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Influence of Cholesterol on Molecular Motions in Spin-Labeled Lipid Bilayers Observed by Stimulated ESE

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Abstract Electron spin echo (ESE) study was performed for spin-labeled lipids 1-palmitoyl-2-stearoyl-(5-D)-*sn*-glycero-3-phosphocholine in 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine phospholipid bilayer. Recently (Isaev and Dzuba in J Phys Chem B 112:13285–13291, 2008), three-pulse stimulated ESE was shown to be sensitive to two types of orientational motion of spin labels in phospholipid bilayers at low temperatures ($\sim$100–150 K). The first one is fast stochastic libration, with correlation time on the nanosecond time scale. The second one is slow rotational motion, developing on the accessible for measurements microsecond time scale in a small range of reorientation angles, $\sim$0.1°–1°. These two types of motions may be easily discriminated by dependences of the echo decay rates on the time delays between the pulses. The presence of cholesterol in lipid bilayers is found to suppress remarkably rotational motions, while on the contrary stochastic librations seem to become somewhat enhanced. These results evidence that cholesterol increases the long-time stability of lipid orientations in the bilayer, with simultaneous increase of fast fluctuations of these orientations. The former may be related to the known condensing effect of cholesterol and to raft formations, while the latter to the ordering effect.

1 Introduction

Cholesterol is a vital component in the membranes of mammalian cells. It determines mechanical properties of membranes [1], increases the ordering of the phospholipid acyl chains (“an ordering effect”) [2–4], decreases the area per lipid

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("condensing effect") [4, 5], promotes formation of lipid rafts [6, 7]. The lipid rafts are more ordered and constitute a lesser fluid region than the rest of the membrane. Cholesterol is also known to influence molecular motions in membranes, especially lateral diffusion of lipids and proteins [4, 8, 9].

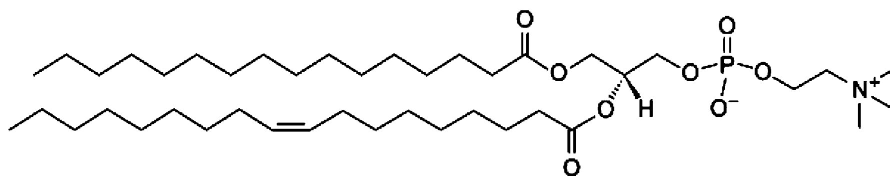
Molecular motions in biological membranes at cryogenic temperatures occur across low-energetic barriers, which are inevitably present, but cannot manifest itself at physiological temperatures. Low-temperature dynamics in membranes have been studied by employing neutron scattering [10, 11], nuclear magnetic resonance (NMR) [12, 13], dielectric relaxation [14], Raman scattering [15], electron spin echo (ESE) of spin labels [16–18], and other techniques.

ESE spectroscopy applied to amphiphilic spin-labeled stearic acids in lipid bilayers [18] has shown that spin labels at low temperatures, between 100 and 150 K, participate in orientational motions of two types. The first one is fast stochastic librations, with correlation time on the nanosecond time scale. The second one is slow rotational motions, developing on the accessible for measurements microsecond time scale in a small range of reorientation angles, $\sim 0.1°–1°$. These two types of motion may be easily discriminated by the dependence on the time delay T between the second and third pulses of the echo decay rate, measured with the increasing time delay τ between the first and the second pulses, with T fixed.

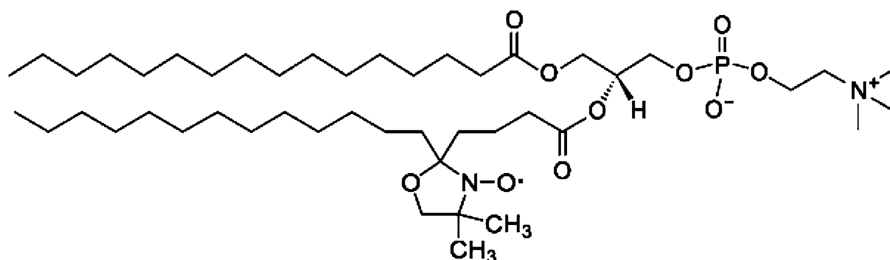
In this work, we apply the approach developed in Ref. [18] to study possible influence of cholesterol addition to lipid bilayers on the lipid molecular motion.

2 Experimental

To prepare lipid bilayers, the phospholipids (obtained from Fluka) 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) of the structure



were employed in this work. A spin-labeled lipid 1-palmitoyl-2-stearoyl-(5-D)-*sn*-glycero-3-phosphocholine (5-D-PSPC) of the structure



was used (obtained from Avanti). 5-D-PSPC was dissolved in trifluoroethanol together with, optionally, POPC at a 1:100 molar ratio or with mixture of cholesterol and POPC at a 1:50:50 molar ratio. The solvent was then removed by evaporation in vacuum. Then the sample was hydrated at room temperature by water vapors of 100% humidity.

