Published in final edited form as:

J Magn Reson. 2013 January ; 226C: 35–44. doi:10.1016/j.jmr.2012.11.001.

Using spin-label W-band EPR to study membrane fluidity profiles in samples of small volume

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Abstract

Conventional and saturation-recovery (SR) EPR at W-band (94 GHz) using phosphatidylcholine spin labels (labeled at the alkyl chain [n-PC] and headgroup [T-PC]) to obtain profiles of membrane fluidity has been demonstrated. Dimyristoyl-phosphatidylcholine (DMPC) membranes with and without 50 mol% cholesterol have been studied, and the results have been compared with similar studies at X-band (9.4 GHz) (L. Mainali, J.B. Feix, J.S. Hyde, W.K. Subczynski *J. Magn. Reson.* 212:418-425 [2011]). Profiles of the spin-lattice relaxation rate (T_1^{-1}) obtained from SR EPR measurements for n-PCs and T-PC were used as a convenient quantitative measure of membrane fluidity. Additionally, spectral analysis using Freed's MOMD (microscopic-order macroscopic-disorder) model (E. Meirovitch, J.H. Freed *J. Phys. Chem.* 88:4995-5004 [1984]) provided rotational diffusion coefficients ($R_{\perp}$ and $R_{\parallel}$) and order parameters (S_0). Spectral analysis at X-band provided one rotational diffusion coefficient, R . T_1^{-1} , $R_{\perp}$, and $R_{\parallel}$ profiles reflect local membrane dynamics of the lipid alkyl chain, while the order parameter shows only the amplitude of the wobbling motion of the lipid alkyl chain. Using these dynamic parameters, namely T_1^{-1} , $R_{\perp}$, and $R_{\parallel}$, one can discriminate the different effects of cholesterol at different depths, showing that cholesterol has a rigidifying effect on alkyl chains to the depth occupied by the rigid steroid ring structure and a fluidizing effect at deeper locations. The nondynamic parameter, S_0 , shows that cholesterol has an ordering effect on alkyl chains at all depths. Conventional and SR EPR measurements with T-PC indicate that cholesterol has a fluidizing effect on phospholipids headgroups. EPR at W-band provides more detailed information about the depth-dependent dynamic organization of the membrane compared with information obtained at X-band. EPR at W-band has the potential to be a powerful tool for studying membrane fluidity in samples of small volume, ~30 nL, compared with a representative sample volume of ~3 μ L at X-band.

Keywords

W-band; spin label; cholesterol; membrane fluidity; saturation-recovery; spin-lattice relaxation time; spin-lattice relaxation rate

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1. Introduction

An intensive development and a broad application of T_1 -sensitive EPR spinlabeling methods began in the 1980s at the National Biomedical EPR Center at the Medical College of Wisconsin [1]. T_1 -sensitive methods include T_1 -sensitive spin-label oximetry (the absolute T_1 method using saturation recovery [SR] [2, 3], continuous wave [CW] saturation [4, 5], passage displays [6], and the multiquantum approach [7, 8]; site-directed spin labeling [9]; ELDOR and SR methods for measurements of lateral diffusion and vertical fluctuations of lipids in membranes [10]; and recently developed methods for measurements of profiles of membrane fluidity that reflect membrane dynamics [11–13]. T_1 -sensitive methods have significant advantages over T_2 -sensitive methods because T_1 (usually, 1 to 10 μ s) is from one to three orders of magnitude longer than T_2 . Additionally, T_1 -sensitive methods can be applied to any system that can be spin-probed or spin-labeled, without the need for a narrow EPR line or the presence of a resolved superhyperfine structure.

Five SR instruments have been constructed at the National Biomedical EPR Center, which allow measurements at different microwave frequencies. SR capabilities exist at S-band (2.54 and 3.45 GHz), X-band (9.15 GHz), K-band (18.5 GHz), Q-band (34.6 GHz), and W-band (94 GHz), resulting in an overall frequency range of almost a factor of 40, which is covered by six discrete frequencies [14, 15]. The development of new loop-gap resonators (LGRs) [16] has allowed not only transfer of the T_1 -sensitive SR methods to higher microwave frequencies, including Q- and W-band, but also permitted SR measurements to be made for very small water-containing samples (~30 nL). In our papers [14, 15], we reported T_1 data acquired using SR at frequencies from 2.54 to 94 GHz. We showed that the T_1 of water-soluble spin labels and lipid-type spin labels in membranes exhibits a maximum value at Q-band. All of our published and unpublished data indicate that the observed “anomalous” T_1 dependence on microwave frequency is independent of the structure of the nitroxide moiety, the structure of the environment of the spin-label, the polarity of the local nitroxide environment (which changes with the depth in the membrane), the rate and anisotropy of motion, and the temperature. Explanation of the nature of the break in the trend of relaxation time vs. microwave frequency requires further investigation. It follows that the best frequency for application of the T_1 -sensitive spin-labeling method is 35 GHz (Q-band). However, W-band has its own advantages over other frequencies, which were discussed earlier [12, 17].

