the bilayer normal of the Dy^{3+}-doped bicelle is aligned perpendicular to the static magnetic field. Conversely, the EPR spectrum of the Tm^{3+}-doped bicelle sample (Fig. 1C) shows a significant decrease in hyperfine splitting (9.1 G) with respect to the unoriented and perpendicularly aligned spectra. This is consistent with macroscopic orientation of the phospholipid bilayers such that their normals (and hence y-axis of associated CLS spin labels) are nearly parallel with the static magnetic field and the EPR spectrum consists of three lines separated by A_{yy} (5.5 G). The experimental hyperfine value of 9.1 G is larger than the theoretical value of A_{yy}. We can attribute this difference to slight variations in uniform alignment between the various magnetically oriented phospholipid bilayers and a restricted random walk motion of the cholestane spin label that occurs perpendicular to the surface of the bilayers [19,21,22]. Studies of mechanically oriented cholestane spin-labeled egg lecithin multilayer studies have also indicated that experimentally measured hyperfine values of the oriented systems deviate from A_{yy} because the long axis of cholestane label is not perfectly aligned with the direction of the static magnetic field and undergoes a restricted random walk motion within a cone of angle whose axis is perpendicular to the bilayers normal [21,22].

3.1.2. Variation of lanthanide ion concentration

The X-band and Q-band EPR spectra of the cholestane spin label incorporated into DMPC/DHPC bicelle samples as a function of lanthanide ion (Dy^{3+} and Tm^{3+}) concentrations at 318 K are shown in Figs. 2A and B, respectively (solid line, perpendicular aligned bicelle; dotted line, parallel aligned bicelle; and dashed line, unaligned bicelle). At X-band (Fig. 2A; solid line), the spectra indicate that the bicelles are completely aligned at the perpendicular orientation with respect to the magnetic field in the presence of 10% molar Dy^{3+} as observed by the resultant line shape and hyperfine splittings. However, it requires only 2.5% of Dy^{3+} to align bicelles in the perpendicular direction for Q-band (Fig. 2B) when compared to X-band.

Figs. 2A and B also display the X-band and Q-band EPR spectra of CLS incorporated into DMPC/DHPC/cholesterol phospholipid bilayers as a function of the concentration of Tm^{3+} (dotted line). At 2.5% molar Tm^{3+} with respect to DMPC, the spectral line shape and hyperfine splitting show that the bicelles are partially aligned with respect to the direction of the static magnetic field. At 5% molar Tm^{3+}, we observed a significant reduction in the hyperfine splitting and the linewidth with respect to those of the unoriented sample and perpendicular aligned spectra. The observed hyperfine splitting ($A_{\|} = 9.4$ G; Fig. 2B) is less than the value obtained from the partially aligned bicelle study performed at X-band ($A_{\|} = 13.6$ G; Fig. 2A). An