A Bruker ELEXSYS E580 X-band Fourier-transform electron paramagnetic resonance (EPR) spectrometer, equipped with a dielectric cavity (Bruker ER 4118 X-MD-5) inside an Oxford Instruments CF 935 cryostat, was used. Duration of microwave pulses in a three-pulse stimulated echo sequence was 16 ns. Their amplitudes were adjusted to provide a $\pi/2$ turning angle, so making the amplitude $\sim$6 G (that is an excitation bandwidth). To remove unwanted echos, a four-step phase cycling was applied.

Cryostat was cooled either by cold nitrogen or by cold helium. The sample temperature was monitored with a calibrated Cu-constantan thermocouple directly placed into the sample tube. Temperature was maintained with an accuracy of ±0.5 K.

3 Results

The line shape of EPR spectra of nitroxides consists of three hyperfine structure components corresponding to three nitrogen spin projections, and is determined by anisotropy of the hyperfine interaction and of a *g*-factor. It is demonstrated in the inset to Fig. 1, where a so-called echo-detected EPR line shape is shown (the amplitude of a two-pulse echo vs. the scanning magnetic field, with the time delay τ between the pulses fixed).

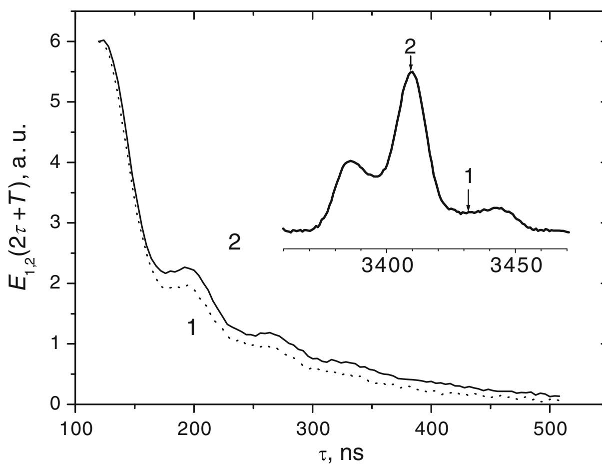


Fig. 1 Echo decay traces versus τ for $T = 2.3$ μs at two field positions shown in *inset*, where echo-detected EPR line shape is shown (a two-pulse echo taken at a constant time delay $\tau = 120$ ns, with the magnetic field scanned). The intensities at the beginning are artificially adjusted approximately to the same value. Temperature is 155 K, the sample is 5-D-PSPC in cholesterol-free POPC bilayer (1:100)

Stimulated ESE is formed after application of three microwave $\pi/2$ pulses to a spin system, stored along the external magnetic field, in a time sequence denoted as $\pi/2$–τ–$\pi/2$–T–$\pi/2$–τ–echo. The echo signal $E(2\tau + T)$ decays because of spin relaxation during the first and the last τ-intervals and during the intermediate T-interval. Two-pulse primary ESE formally corresponds to the case when T is zero. To discriminate spin relaxation processes induced by orientational motion from other sources of relaxation (spin flips of nearby nuclei, etc.), in our experiments echo signal was recorded for two positions in the EPR spectrum, differing by the anisotropy of magnetic interactions (see Fig. 1). These positions are indicated by arrows in the inset of Fig. 1, they correspond to two limiting cases of anisotropy; the highest one for the first position, and the lowest one for the second position. So the comparison of the decays for these two positions would allow refining the pure contribution of orientational motion of the nitroxide itself.

Note that selection between these two positions is possible only when the excitation bandwidth is much less than the total EPR line width (in our case, these two values are 6 and 70 G, respectively).

Echo decays given in Fig. 1 show that larger anisotropy results in a faster decay. Also, noticeable oscillations are seen at the decay curves (it is known as ESE envelope modulation or ESEEM).

To refine a pure contribution of relaxation induced by anisotropy of magnetic interactions (anisotropic relaxation), we divided the time traces for the decays at the first field position by those at the second one. The results are given in Fig. 2 on a semilogarithmic scale, for three different T delays. Note that after division ESEEM is essentially damped (which means that ESEEM is field independent).

One can see that echo decay in Fig. 2 is satisfactorily fitted by straight lines, so the decays are exponential. Hereafter, we denote the slope of the line as $W(T)$.