We use EPR spin-labeling methods, including the SR approach, to study the organization and dynamics of model and biological membranes (see reviews [11, 18, 19]). Our major aim is to understand how the major lipid component of these membranes, namely cholesterol, affects lateral organization of lipids and induces formation of coexisting membrane phases and domains [20–24]. T_1 -sensitive EPR spin-labeling methods were used to determine the lateral organization of lipid membranes, including coexisting membrane domains and phases. In addition, measurements were made of the oxygen diffusion-concentration product (called the oxygen transport parameter) [2] and of the collision rate of nitroxides with other spin-lattice relaxation agents [21, 23–25] as a function of membrane depth. In these experiments, membrane organization (fluidity) is reported based on the motion of small molecules (oxygen or relaxation agent) within the membrane but not directly on the motion and organization of alkyl chains. Profiles of membrane fluidity obtained by these methods [2, 20, 21] differ from typical profiles of membrane fluidity reported by the alkyl chain molecular order parameter [13, 22, 26, 27]. Additionally, they reveal more features and can differentiate effects of cholesterol at different depths [20, 21]. They also exhibit much greater spatial sensitivity and can differentiate the effects of cholesterol at atomic resolution [20, 21]. During these measurements, we recognized that the spin-lattice relaxation time of

the phospholipid-type spin labels measured in the absence of oxygen reveal features in profiles across the membrane that are similar to oxygen transport parameter profiles.

In a review paper in 2010 [11], we reported T_1 profiles across model POPC membranes with and without 50 mol% cholesterol and across lens lipid membranes isolated from the cortex and nucleus of two-year-old cow eyes. Because T_1 depends primarily on the rate of rotational motion of the nitroxide moiety within the lipid bilayer [28–30], we proposed that T_1 can describe the dynamics of the membrane environment at the depth at which the nitroxide fragment attached to the alkyl chain is located. In 2011, we developed in greater detail this T_1 -sensitive EPR spin-labeling method for studies of profiles of membrane fluidity [13]. We showed that the spin-lattice relaxation rate (T_1^{-1}) of lipid-analog spin labels can be used as a convenient, quantitative measure of membrane fluidity that is sensitive to the averaged rate of nitroxide motion. We confirmed that the measurement of T_1^{-1} for a series of n-PC (or n-SASL) as a function of label position provides a fluidity profile that reflects averaged local membrane dynamics across the membrane. Such T_1^{-1} profiles, which were obtained first at X-band [13], can also be obtained at other frequencies. We predicted that at higher frequencies these profiles could be obtained with increased sensitivity. We indicated that application of this method at Q- and W-band has the potential to be a powerful tool for studying membrane fluidity in samples of small volume (e.g., ~30 nL), which can be especially significant for studies of isolated biological membranes from cell cultures and human samples.

In the present research, we confirm that this is possible. We make SR measurements at W-band for the same samples as were studied earlier at X-band, namely DMPC membranes with and without 50 mol% cholesterol [13]. We extend profiles of the spin-lattice relaxation rate (T_1^{-1}) to the polar headgroup region of the membrane using a phospholipid spin label labeled at the headgroup (T-PC) (see Fig. 1). Because at W-band phospholipid spin-label EPR spectra are in the very slow motion regime, we were able to obtain both rotational diffusion coefficients ($R_{\perp}$ and $R_{\parallel}$) from spectral analysis using the MOMD (microscopic-order macroscopic-disorder) model [31–33]. Spectral analysis at X-band provided only one rotational diffusion coefficient, namely $R_{\perp}$ [13] (see Fig. 1 where orientations of nitroxide axes and axes of spin-label molecules are indicated). Here, we believe we have successfully developed T_1 -sensitive EPR spin-labeling methods for studies of profiles of membrane fluidity in samples of small volume, which can be used at Q- and W-band.

2. Materials and Methods

2.1. Materials

One-palmitoyl-2-(n-doxylstearoyl)phosphatidylcholine (n-PC, n = 5, 7, 10, 12, or 14), tempocholine-1-palmitoyl-2-oleoylphosphatidic acid ester (T-PC), dimyristoylphosphatidylcholine (DMPC), and cholesterol were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Other chemicals, of at least reagent grade, were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Sample preparation

The membranes used in this work were multilamellar dispersions of lipids containing 1 mol % n-PC in DMPC or DMPC/cholesterol (1:1 molar ratio) and were prepared as described earlier [13].

2.3. EPR measurements

The membranes were centrifuged briefly, and the loose pellet was used for EPR measurements. For EPR measurements at W-band, samples were first placed in a 0.6mm i.d.

capillary made of gas-permeable methyl pentene polymer, called TPX and equilibrated with nitrogen (deoxygenated) at room temperature outside the resonator. Then, they were transferred to a quartz capillary (i.d. 0.15 mm), and positioned in the resonator of the W-band spectrometer, which is equipped with a temperature control system. Care was taken to avoid contact of the sample with air [12]. The spectrometer and LGR used for W-band measurements, including SR capabilities, have been described previously [15, 17]. Other benefits of SR EPR at W-band include a higher resonator efficiency parameter and a new technique for canceling free induction decay signals [17].

The spin-lattice relaxation times, T_1 s, of the spin labels were measured using the SR capabilities of the W-band EPR spectrometer. They were determined by analyzing the SR signal of the low-field line obtained by short-pulse SR EPR. At W-band, the low-field hyperfine line is most intense (Fig. 2). For lipid spin labels in membrane suspensions, the pulse duration was 0.3–1 μ s. For these samples, motion of spin labels was sufficiently slow that the nitrogen nuclear relaxation times were shorter than the electron T_1 values, resulting in strong coupling of the three hyperfine lines (see also section 2.3 in Ref. [17]).

Control experiments to verify that measured T_1 values do not decrease from true values by use of an observe microwave power that is too high were carried out. Results for representative samples, namely those with the shortest and the longest T_1 are presented in Fig. 3. It is clear that the observe microwave power of –13.5 dBm at which saturation-recovery measurements were performed is sufficiently low.

