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LETTERS

A Multifrequency ESR Study of the Complex Dynamics of Membranes

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A combined 250 and 9 GHz ESR study was performed on membrane vesicles composed of pure lipid (DPPC) and of DPPC:cholesterol in a 1:1 molar ratio using the end chain labeled lipid, 16-PC. It is shown that the 250 GHz spectra represent a "fast time-scale" such that the overall restricted motion of the lipid in the membrane is frozen out, but it is sensitive to the internal dynamics of the end chain. The 9 GHz spectra are, however, sensitive to the overall motion as well. This combined study thus permitted the separation of both types of motion. It is shown that the addition of cholesterol has a large effect on the end chain dynamics by restricting its motion while increasing motional rates, whereas its effects on the overall lipid motion are modest. This leads to a clearer characterization of the dynamic structure of the cholesterol-rich liquid-ordered phase as compared to the liquid crystalline phase. This study thus shows the significant potential of a multifrequency ESR approach to the complex dynamics of membranes.

Introduction

ESR has been extensively used to study membrane structure and dynamics with the aid of spin-labeled lipid additives.[1−3] By means of careful line shape analysis, one can obtain detailed information on the ordering and motion of the lipids in the membrane.[4−7] Also, recent studies on membranes have shown that high-frequency ESR provides improved orientational resolution.[8,9] It is certainly true that the dynamical structure of lipid membranes is very complex. The lipid molecules are locally ordered and engaged in overall reorientation. In addition, the internal motions of the chain segments around the many C−C bonds leads to complex dynamics.[10] It could be expected that a combined study at a low frequency (9 GHz) and a high frequency (250 GHz) would enable one to distinguish between the overall motion of the lipids and the internal modes of motion affecting the local site to which the spin label is attached (cf. Figure 1A,B).

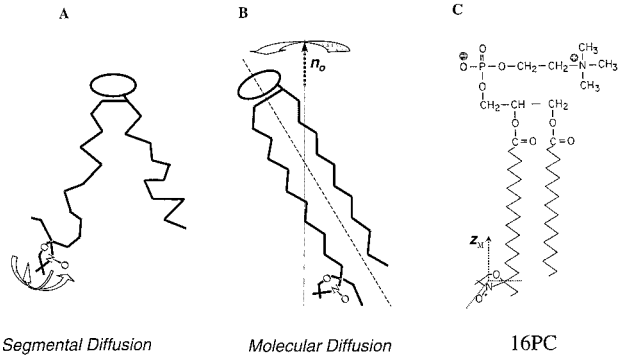

Figure 1. (A) and (B) respectively show sketches of segmental motion affecting the spin-labeled end chain and the overall motion (or molecular diffusion) of the spin-labeled lipid relative to its preferred orientation, along $\mathbf{n}_o$, in the membrane. (C) shows the chemical structure of 16-PC.

Recently, such a multifrequency ESR approach was applied to study the complex dynamics of spin-labeled proteins[11,12] and polymers.[13] This approach is based on the fact that ESR at higher

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frequencies is more sensitive to the faster dynamic modes, whereas ESR at lower frequencies is more sensitive to the slower modes.[14,15] It was shown that the faster time scale of high-frequency ESR was sensitive to the more rapid internal modes of motion but "sees" the slower overall tumbling of the macromolecule as "frozen out". However, low-frequency ESR, with its slower time scale, retains sensitivity to the overall tumbling. Thus it was possible to separate the internal and overall modes of motion by combining a 9 GHz study with a 250 GHz one.[12]

The analysis of ESR spectra in terms of the overall and internal modes of motion is based on the use of the slowly relaxing local structure (SRLS) model, wherein the nitroxide spin probe is taken as reorienting in a restricted local environment, which itself is relaxing on a longer time scale.[11,16] For the present study of dynamics in membranes this faster motion approximately describes the internal acyl chain dynamics (cf. Figure 1A) whereas the slower motion describes the overall tumbling of the lipid molecule (cf. Figure 1B). When the global tumbling is very slow on the ESR time scale, i.e., in the rigid limit, then this SRLS model simplifies into the microscopic order−macroscopic disorder (MOMD) model.[11,12,17]