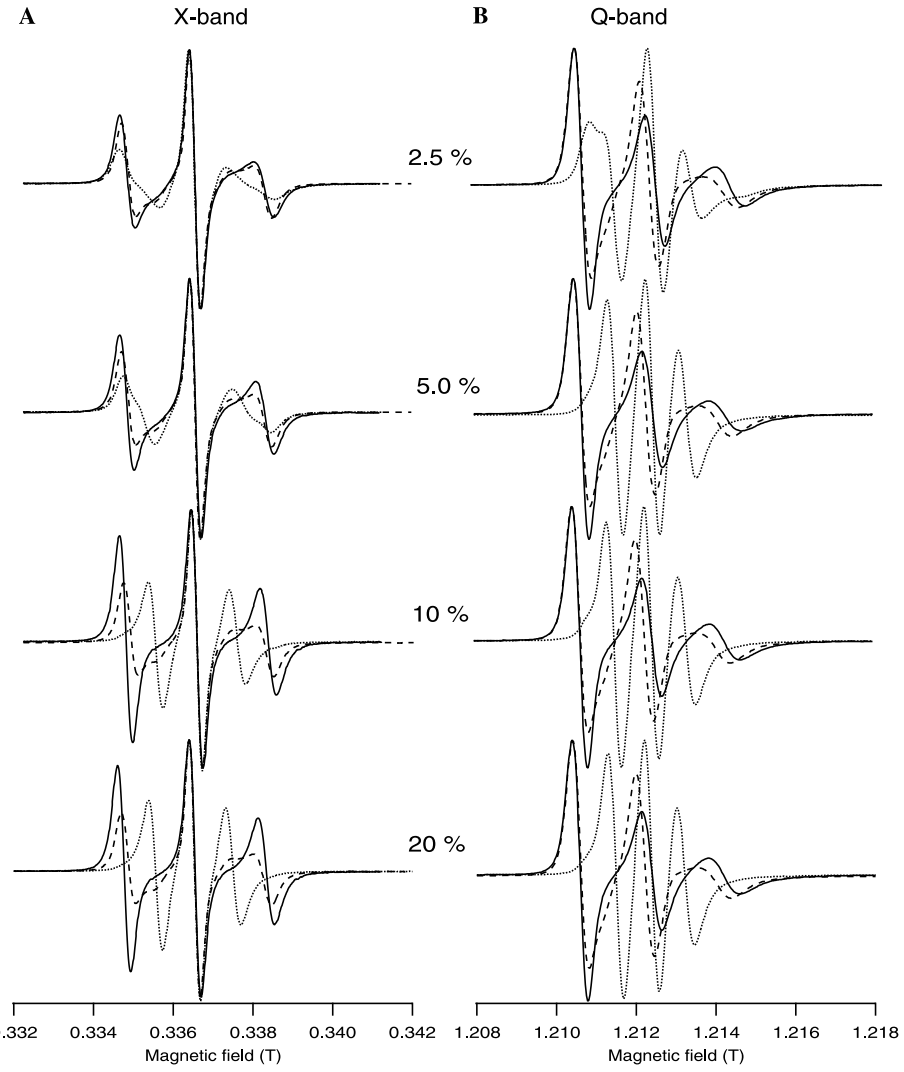

Fig. 2. (A) X-band EPR spectra taken at 318 K of the cholestane spin label incorporated into oriented and randomly dispersed DMPC/DHPC/cholesterol bicelles at various concentrations of Dy^{3+} and Tm^{3+} (2.5, 5, 10, and 20 mol% with respect to DMPC). The temperature was ramped from 298 to 318 K in the presence of a static magnetic field strength of 0.63 T. (B) Same as described in (A) except the experiments were performed with Q-band at the static magnetic field of 1.25 T. The dashed line spectra represent the randomly dispersed bicelles without adding lanthanide ions. The solid line spectra represent the Dy^{3+}-doped bicelle samples. The dotted line spectra represent the Tm^{3+}-doped bicelle samples.

improvement in the alignment for both X-band and Q-band is observed as the concentration of Tm^{3+} increases. At all the concentrations of Tm^{3+}, the observed hyperfine splitting values for Q-band experiments are less than X-band. This result reveals that the amount of Ln^{3+} required to align bicelles for the Q-band EPR studies is less than that required for X-band.

Fig. 3 summarizes the results from Figs. 2A and B by showing the hyperfine splitting values obtained from a series of Tm^{3+} and Dy^{3+} bicelle titration experiments. The hyperfine splitting values from various DMPC/DHPC bicelle samples are plotted versus the concentration of Tm^{3+} ($A_{\|}$, Fig. 3A) and Dy^{3+} ($A_{\perp}$, Fig. 3B) with respect to DMPC. In our Q-band EPR experiments, the splitting corresponding to the bicelles oriented with the director parallel to the magnetic field ($A_{\|}$) are marginally lower than the values observed for X-band alignment for all the concentrations of Tm^{3+}. Conversely, the hyperfine splitting values for the perpendicular oriented phospholipid bilayers ($A_{\perp}$), are higher for Q-band experiments when compared to X-band at all the concentrations of Dy^{3+}.