2.4. Saturation-recovery data acquisition and processing

Typically, 10^6 decays were averaged, half of which were off-line and differenced for baseline correction, with 1024 data points per decay. Sampling intervals depended on sample and temperature and were from 1 to 40 ns. Total accumulation time was about 2–5 min. Recovery curves were fitted by single and double exponentials and compared. Results showed that, for all of the recovery curves obtained in this work, there was no substantial improvement in fitting when the number of exponentials was above one. This finding established that recovery curves can be analyzed as single exponentials. Decay time constants were determined with accuracy better than $\pm 3\%$.

2.5. Simulation of EPR spectra

Nonlinear least squares (NLLS) analyses of the continuous wave (CW) EPR spectra of n-PC were performed using the fitting program of Budil et al. [34] based on the stochastic Liouville equation developed by Freed and coworkers [31, 35]. The hyperfine A -tensor and the g -tensor components for the n-PC spin labels were obtained from rigid limit spectra (-165°C) in either DMPC or DMPC/cholesterol measured at X-band (see Table 1 in Ref. [13]) and were used in the simulation of fluid-phase membrane spectra at W-band. The spectral simulations for spin labels in multilamellar dispersions of lipids used in the NLLS fittings were obtained using the MOMD (microscopic order and macroscopic disorder) model [31–33] (see Ref. [13] for more details).

Superimposed experimental and simulated W-band spectra of rapidly rotating n-PC spin labels in DMPC and DMPC/cholesterol membranes are shown in Fig. 2. In the simulations, the motion of the nitroxide moiety attached to the C_n position of the alkyl chain is treated as Brownian diffusion of the (C_{n-1}, C_{n+1}) vector within the confines of a cone with a semi-cone angle, θ_C . The ordering potential (spatial constraints imposed by the membrane) is related to the order parameter, S_0 , which measures the angular extent of the rotational diffusion of the nitroxide moiety relative to the membrane normal, and was extracted from the simulation. In the present work, using this approach, we calculated profiles of the order

parameter for n-PC. Because of the different structure of the nitroxide moiety of T-PC and its different location on the PC molecule, this spin label is not included in these profiles and simulations of T-PC spectra were not performed.

In this model, one can consider two independent modes of motion: (1) axial rotation of n-PC about the long axis with a rotational diffusion coefficient $R_{\parallel}$ irrespective of the orientation of the long axis, and (2) wobbling of the long axis itself within a cone (i.e., the (C_{n-1}, C_{n+1}) vector reorients rapidly with a rotational diffusion coefficient $R_{\perp}$ within the confines of a cone with a semi-cone angle of θ_C). Although, the NLLS analysis for the n-PC spin labels at X-band was found to be insensitive to $R_{\parallel}$ [13], simulation at W-band yields both rotational diffusion coefficients $R_{\perp}$ and $R_{\parallel}$. The rotational diffusion coefficient R is related to the rotational correlation time τ by $R = 1/\tau$. The profiles of the rotational diffusion coefficients were reported earlier [36, 37] for 40 mol% cholesterol. To the best of our knowledge, for the first time we are able to report here the fluidizing and rigidifying effect of cholesterol with both rotational diffusion coefficients ($R_{\perp}$ and $R_{\parallel}$). These results from the simulation are supported by our experimental results (i.e., the profiles of T_1^{-1} obtained at W-band [compare Fig. 5 and Figs. 6 and 7]).

The fits shown in Fig. 2A are appropriate for fluid phase membranes, where the assumptions of rapid anisotropic motion (described above) are satisfied. Attempts to simulate these spectra with only one rotational diffusion coefficient (namely $R_{\perp}$) and/or without an ordering potential produced poor fits.

2.6. Rapid rotation about a molecular axis

Structures of spin labels showing principal axes of the nitroxide moiety and molecular axes are shown in Fig. 1. The nitroxide x -axis is along the N-O bond, z is parallel to the nitrogen and oxygen 2p orbitals containing the unpaired electron, and y is perpendicular to the xz plane. In order to describe motion and orientation of spin labels in membranes it is convenient to relate the nitroxide principal axes with molecular (spin label) and system (lipid bilayer membrane) axes [38]. The molecular axes refer to the geometry of the lipid spin labels which usually have a well-defined long axis, referred as R , which is rapidly wobbling about the lipid bilayer normal (the system axis). Analysis of the EPR spectra is considerably simplified in special cases where one of the principal nitroxide axes coincides with the long axis of the lipid spin label. This is the case for n-PC where the z -axis coincides with R and in the case of T-PC where the x -axis coincides with R (Fig. 1). The relationship between the molecular axes and the nitroxide axes is fixed by the geometry of the lipid spin label and both axis systems rotate rapidly in the sample coordinate system (lipid bilayer). For lipid spin labels in fluid phase membranes, anisotropic motion is assumed, with the rapid rotation about the long axis R (with rotational diffusion coefficient $R_{\parallel}$) and the wobbling of the long axis R (with a rotational diffusion coefficient $R_{\perp}$) within the confines of a cone imposed by the membrane environment (defined by the order parameter S_0). In the case of n-PC spin labels, rapid rotational motion about the long axis R (fast $R_{\parallel}$) tends to average x and y components of the anisotropic g and A tensors, while at ordered motion (high S_0) the z component is less affected (Fig. 2A). In the case of T-PC spin label, rapid rotational motion about axis R (fast $R_{\parallel}$) tends to average y and z components, while at ordered motion the x component is less affected (Fig. 2B). These averaging and separations are seen in EPR spectra at W-band, giving clear qualitative pictures of spin labels motions (see section 3.1), while spectral simulation support these pictures by values of rotational diffusion coefficients and order parameters (see sections 3.4 and 3.5).