Herein we study model membrane vesicles consisting of dipalmitoylphosphatidylcholine (DPPC) in excess water. It exhibits a range of phase structures. In particular, the gel to liquid crystalline (LC) phase transition occurs at about 41 °C, and it involves changes of the hydrocarbon chains from an extended, mainly all-trans conformation, to a relatively disordered conformation, which contains a number of gauche conformers and exhibits increased motion.[18] Cholesterol is an important regulator of the physical properties of biological membranes and membrane function.[19] It affects the phase transitions of the pure DPPC, as well as the rotational and translational motions of the DPPC molecules including their internal motions and conformations.[20] Addition of cholesterol generally leads to a more conformationally ordered state of the acyl chains. At high enough concentrations of cholesterol (ca. 25% of total lipids and greater) there is a phase transformation to a liquid ordered (LO) phase.[21] This LO phase is believed to play an important role in facilitating phase segregation in cell membranes, wherein important biological processes may occur.[7,22]

By means of the multifrequency ESR approach, we explore in this Letter the differences between the normal gel and LC phases of DPPC vs the LO phase using 50−50 molar mixtures of DPPC and cholesterol. We are able to distinguish the extent to which the differences in dynamic structure in these phases are due to internal vs overall molecular degrees of freedom. We note that recent extensive phase studies in vesicles including DPPC/cholesterol mixtures have shown that these compositions exist as only a single phase,[21] and this is confirmed by our extensive ESR studies.[7,14,15]

Experimental Section

The lipid DPPC and the spin label (cf. Figure 1C) chain labeled lipid 1-palmitoyl-2-(16-doxylsteroyl)phosphatidylcholine (16-PC) were purchased from Avanti Lipids (Alabaster, AL) and were used without further purification. The dispersions of multilamellar vesicles in excess water used in this study were prepared as described elsewhere.[7]

ESR spectra were taken on a home-built 250 GHz spectrometer using a specially designed aqueous sample holder, described in detail elsewhere.[23] The disk-shaped vesicles samples were about 0.02 mm in thickness and contained between thin quartz disks. The 9 GHz ESR spectra were taken on a Bruker ER200 spectrometer using standard 1.5 mm i.d. capillary tubes as described previously.[7] The magnetic **g**-tensor and **A** tensor components used for the spectral simulations were published values.[7]

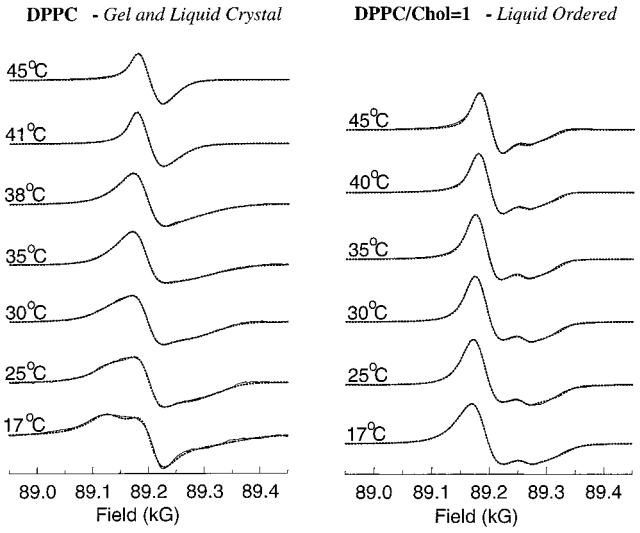

Figure 2. 250 GHz ESR spectra of 16-PC in membrane vesicles for a range of temperatures (solid lines), and their fits to the MOMD model (dotted lines). Left side: pure DPPC vesicles. Right side: DPPC/Chol at 50:50 molar ratio.

Results and Analysis

The 250 GHz ESR spectra of 16-PC dissolved in pure DPPC (Figure 2A) and DPPC/Chol = 1 (molar ratio) (Figure 2B) vesicles were recorded from well below to above the 41 °C gel to the liquid crystalline phase transition of DPPC. The ESR line shapes from 16-PC in DPPC show somewhat greater variation with temperature than those using DPPC/Chol.

In vesicles, the lipid chains are oriented within a local bilayer region, but these regions are randomly distributed in space. That is, the lipids in the vesicle are microscopically ordered, but overall the vesicles are macroscopically disordered. Thus, the MOMD model was used to fit[24] the 250 GHz ESR line shapes.