Fig. 4 shows the molecular orientational order parameter (S_{mol}) of cholestane embedded into DMPC/DHPC bicelles at 318 K derived from the parallel and perpendicular oriented EPR spectra with respect to the magnetic field as a variation of lanthanide ion concentration for X-band and Q-band. S_{mol} was calculated using Eqs. (1) and (2) as described in Section 2. The increase in the order parameter for Q-band studies when compared to X-band suggests that the ordering of phospholipid bilayers is increased. At Q-band (1.25 T),

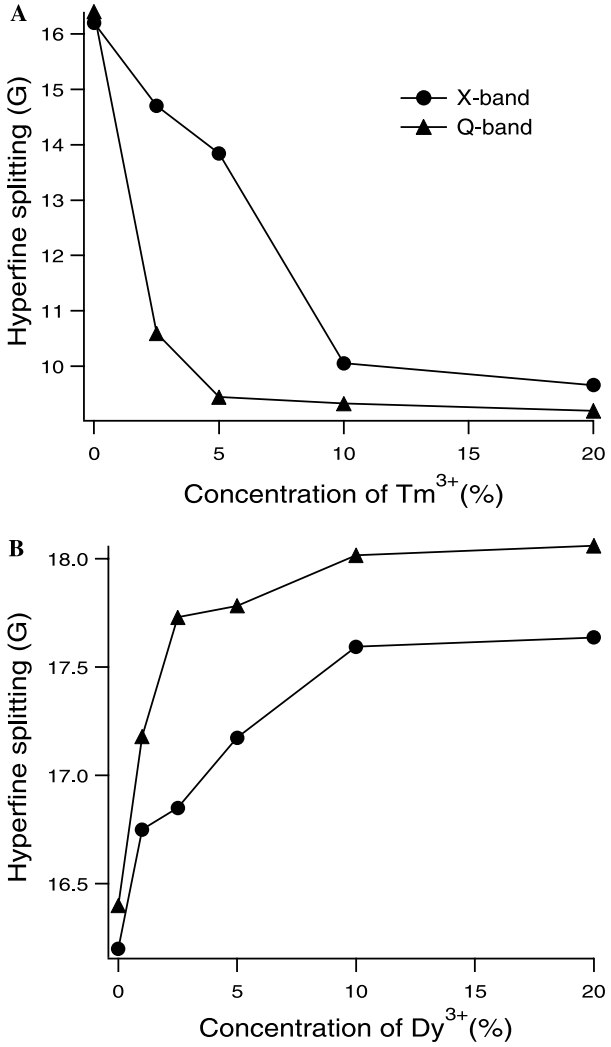

Fig. 3. Diagram showing the dependence of the hyperfine splitting values on the concentration of (A) Tm^{3+} and (B) Dy^{3+} lanthanide ions added to DMPC/DHPC phospholipid bicelles. The circles represent the hyperfine splitting values measured from the X-band EPR spectra and the triangles are the resultant hyperfine splitting obtained from the Q-band EPR spectra. The data are presented as mean values ($n = 3$).