3. Results and discussion

3.1. Conventional EPR spectra

W-band spectra of all spin labels used in this work for DMPC membranes with and without 50 mol% cholesterol are shown in Fig. 2. Shapes of spectra indicate that high (saturating) amounts of cholesterol significantly affect the order and rate of motion for all spin labels. The simulated spectra for n-PCs are superimposed on experimental spectra in Fig. 2A, while in Fig. 2B, experimental spectra for T-PC are presented. Because of the good separation of the g_{xx} , g_{yy} , and g_{zz} components of EPR spectra at W-band, a few significant conclusions can be immediately drawn from these spectra.

In n-PC spin labels, the nitroxide moiety is rigidly attached to the C_n carbon of the alkyl chain of the phospholipid molecule. Therefore, the dynamics and order parameters extracted from the EPR spectra (Fig. 2A) reflect the dynamics and order parameters of alkyl chains. In the absence of cholesterol, the motion of n-PCs is anisotropic near the membrane surface (5-, 7-PC) and become isotropic at the membrane center (see spectrum for 14-PC where all g components are mixed). Interestingly, g_{xx} and g_{yy} components are mixed even close to the membrane surface. The rotational motion ($R_{||}$) about the long axis of the spin label (z -axis) is fast enough to average g_{xx} and g_{yy} values even close to the membrane surface (see Fig. 1 to relate nitroxide axes with the molecular axes of anisotropic rotation in a membrane). However, the rate of wobbling motion ($R_{\perp}$) is much smaller, allowing separation of the g_{zz} component. The presence of a saturating amount of cholesterol drastically changes the shapes of these spectra, allowing motion at all depths to become more anisotropic with preferential fast rotation about the long axis (z -axis) of the spin label. However, this motion is fast enough to average the g_{xx} and g_{yy} components of the EPR spectra for 10-, 12-, and 14-PC, while for 5- and 7-PC the separation of g_{xx} and g_{yy} is very good. Thus, the rotation rate about the long axis is strongly reduced only to the depth to which the rigid cholesterol ring structure is immersed into the bilayer. These conclusions were confirmed and strengthened by simulation of spectra (see sections 3.4 and 3.5).

The T-PC spin label is not rigidly attached to the choline group of the phospholipid molecule, and there is freedom for rotation relative to the entire headgroup. Therefore, the interpretation of the EPR spectra (Fig. 2B) in terms of the motion of the entire headgroup is less clear. However, as indicated by Ge and Freed [39], because of the large damping effect of the aqueous medium, a high correlation between the motions of the nitroxide and the whole headgroup can be assumed. In the absence of cholesterol the motion of the nitroxide moiety is anisotropic with preferential fast rotation about the x -axis of the nitroxide moiety of T-PC. This motion is fast enough to average g_{yy} and g_{zz} values (see Fig. 1 to relate nitroxide axes with the molecular axes of anisotropic rotation in a membrane). However, the amplitude of the wobbling motion of the x -axis should be restricted and the rate of wobbling motion should be smaller, allowing separation of the g_{xx} component. In contrast with n-PC, the addition of saturating amount of cholesterol decreases anisotropy of the motion of the nitroxide moiety of T-PC, which is indicated by averaging all g values. The EPR spectrum of T-PC becomes as isotropic as that recorded for 14-PC. We interpret these results as a motion of entire phospholipid headgroups. In the absence of cholesterol, headgroups are tightly packed and oriented toward the water phase with somewhat restricted anisotropic motion. Saturating amounts of cholesterol greatly separate headgroups, increasing the amplitude of wobbling motion and the rate of motion. Our conclusions are in agreement with those drawn based on X-band spectral simulation of the headgroup spin-labeled dipalmitoylphosphatidylcholine [40]. They showed that increased cholesterol content increases the amplitude and the rate of the wobbling motion of the phosphatidylcholine headgroups, implying a greater range of motion away from the bilayer normal and towards

the bilayer surface in cholesterol-containing membranes. For polar headgroups, the magnetic x -axis of the nitroxide moiety is aligned with the bilayer normal.

3.2. Saturation-recovery measurements

Figure 4 shows representative SR signals of T-PC (A), 5-PC (B), and 14-PC (C) in DMPC membranes without and with 50 mol% cholesterol obtained from measurements performed on deoxygenated samples. The residuals indicate that the SR signals from T-PC, 5-PC, and 14-PC and from all other phospholipid spin labels used in this work were satisfactorily fit to a single exponential function. SR measurements were carried out systematically as a function of the location of the nitroxide moiety of spin labels at different depths in the membrane. The unique localization of the phospholipid-type spin labels (T-PC and n-PC) in DMPC membranes ensures that profiles of membrane properties obtained with these spin labels describe properties of major membrane regions, including the polar headgroup region. As can be seen from Fig. 4, incorporation of cholesterol increases T_1 for 5-PC, which is located in the most ordered region of alkyl chains, decreases T_1 for 14-PC, which is located in the membrane center, and also decreases T_1 for T-PC, which is located in the membrane polar headgroup region. The presence of cholesterol decreases the averaged rate of spin-label motion in the hydrocarbon region near the bilayer surface, while increasing motion near the center of the bilayer. Cholesterol also increases the average rate of spin-label motion in the polar headgroup region.