The structure of the 16-PC molecule in its all-trans configuration is such that the magnetic tensor $\mathbf{z}_A$ axis (defined as parallel to the $2p_z$ orbital of the nitroxide N atom) is itself parallel to the extended acyl chains which define the principal axis, $\mathbf{z}_M$ for molecular diffusion (and ordering)[5,7] (cf. Figure 1C). The primary motions are then usually represented by an axially symmetric rotational diffusion tensor with components $R_\perp$ and $R_\parallel$, representing reorientation of the $\mathbf{z}_M$ axis and rotation about this axis, respectively. The ordering potential experienced by the lipid molecules restricts their rotational motion. This orienting potential $U(\Omega)$ is usually expanded in generalized spherical Harmonics, $D^L_{mm'}(\Omega)$, where Ω represents the Euler angles for the transformation between the preferred orientation and the molecular diffusion frames.[4,11,24] Usually only the lowest order terms are kept, yielding

$$-U(\Omega)/kT = c_0^2 D_{00}^2(\Omega) + c_2^2[D_{02}^2(\Omega) + D_{0-2}^2(\Omega)] \quad (1)$$

where c_0^2 and c_2^2/c_0^2 determine respectively the strength and asymmetry of the potential. If $c_0^2 > 0$ and $c_2^2 = 0$, the lipid chain with its nitroxide label prefers to align in the all-trans configuration, in an axially symmetric fashion. One typically describes the ordering by the ordering tensor with compo-

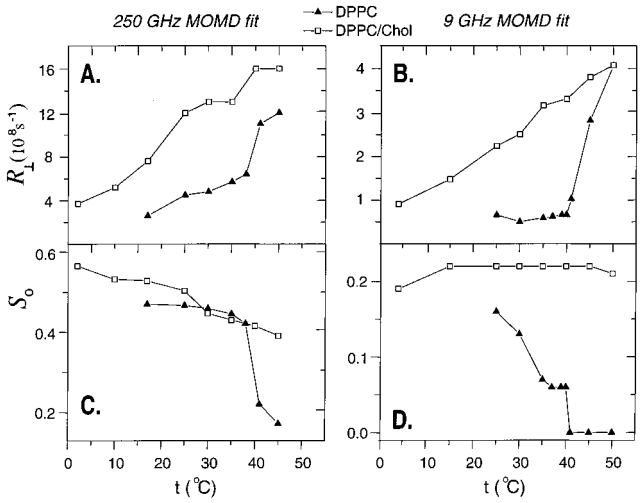

Figure 3. Diffusion coefficient ($R_\perp$) and order parameter (S_0) from 16-PC in membrane vesicles vs temperature from 250 GHz (A and C) and 9 GHz (B and D) ESR spectra obtained from their respective MOMD fits.

nents: $S_0 \equiv \langle D_{00}^2[\Omega(t)]\rangle$ and $S_2 \equiv \langle D_{02}^2[\Omega(t)]\rangle + \langle D_{0-2}^2[\Omega(t)]\rangle$, where the angular brackets imply ensemble averages, using the potential of eq 1.[4,11,24]

Based on this MOMD model the best fit simulated spectra for 250 GHz are compared with the experiment in Figure 2, showing good agreement. The best fit $R_\perp$ and S_0 parameters vs temperature are shown in Figure 3A,C, respectively.

As shown in Figure 3C, there is a sharp decrease in S_0 for 16-PC in DPPC on going from 40 to 41 °C, indicative of the gel to LC phase transition of DPPC bilayers. In contrast, S_0 for 16-PC in DPPC/Chol does not show a phase transition. Also, these S_0 values are somewhat greater than those for pure DPPC. As shown in Figure 3A, there is a sharper increase in $R_\perp$ of 16-PC in DPPC from 40 to 41 °C, a further indication of the gel to LC phase transition of DPPC bilayers. The values of $R_\perp$ for 16-PC in DPPC/Chol are greater than those for 16-PC in pure DPPC dispersions and show no phase transition. These are definitive characteristics of the LO phase.[7]

The values of S_2 were found to be close to zero for both types of sample over the whole temperature range, implying axial alignment of the acyl chains. The values of $R_\parallel$ ranged from 1.6 to 3.9 $\times$ 10^8 s^{-1} for 16-PC in pure lipid, and from 2.4 to 11 $\times$ 10^8 s^{-1} for DPPC/Chol over the same temperature range of 17−45 °C.