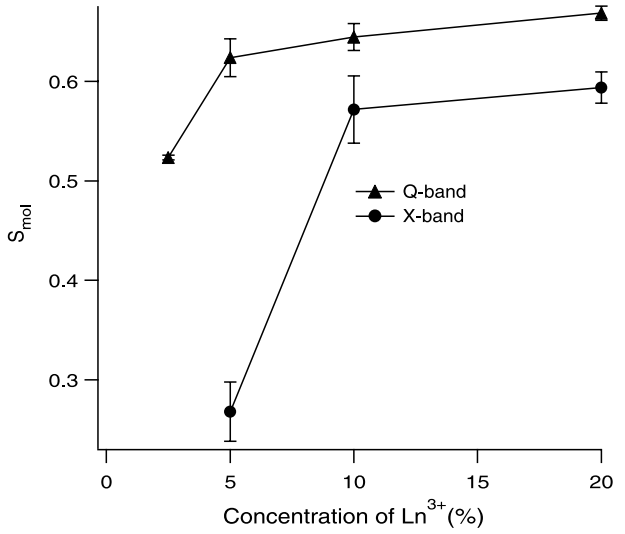

Fig. 4. The molecular order parameter (S_{mol}) calculated from the spin-labeled cholestane EPR spectra at 318 K in magnetically aligned DMPC/DHPC/cholesterol bicelles at lanthanide ion concentrations of 5, 10, and 20 mol% with respect to DMPC. Each data point represents means ± SD of three experiments performed on different bicelle samples.

the cholestane spin label has a higher degree of ordering than the same sample composition at X-band (0.63 T). This could be explained based on the fact that the degree of ordering depends on the square of the magnetic field [2,6].

3.1.3. Effect of temperature

In previous X-band EPR spectroscopic studies of magnetically aligned phospholipid bilayers, Tm^{3+}-doped bicelles magnetically aligned in the presence of a static magnetic field of 0.63 T, when the magnetic alignment procedure was performed on the sample with a temperature ramping time of approximately 15 min with initial and final temperatures being 298 and 318 K, respectively [29,30]. The conclusion of the previous X-band study is that the minimum final temperature of the optimized magnetic alignment procedure was 318 K to get well-aligned bicelles [29,30]. To determine the minimum final temperature for Q-band EPR bicelle studies that results in well-aligned bicelles, the magnetic alignment procedure was performed on a Tm^{3+}-doped bicelle sample with the temperature being raised slowly (1 K/min) from the initial temperature of 298 K to a variable final temperature between 298 and 318 K. The temperature ramping is performed in the presence of a static magnetic field at 0.63 and 1.25 T for X- and Q-band, respectively. Fig. 5 shows X-band and Q-band EPR spectra of a Tm^{3+}-doped bicelle (20% molar Tm^{3+} with respect to DMPC) sample at final temperatures of 298, 304, 308, 313, and 318 K. At all the final temperatures, there is a significant difference in line shape and hyperfine splitting values between the X-band and Q-band bicelle system. At 298 K, both the X-band and Q-band EPR spectra are similar to randomly dispersed bicelles. The increase in the final temperature showed an improvement in the alignment of bicelles at the parallel orientation. The inspection of the line shape and hyperfine splitting values of X-band EPR spectra indicate that the bicelles begin to align at 308 K and well-aligned bicelles are obtained only at 318 K, which agrees well with our previous X-band work for the temperature dependence on the bicelle alignment. However, at a final temperature of 304 K, the Q-band EPR spectrum shows a significant decrease in the hyperfine splitting value (8.9 G), which is smaller than the randomly dispersed bicelles (16.1 G). This suggests that the bicelles start to align at 304 K and the alignment in the parallel orientation increased as the final temperature increased. At