3.3. Profiles of the spin-lattice relaxation rate (fluidity profiles)

In previous papers [11–13], we proposed that T_1 can be used as a convenient quantitative measure of membrane fluidity. Profiles of T_1 across the membrane were constructed from data obtained at X-band [11] and W-band [12]. However, later we realized that the best display of membrane fluidity should indicate the rate of motion and, therefore, should display the spin-lattice relaxation rate (T_1^{-1}) [13]. We constructed fluidity profiles obtained from measurements at W-band based on T_1^{-1} as a function of membrane depth for DMPC membranes with and without 50 mol% cholesterol (Fig. 5). We made these displays in the form of profiles across the entire membrane adding points obtained in the polar headgroup regions of the DMPC membrane obtained with T-PC. Because T-PC contains a different type of the nitroxide moiety (tempo) than n-PC (doxyl), we did not connect profile lines from headgroup region with those from hydrocarbon region, leaving the data somewhat separated. However, the effect of cholesterol on mobility of nitroxide moieties in different membrane regions is clearly seen.

Profiles presented in Fig. 5 show that cholesterol decreases fluidity of alkyl chains close to the membrane surface and increases fluidity near the membrane center. The transition between the rigidifying and fluidizing effects of cholesterol lies at approximately the C9 position, the depth to which the rigid ring structure of cholesterol is immersed into the bilayer. Profiles of T_1^{-1} obtained at W-band show at all depths lower spin-lattice relaxation rates than those obtained at X-band [13]. This is in agreement with the finding that spin-lattice relaxation times for lipid-type spin labels in membranes measured at Q-band and W-band are greater than those measured at X-band. Thus T_1 -sensitive methods should have greater sensitivity at Q- and W-band than at X-band (see Discussion in [17]). All T_1^{-1} displays of membrane fluidity are in good agreement with our previous work utilizing another dynamic parameter—namely, the oxygen transport parameter—which clearly differentiates between the membrane region occupied by the cholesterol ring structure and deeper regions of the bilayer containing the aliphatic isooctyl chain of cholesterol (see Refs. [20, 21, 41]).

Because, as we stated in the Introduction, T_1 can be measured for any system that can be spin-probed or spin-labeled, we extended saturation-recovery measurements into the polar headgroup region obtaining T_1^{-1} data for T-PC. Results presented in Fig. 5 indicate that cholesterol significantly increases fluidity of that membrane region. This is in agreement with the analysis of the conventional EPR spectra for T-PC performed in section 3.1 and with a recent study [40]. These data are valuable and new because there are no easy obtainable information about organization and dynamics of this membrane region.

Based on earlier measurements, we concluded that cholesterol separates polar headgroups, which increases water accessibility to that region [25]. We also reported, based on SR measurements with T-PC, that the oxygen transport parameter in the polar headgroup region of phospholipid bilayers is decreased in the presence of cholesterol [20, 21]. The later result is in seeming contrast to the fluidizing effect of cholesterol in the headgroup region reported here. Interpretation of the SR EPR data obtained with T-PC needs some clarification. The oxygen transport parameter is a monitor of membrane fluidity that depends on both oxygen solubility and the oxygen diffusion coefficient. Increased water accessibility should decrease the solubility of hydrophobic oxygen and the presence of boundary water may decrease oxygen diffusion. The T_1^{-1} of T-PC reports on the rate of rotational motion of the phosphatidylcholine headgroup in the DMPC bilayer, which is enhanced by the separation of headgroups. Thus, we need to clearly understand and indicate what we are reporting using the term “fluidity”.

3.4. Profiles of the rotational diffusion coefficients

Profiles of the rotational diffusion coefficients of alkyl chain perpendicular to the bilayer normal, $R_{\perp}$, and parallel to the bilayer normal, $R_{\parallel}$, obtained from spectral simulations of the conventional EPR spectra for n-PC spin labels in DMPC membranes with and without 50 mol% cholesterol are shown in Figs. 6 and 7. As expected, $R_{\parallel}$ is significantly (~2.5 times) greater than $R_{\perp}$, however, both show similar behavior as a function of membrane depth in the absence and presence of cholesterol. Both rotational diffusion coefficients, $R_{\perp}$ and $R_{\parallel}$, increase with increasing depth in the membrane. In the pure DMPC membranes, the range of change of $R_{\perp}$ and $R_{\parallel}$ is similar, and between 5-PC and 14-PC, is approximately a factor of 1.8. In the presence of cholesterol, both diffusion coefficients are substantially decreased near the membrane surface and increased near the membrane center. However, the effect of cholesterol on $R_{\perp}$ is significantly greater than on $R_{\parallel}$. 50 mol% cholesterol decreases $R_{\perp}$ close to the membrane surface by 40% and increases it in the membrane center by 20%. Changes in $R_{\parallel}$ are 20% and 12%, respectively. In all displays of membrane dynamics (as a profile of T_1^{-1} , $R_{\perp}$, or $R_{\parallel}$), the transition from the rigidifying to the fluidizing effect of cholesterol occurs at approximately the same depth, the C9 position, which corresponds to the rigid ring structure of cholesterol immersed into the bilayer. Similar results showing increase in $R_{\perp}$ at the end of the alkyl chain with increasing cholesterol content was also observed for different model membranes [40, 42]. Comparison of $R_{\perp}$ obtained from spectral simulation at X- [13] and W-band show good agreement not only in the qualitative behavior of $R_{\perp}$ as a function of membrane depth and the effect of cholesterol, but also good quantitative agreement between values of $R_{\perp}$.