We now make the assignment (to be justified below) that the results of $R_\perp$ and S_0 in Figures 3A,C (and S_2 and $R_\parallel$) represent the internal dynamical modes of motion at the end of the acyl chain where the nitroxide resides in 16-PC. Thus the Ω in eq 1 represents the instantaneous orientation of the end chain (cf. Figure 1A) relative to its all-trans configuration. We are thus assuming that the overall tumbling of the lipid molecules in the membrane vesicle (cf. Figure 1B) is too slow to affect the 250 GHz spectra, since they are only sensitive to faster motions, (corresponding to $R > 10^7$ s^{-1}).[11,12,14,15] Studies at the much lower frequency of 9 GHz are likely to be sensitive to both modes of motion.[11,12,14,15,25]

The 9 GHz spectra of 16-PC in DPPC and DPPC/Chol are shown in Figure 4. Following past studies,[5,7,12,13,17,24] the MOMD model was initially used to fit the spectra, and results for $R_\perp$ and S_0 are shown in Figures 3B,D, respectively.

There are some common features between the parameters obtained by the MOMD fits to the 9 and 250 GHz spectra. First

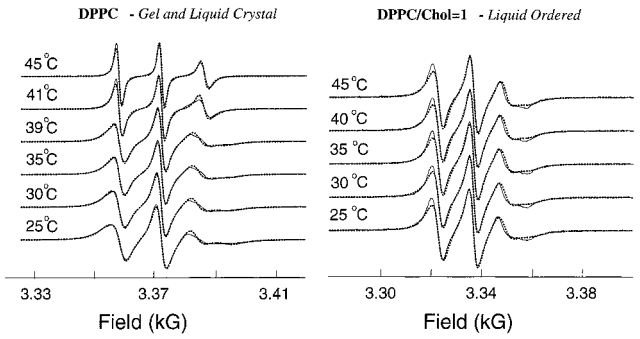

Figure 4. SRLS fits (dotted lines) to 9 GHz ESR spectra of 16-PC in membrane vesicles for a range of temperatures (solid lines). The internal dynamic and ordering parameters were obtained from the 250 GHz MOMD fits. Only the overall motional parameters were fit. Left side: pure DPPC vesicles. Right side: DPPC/Chol at 50:50 molar ratio.

of all, both sets of results show the gel to LC phase transition of DPPC at 40−41 °C, while for DPPC/Chol this is absent. Second, the S_0 values found for DPPC/Chol are greater than those for pure DPPC. Last, values of $R_\perp$ for DPPC/Chol are much larger than those for DPPC. However, there are serious discrepancies between these two sets of parameters. The values of $R_\perp$ from the former are about 4 times smaller than those from the latter. The S_0 from the former are also much smaller than those from the latter. An additional discrepancy is large nonzero values for S_2 from the MOMD analysis of the 9 GHz spectra, which would imply substantial biaxial alignment of the acyl chains.

These discrepancies shown in Figure 3 are quite similar to those observed in the recent studies of a spin-labeled protein[12] and of a spin-labeled polymer,[13] so we believe the cause to be similar.[14,15] As we already mentioned, the overall motion of the lipid in its membrane environment is probably too slow to affect the 250 GHz spectra but not the 9 GHz spectra. Thus, while the MOMD model is appropriate for the 250 GHz spectra, the SRLS model should be more suitable for analyzing the 9 GHz spectra. To achieve consistency in the SRLS analysis of the 9 GHz spectra with the MOMD analysis of the 250 GHz spectra, we follow the procedure of utilizing the ordering and motional parameters for the internal end-chain motions obtained from the MOMD fit to the 250 GHz spectra (given in Figures 3A,C), and we only vary the overall motional and ordering parameters of the lipid molecule to obtain a SRLS fit to the 9 GHz spectra. This is analogous to what was done in the protein study,[12] except in the present case the external medium is a microscopically ordered membrane, whereas the protein was in an isotropic aqueous phase.