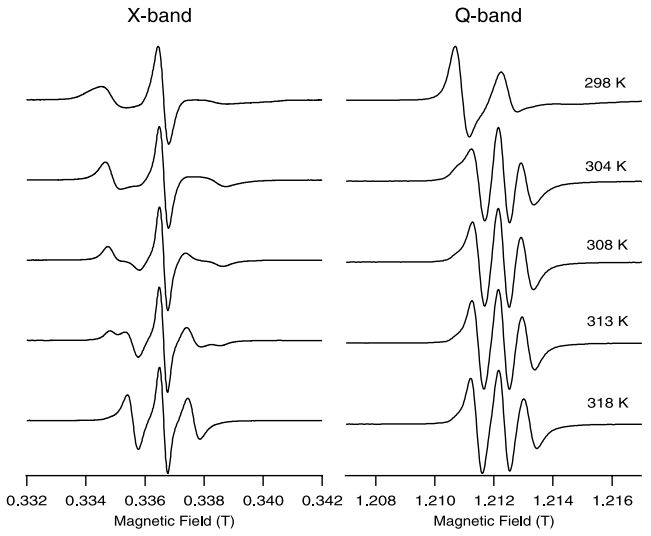

Fig. 5. X-band and Q-band EPR spectra of Tm^{3+}-doped bicelles (20% molar Tm^{3+} with respect to DMPC) showing the final temperature dependence on the magnetic alignment process. The initial temperature of the magnetic alignment procedure was 298 K and the final temperature was varied between 298 and 318 K. The temperature ramping rate was 1 K/min for all the experiments in the presence of a static magnetic field of 0.63 and 1.25 T for X- and Q-band, respectively. The sample was equilibrated at each temperature for 5 min before the EPR spectra recorded.

308 K, the Q-band EPR spectrum had a hyperfine splitting value of 8.6 G and had no detectable coexisting components indicating that the bicelles are well-aligned in the parallel orientation with respect to the direction of the static magnetic field. This result reveals that the bicelle alignment at Q-band can be performed at 308 K.

4. Discussion

The dynamics and ordering of cholestane in phospholipids membrane have been well studied by conventional X-band EPR spectroscopy [16–18,23,24]. However, only few Q-band EPR spectroscopic studies have been reported on the orientation of phospholipid bilayers on glass plate using cholestane as a spin label [22,36]. In our laboratory, previous studies have described the effect of lanthanide ions and experimental conditions necessary to magnetically align phospholipid bilayers in a static magnetic field for X-band EPR spectroscopic experiments [27,29–32]. In this paper, for the first time we present the advantages of using Q-band to study magnetically aligned phospholipid bilayers.

In the aligned spectra, the orientation that the spin label makes with respect to the magnetic field and the motion about its long molecular axis determine the observed hyperfine splitting and line shapes. Figs. 2A and B illustrate the effect of lanthanide ions (Dy^{3+} and Tm^{3+}) on the bicelle alignment (perpendicular and parallel) for X-band and Q-band EPR spectra, respectively. Figs. 2 and 3 show the effect of lanthanide ions on the observed line shape and hyperfine splitting values. Based upon the line shape and hyperfine splittings, both X-band and Q-band spectra show an improvement in the alignment with respect to the unoriented samples (dashed line) as the Ln^{3+} concentration increases. However, the amount of lanthanide ion required for complete alignment either at the parallel or perpendicular orientations varies between X-band and Q-band. In the parallel orientation, the normal to the bicelle and the long molecular axis of cholestane are parallel to the magnetic field; thus, the y-axis of cholestane is nearly parallel with the direction of static magnetic field and the EPR spectrum consist of three lines separated by approximately 5.5 G (A_{yy}). The reduced linewidth and hyperfine splitting values are indicative of macroscopic orientation of the phospholipid bilayers. At X-band with 2.5 mol% Tm^{3+} with respect to DMPC, we observed an unaligned EPR spectrum whereas a partially aligned spectrum was observed with Q-band (Fig. 2). Inspection of the line shape and hyperfine splitting values of the X-band EPR experiments (0.64 T) shown in Figs. 2A and 3 indicates that the bicelles are not aligned at the parallel orientation until 10% and fully aligned with 20% molar Tm^{3+} with respect to DMPC is added to the bicelle sample. However, for the high field Q-band EPR experiments (1.25 T), alignment of the bicelles at this orientation requires only 5% molar Tm^{3+} with respect to DMPC (Figs. 2B and 3).