The $R_{\parallel}$ diffusion coefficient of n-PC spin labels is typically very fast, and when the rotational rate is much greater than the anisotropy of nitroxide g and A tensors, the fitting is not very sensitive to that parameter [43–46]. This was the case for simulations of EPR spectra of n-PCs in DMPC membranes at X-band, which were found to be insensitive to $R_{\parallel}$ [13]. However, simulation at W-band, where the separation of g components of EPR spectra is ten times greater than at X-band, yields both rotational diffusion coefficients, $R_{\perp}$ and $R_{\parallel}$. Simultaneous multifrequency fits as performed by Freed and co-workers [47] were not performed here for X- and W-band EPR spectra.

Although the fit of X-band EPR spectra of n-PC is not very sensitive to changes in the parallel rotational diffusion [13, 43–46], Marsh and co-workers [36, 37] attempted to obtain values of $D_{\parallel}$ from simulation of EPR spectra obtained at X-band for DMPC membranes containing 40 mol% cholesterol. Having in mind that rotational diffusion coefficients calculated in Marsh's papers are related to those calculated in this work as $D = 1/6\tau = R/6$, values presented by Marsh and in our paper for DMPC containing 50 mol% cholesterol for both, parallel and perpendicular rotational diffusion coefficients are close. However the change of the anisotropy of rotational motion, $R_{\parallel}/R_{\perp}$ in our work and $D_{\parallel}/D_{\perp}$ in Marsh's paper [36], with depth in the membrane, show opposite tendencies. Our data (Fig. 6 and 7) indicate that this ratio decreases with depth in the membrane (from 2.7 for 5-PC to 2.1 for 14-PC). Data presented in [36] show significant increases of $D_{\parallel}/D_{\perp}$ ratio (from 1.0 for 4-PC to 6.5 for 12-PC). Because segmental motion of the alkyl chain is most anisotropic close to the membrane surface and becomes more isotropic in the membrane center, its tendency observed in our work is reasonable. Marsh's results are possibly affected by the insensitivity of spectral simulation at X-band to fast parallel rotation, especially close to the membrane center.

3.5. Profiles of the order parameter

Profiles of the order parameter, S_o , obtained from spectral simulations of the W-band EPR spectra for n-PC spin labels in DMPC membranes using Freed's model without and with 50 mol% cholesterol are shown in Fig. 8. Both profiles show a gradual decrease in alkyl chain order with increasing depth in the membrane. This trend has been observed before by EPR [44, 48] and 2H NMR [49–51]. The differences in reported order parameter values are usually attributable to different time-scales of the methods. Values of the order parameter measured at the same depth are always significantly greater for the DMPC/cholesterol membrane than for the pure DMPC membrane. Thus, an ordering effect of cholesterol in fluid-phase DMPC is observed at all depths from membrane surface to membrane center. Such results have traditionally been used to suggest that cholesterol decreases membrane fluidity throughout the membrane bilayer. The same trends in the ordering effects of cholesterol are also revealed in profiles of the order parameter obtained through molecular dynamics simulation of lipid bilayer membranes without and with cholesterol [52, 53].

Explanation is needed to clarify the seeming discrepancy between the well-established condensing effect of cholesterol (manifesting itself by the ordering of alkyl chains), which extend to the whole alkyl chain of the lipid bilayer [53–57] and the cholesterol effect on membrane dynamics, which is suppressed only to the depth of the C9 and increased at deeper locations. There is general agreement that membrane order and membrane dynamics are correlated (which is however not always true [58]). Thus, profiles of the order parameter and profiles of dynamic parameters (T_1^{-1} , $R_{\perp}$, $R_{\parallel}$) can be used interchangeably. We think that in the cases of the liquid-ordered phase, and especially in membranes saturated with cholesterol, dynamics and ordering are less correlated [20, 22, 59]. Deviations of the alkyl chain segment direction from the bilayer normal accumulate as one proceeds from the bilayer surface to the membrane center, a result of the effective tethering of the alkyl chain at the bilayer surface. Consequently, ordering of the alkyl chain induced by the steric contact with the plate-like ring structure of cholesterol will also cause ordering of the distal fragment of the alkyl chain, even though free volume in the membrane center is created because of the isooctyl chain of cholesterol has a cross-section that is much smaller than that across the cholesterol ring structure. This free volume provides additional opportunity for trans-gauche isomerization of the alkyl chains of neighboring phospholipids, and thus facilitates rate of motions in that region.

In a recently published paper [60], Freed and co-authors use the free volume argument to explain their high frequency EPR data of n-PC spin labels in frozen lipid bilayers. They

argue that the two-component 240 GHz EPR spectra observed in frozen lipid membranes for n-PC spin labels (with $n > 7$) arise from nitroxide moieties that are located not only in the hydrocarbon core of the membrane (fully extended conformation), but also close to the membrane surface (bent conformation). The free volume created by cholesterol in the membrane center can more easily accommodate a structure-disturbing nitroxide moiety than compactly packed alkyl chains in the absence of cholesterol, thus, causing a change in the component ratio. The concept of the redistribution of nitroxide between two locations is closely related to the concept of the vertical fluctuations of nitroxide moieties toward the membrane surface [59, 61]. Free volume created by cholesterol in the membrane center increases not only the rate of rotational motion, but also the fraction of time that spin label are in the fully extended conformation.