In the SRLS model the overall tumbling of the lipid relative to its preferred alignment in the membrane is represented by fluctuations in Euler angles Ω^C (cf. Figure 1B).[16] Such motion appears frozen on the 250 GHz time scale, but its effects are noticeable at 9 GHz. We were able to obtain good SRLS fits to the 9 GHz spectra, as shown in Figure 4. These fits yielded the dynamic and ordering parameters for the *overall motion* (indicated with a superscript c). We found that over the temperature range of 25−45 °C, $R_\perp^C$ varied from 1.4 to 1.9 $\times$ 10^7 s^{-1} for DPPC vesicles and 1.4 to 2.1 $\times$ 10^7 s^{-1} for DPPC/Chol vesicles, i.e., only a small change. (Similar conclusions apply to $R_\parallel^C$, which was in the range (6−7) $\times$ 10^6 s^{-1} for both samples). S_0^C varied from 0.63 to 0.55 in the gel phase of pure DPPC vesicles with a drop to 0.48 in the LC phase, while it remained fairly constant above 26 °C at about 0.64 for DPPC/Chol vesicles. Thus the differences in S_0^C for the two cases are

not large. Also S_2^C was found to be extremely small in both cases (-0.06 and -0.08).

Note that values of $R_\perp^C$ and $R_\parallel^C$ are indeed so small as to be in the rigid limit for the 250 GHz spectra but not for the 9 GHz spectra.[11] Thus we have achieved a self-consistent time-scale separation of the slow tumbling of the overall lipid molecule from the faster internal chain dynamics, with the combined use of 250 and 9 GHz ESR.

Discussion

It follows from our results that (i) the effect of cholesterol is primarily to restrict the end-chain fluctuations, but within their restricted range of motion to allow for faster motion, and (ii) the cholesterol has only a modest effect on the overall restricted motion of the lipid molecules.

It is instructive to compare our results to a previous 9 GHz ESR study on 16-PC in macroscopically oriented pure DMPC membrane multilayers in the LC phase.[10] There, a very sophisticated dynamic rotational isomeric state model was used involving transitions between all 3^{15} conformers of the acyl chain not forbidden by excluded volume considerations (ca. 40,000). Transition rates between conformers were estimated theoretically, so only S_0^C and $R_\perp^C$ (and $R_\parallel^C$) for the overall motion needed to be fit to the experiments. It was found that $S_0^C \approx 0.4$ and $R_\perp^C \approx (1-2) \times 10^7$ s^{-1} for 16-PC in DMPC in the LC phase at 25 °C, in rather good agreement with our results for 16-PC in DPPC, where all parameters have been fit experimentally, and we have approximated the complex internal dynamic modes by a single set of effective ordering and diffusion tensors.

We can now interpret the discrepancies between the MOMD fits at 250 and 9 GHz in light of the full SRLS analysis of the 9 GHz spectra. Whereas the ordering and motional rates from the 250 GHz spectra just characterize the faster internal modes, the estimates from the 9 GHz MOMD fits represent some mean of the slower overall motional and faster internal rates, thus yielding a net slower motional rate compared to the 250 GHz results. The effective order parameter, S_0^E from the 9 GHz MOMD fits includes effects of both the true S_0 (internal motions) and the S_0^C (the overall motions). In general, one can introduce a total ordering parameter $S_T \cong S_0^C \cdot S_0$ (which is about 0.23 at 38 °C and 0.08 at 45 °C for pure DPPC and 0.26 at 40 °C for DPPC/Chol). Although S_T is closer to the S_0^E obtained from the 9 GHz MOMD fits, a direct comparison is not appropriate, since in the 9 GHz MOMD fit case there are also large nonzero values for S_2, which is not borne out by the more complete analysis.

Our results are consistent with the accepted picture of the effect of cholesterol in the liquid-ordered phase;[20] viz. there is high acyl chain ordering[26,27] yet relatively rapid acyl chain mobility.[28] With the use of 9 and 250 GHz ESR, not only have we quantified these features but we have also clearly distinguished them from the overall ordering and rotational mobility of the lipid molecules, which is less affected by the addition of cholesterol. We note that NMR relaxation (e.g., T_1 and T_2) has also been used to study membrane dynamics.[29−31] Since NMR is at a longer time scale (due to much smaller magnetic tensors) than ESR, these motions are typically in the motional narrowing regime.[32]

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(32) It is not straightforward to compare our ESR results with those from NMR (e.g., refs 29 and 30), since somewhat different models were used in the analyses, and the much longer NMR time scales make them very sensitive to collective director fluctuations, which are unimportant on the ESR time scale.[31]