The difference in the influence of bicelle alignment between X-band and Q-band can be clearly seen in Figs. 3A and B that compares the resultant hyperfine splitting of cholestane with varying concentrations of Dy^{3+} and Tm^{3+}. The theoretical hyperfine splitting value for the perpendicular and parallel orientations is 19 and 5.5 G, respectively. If the hyperfine splitting values are closer to these theoretical values, then it is an indication of more ordering and better alignment. Figs. 3A and B clearly show that there is a significant difference in the hyperfine splitting value between X-band and Q-band at the lower concentrations and approach a closer value at higher molar concentrations of Dy^{3+} and Tm^{3+} with respect to DMPC. At Q-band, the spectra indicate that the bicelles are completely aligned at 2.5% molar Dy^{3+} and 5% molar Tm^{3+} with respect to DMPC as observed by the line shape and hyperfine splitting values. At X-band, we observe complete alignment only at the higher concentration of Dy^{3+} (10%) and Tm^{3+} (20%). This difference in the alignment between X-band and Q-band studies can be explained based on the increase in the magnetic field strength in the Q-band (1.25 T) as compared to X-band (0.63 T). At X-band, we performed these experiments by ramping the magnetic field to 0.63 T to magnetically align the DMPC/DHPC phospholipid bilayers and then lowered to 0.34 T to

observe the $g = 2$ nitroxide spin label EPR signal. The jump in the magnetic field may cause slight miss alignment of the bicelles, thus, resulting in inhomogeneous broadening. The advantage of Q-band is that the bicelles align in the 1.25 T magnetic field and the aligned spectra are recorded at the same magnetic field strength at which they are aligned.

The temperature dependence on the alignment of the DMPC/DHPC phospholipid bilayers is shown in Fig. 5 over the temperature range from 298 to 318 K. At a final temperature of 308 K the cholestane spin-labeled Q-band EPR spectrum had a hyperfine splitting of 8.6 G and no detectable coexisting component in the EPR spectrum. This is in good agreement with well-aligned Tm^{3+}-doped bicelles in the parallel alignment at a temperature of 308 K. However, at a final temperature of 308 K, the X-band EPR spectrum shows the presence of more than one spectral component and a hyperfine splitting value of 11.3 G indicating that bicelles are not perfectly aligned. Previous X-band studies optimized the maximum initial temperature (307 K) and minimum final temperature (318 K) of the magnetic alignment procedure for X-band EPR studies of Tm^{3+}-doped bicelles. A comparison with ^2H NMR phase transition studies of a Tm^{3+}-doped bicelle sample indicated that the macroscopic reorientation of the bicelle starts in the liquid crystalline nematic phase (307 K). The alignment process continued to increase upon slowly raising the temperature and was well aligned in the liquid crystalline lamellar smectic phase (318 K) in the presence of an applied magnetic field [32]. The magnetic alignment of the bicelle system was well-aligned and stabilized by the smectic phase at 318 K. However, for Q-band EPR experiments, the magnetic alignment of the bicelle system was stabilized even at a nematic liquid crystalline phase, i.e., 308 K. When the sample temperature was raised further from 308 to 318 K, the measured hyperfine splitting increased from 8.6 to 9.5 G. We attribute the increase in hyperfine splitting value to an enlargement in the amplitude of the random walk motion and a decrease in the overall alignment of the oriented bicelles caused by the increase in temperature. So the minimum final temperature to probe bicelles at Q-band is 308 K. This is advantageous because the bicelle alignment can be performed at a more physiologically relevant temperature.

5. Conclusion

At Q-band (1.25 T), we were able to magnetically align phospholipid bilayers so that the membrane normal is perpendicular or parallel with the direction of the static magnetic field with lower concentration of Dy^{3+} and Tm^{3+}, respectively, when compared to the required amount of lanthanide ion for X-band EPR studies (0.63 T). Thus, Q-band experiments will be more useful than X-band for investigating the structural and dynamical properties of membrane proteins with site-specific spin-labeled EPR studies with the smallest amount of paramagnetic lanthanide alignment reagents as possible to minimize potential lanthanide ion–protein interactions. At Q-band (1.25 T), the cholestane spin label has a high degree of ordering than the corresponding same sample composition at X-band (0.63 T).

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