4. Conclusion

The similarity of the profiles presented in Figs. 5, 6, and 7 supports the use of the spin-lattice relaxation rate (T_1^{-1}) as a convenient quantitative measure of membrane fluidity that indicates the average motion of phospholipid alkyl chains (or nitroxide free radical moieties attached to those chains) [13]. The agreement between the experiment and the theoretical model proposed by Robinson et al. [29], which is based on the isotropic rotational diffusion of nitroxides and is dominated by the so-called electron-nuclear dipolar mechanism, provides the fundamental basis for use of T_1^{-1} as a fluidity parameter. Later, this group extended this model to anisotropic motions of lipid spin labels in membranes [30], showing that for multifrequency T_1 data, consideration of anisotropic nitroxide motion is necessary. T_1^{-1} profiles presented in Fig. 5 possess the same features as profiles of $R_{\perp}$, and $R_{\parallel}$ presented in Figs. 6 and 7 which indicates that both components of the anisotropic rotational diffusion of the nitroxide moiety contribute to the spin-lattice relaxation process. Further discussion of mechanisms for spin-lattice relaxation of nitroxide spin labels requires more experiments.

Use of T_1^{-1} as a convenient quantitative measure of membrane fluidity is appropriate only when SR measurements are carried out on deoxygenated samples. Collisions with molecular oxygen introduces an additional relaxation mechanism which depends on the local oxygen diffusion-concentration product around the nitroxide and can drastically change the profile of measured T_1^{-1} [2]. The fact that T_1 values for lipid spin labels in membranes obtained at higher microwave frequencies (K-, Q-, and W-band) are longer than T_1 values obtained at X-band [14, 15] provides certain advantages for application of T_1 -sensitive methods at W-band. These advantages have been demonstrated in T_1 -sensitive spin-label oximetry measurements [17], in SR studies of oxygen diffusion in membranes, and in studies of bimolecular collision rates between spin-labeled lipids and water soluble relaxation agents [12]. Other benefits, which include a new technique for canceling free induction decay signals, a higher resonator efficiency parameter, a low resonator Q value, and the ability to use high observing power in SR EPR measurements, were already discussed [17]. However, the most significant advantage of measurements at W-band is the potential to be an extremely powerful tool for studying membrane fluidity within and across biological membranes when the amount of a sample is limited.

Data presented here support the validity of Freed's MOMD model [31, 32], which is based on the anisotropy of nitroxide g - and A -tensors. Analysis of EPR W-band spectra provided more details about the spin-label motion than analysis of X-band spectra. Our data support standard usage of W-band to characterize membrane structure and dynamics.

Figure 9 graphically summarize our results on membrane structure and dynamics, indicating different effects of cholesterol at different membrane depths. Clear rigidifying and

condensing effects of cholesterol are seen only in the membrane hydrocarbon region to the depth of the C9, which is the depth to which the rigid ring structure of cholesterol is immersed into the bilayer. Thus, contact with the flat rigid ring structure of cholesterol is responsible for the condensing effect (reported as a decreased order parameter in Fig. 7), which is accompanied by decreased hydrocarbon chain mobility (reported in profiles of T_1^{-1} , $R_{\perp}$, and $R_{\parallel}$ (Figs. 5, 6, 7)) and decreased free volume in that region (reported by profiles of the oxygen transport parameter [20]). At deeper locations occupied by the aliphatic isooctyl chain of cholesterol, the fluidizing effect of cholesterol is observed as indicated by the increased rates of alkyl chain mobilities (reported by profiles of T_1^{-1} , $R_{\perp}$, and $R_{\parallel}$ (Figs. 5, 6, 7)). Because of the difference in the cross section of the ring structure and isooctyl chain of cholesterol the free volume is created in the membrane center (reported by profiles of the oxygen transport parameter [20]). However, the order parameter is significantly increased in that region (see Fig. 8) which indicates that the amplitude of the wobbling motion of alkyl chains decreases. It can be visualized as a decreased semi-cone angle of a cone confining the wobbling motion of alkyl chains. Thus, the free volume created by cholesterol in the membrane center decrease barriers for all type of motions in that region. The condensing effect of cholesterol on alkyl chains of phospholipids extends to the membrane center (see discussion in section 3.5). Cholesterol also causes separation of polar headgroups, causing increased mobility (reported by T_1^{-1} values in Fig. 5) and decreased order (reported by EPR spectra in Fig. 2). Thus, five distinct regions across the entire membrane saturated with cholesterol were indicated (Fig. 9). Existence of these regions were already predicted based on oxygen transport parameter profiles which report membrane fluidity on translational motion of small molecules (molecules of oxygen) within the membrane. Here, these predictions were confirmed based on measurements of orientations and motions of the phospholipids molecule.

The present research complements papers describing the physical properties of the liquid-ordered phase [20–22] focusing on the conditions when the liquid-ordered phase is saturated with cholesterol. It also contributes to a better understanding of the functions of cholesterol that are specific to fiber-cell plasma membranes of the eye lens. The most unique feature of these membranes is their extremely high cholesterol content. Cholesterol saturates the bulk phospholipid bilayer and induces formation of immiscible cholesterol bilayer domains (CBDs) within these membranes [62, 63]. The presence of the CBD ensures that the surrounding phospholipid bilayer is saturated with cholesterol. The appearance of these domains is usually a sign of pathology [64]. However, in the eye lens can CBDs play a positive physiological function, maintaining lens transparency and, therefore, possibly protecting against cataract formation [63, 65].

We found that the commonly accepted statement that the properties of the liquid-ordered-phase domains lie between those for the liquid-disordered and solid-ordered domains [20] is true for membranes when the liquid-ordered domain coexists with the liquid-disordered or solid-ordered domain. However, at saturating cholesterol concentrations, the properties of the liquid-ordered domain are similar to that in the solid-ordered domain in the upper part of the hydrocarbon chains (to the depth of the C9) and like that in the liquid-disordered domain in the central region of the membrane (at depths deeper than the C9). This conclusion was formulated by us earlier based on measurements of oxygen transport parameter profiles [20] and confirmed here based on profiles of hydrocarbon chain mobility. In the bilayer saturated with cholesterol, the oxygen transport parameter from the membrane surface to the approximate depth of C9 was as low as in a gel-phase membrane, and at locations deeper than C9, it was as high as in the fluid-phase membrane.

In membranes saturated with cholesterol, oxygen transport parameter profiles can indicate abrupt change of the membrane properties within the distance of one carbon–carbon bond,

between the C9 and C10 positions (i.e., 1.3–1.5 Å) [20, 21]. This is possible because very small probes (i.e., molecular oxygen and the nitroxide moiety) are used and membrane fluidity is reported in terms of translational diffusion of small oxygen molecules [2]. This is excellent method to detect cholesterol-induced creation of free volume in the membrane center. Although, the differences in oxygen transport parameter between the polar headgroup region and the hydrocarbon region occupied by the rigid ring structure of cholesterol is negligible. The T_1^{-1} profiles allowed clear separation of all regions, showing the mobility of the distinct fragments of phosphatidylcholine molecules, namely polar headgroups, as well as the upper and the lower fragments of hydrocarbon chains. Both methods are based on SR EPR, which provides data on the spin-lattice relaxation rate of lipid spin labels in the absence and presence of oxygen, and allows detailed description of lipid bilayer membranes. We think that profiles of the spin-lattice relaxation rate of lipid spin labels will be accepted as a good way to display membrane dynamics and to recognize effects of membrane modifiers on this dynamics.

Acknowledgments

This work was supported by grants EY015526, EB002052, and EB001980 of the National Institutes of Health. We thank Theodore Camenisch for engineering assistance.

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Highlights

- A new convenient quantitative measure of membrane fluidity is presented
- Profiles of spin-lattice relaxation rates reflect dynamics of lipid alkyl chains
- EPR at W-band is a powerful tool for studying samples of small volume

Fig. 1. Chemical structures of 5-PC (representative for n-PC spin labels) and T-PC used in this work. Chemical structures of DMPC and cholesterol are indicated to illustrate approximate location and orientation of spin labels in the bilayer. Nitroxide principal axes (xyz) are indicated, together with the molecular axis (R) and bilayer normal (n) (see section 2.6 for more details).

Fig. 2. (A) Experimental (solid line) and simulated (broken line) W-band EPR spectra of n-PC in DMPC and DMPC/50 mol% cholesterol membranes at 27°C (fluid phase membranes). g_{xx} , g_{yy} , and g_{zz} are indicated to show positions of these components at W-band. (B) Experimental W-band EPR spectra of T-PC in DMPC and DMPC/50 mol% cholesterol membranes at 27°C.

Fig. 3.

A plot of the observed spin-lattice relaxation rate (T_1^{-1}) as a function of observed microwave power for representative samples with the longest and the shortest spin-lattice relaxation times.

Fig. 4.

Representative SR signals with fitted curves and the residuals (the experimental signal minus the fitted curve) obtained at W-band for T-PC (A), 5-PC (B), and 14-PC (C) in DMPC membranes with and without 50 mol% cholesterol at 27°C. The fits to the single-exponential curves for T-PC give recovery times of 3.46 μ s and 2.99 μ s, for 5-PC 5.63 μ s and 6.34 μ s, and for 14-PC of 3.67 μ s and 3.15 μ s for membranes without and with cholesterol, respectively. All samples were equilibrated with nitrogen. Upper residuals are for membranes without cholesterol and lower residuals are for membranes with cholesterol. These results indicate that the effects of cholesterol on spin-lattice relaxation time can be quite different in different membrane regions.

Fig. 5. Profiles of T_1^{-1} (spin-lattice relaxation rate) obtained at W-band for n-PC and TPC spin labels in DMPC membranes without and containing 50 mol% cholesterol as a function of nitroxide moiety position “n” on the alkyl chain.

Fig. 6. Profiles of the best fit values of the rotational diffusion coefficient ($R_{\perp}$) obtained at W-band for n-PC in DMPC membranes without and containing 50 mol% cholesterol as a function of nitroxide moiety position “n” on the alkyl chain.

Fig. 7.

Profiles of the best fit values of the rotational diffusion coefficient ($R_{||}$) obtained at W-band for n-PC in DMPC membranes without and containing 50 mol% cholesterol as a function of nitroxide moiety position “n” on the alkyl chain.

Fig. 8. Profiles of the best fit values of the order parameter (S_o) obtained at W-band for n-PC in DMPC membranes without and containing 50 mol% cholesterol as a function of nitroxide moiety position “n” on the alkyl chain.

Fig. 9.

Schematic drawing of the phospholipid bilayer membrane without (A) and saturated without cholesterol (B). Arrows on the left side indicate positions of spin labels across the membrane. Different effects of cholesterol at different membrane depths are summarized on the right side. The five distinct regions across the entire membrane saturated with cholesterol are clearly visualized.