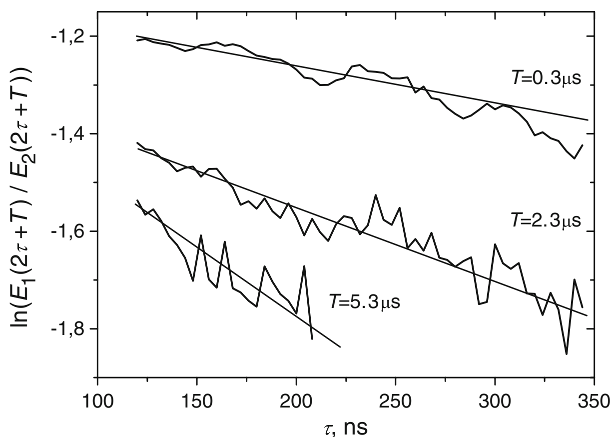


Fig. 2 Semilogarithmic plot of the ratio of the traces taken at the positions 1 and 2 (see Fig. 1), at different time delays T shown in picture. The *straight lines* are linear approximations of these data. For convenience, data are shifted arbitrarily along the vertical axis. Temperature is 155 K, the sample is 5-D-PSPC in cholesterol-free POPC bilayer (1:100)

Figures 3 and 4 show $W(T)$ as a function of T for cholesterol-free and cholesterol-containing samples, respectively. One can see that all data may be satisfactorily approximated by straight lines. At low temperatures for both samples, these lines are nearly parallel to the horizontal axis. Increase of temperature results in appearing of the initial (when $T = 0$) intercepts. Temperature dependence of the line slopes is remarkably different for cholesterol-free (Fig. 3) and for cholesterol-containing (Fig. 4) samples. In the former case, the slope noticeably increases with temperature increase, while in the latter, it is almost temperature independent.

The echo signal for the two samples studied was found only below 154 K. Above this temperature the echo decay was too fast for the signal to be observed.

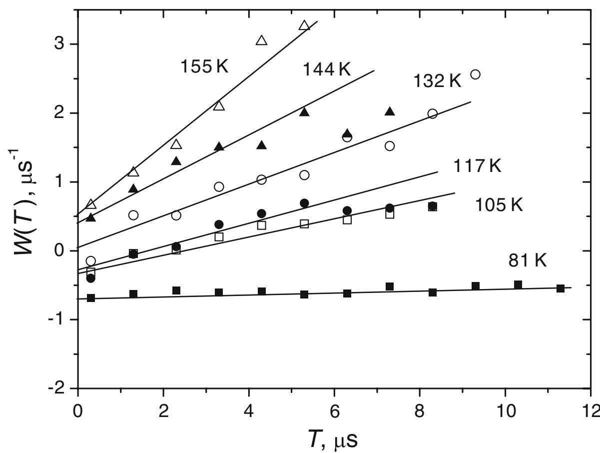


Fig. 3 The relaxation rates $W(T)$ as a function of the time delay T at different temperatures. The *straight lines* are linear approximations of these data. The sample is 5-D-PSPC in cholesterol-free POPC bilayer

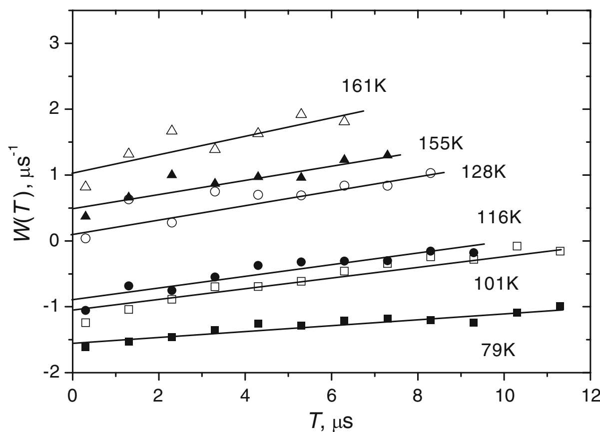


Fig. 4 The same as in Fig. 3, except that the sample is 5-D-PSPC in cholesterol-containing POPC bilayer

The negative $W(T)$ values seen in Figs. 3 and 4 at low temperatures may be readily explained by a so-called instantaneous diffusion process in ESE. This process is induced by alternation of the sign of dipole–dipole interaction between different spin labels under the action of microwave pulses. For nitroxide spin labels, the influence of instantaneous diffusion process was quantitatively described in Ref. [19]. The rate of the additional echo decay due to this process is proportional to the label concentration. It is also proportional to the portion of the excited spins, so the rate is the highest for excitation of the central component (position 2 in Fig. 1), which does explain the appearance of the $W(T)$ negative values.

4 Discussion

For cholesterol-containing sample, $W(T)$ at low temperatures is more negative than that for cholesterol-free sample (cf. Figs. 3, 4). As the total spin concentrations in both cases are close (see Sect. 2), this means that local label concentration in the presence of cholesterol is higher. This fact could be explained by the known property of cholesterol to induce a phase separation in the membrane [20]. So, probably spin-labeled 5-D-PSPC lipids tend to form in the POPC bilayer a phase enriched by this type of lipids.

Increase of temperature results in increase of $W(T)$ values (see Figs. 3 and 4). This may be attributed to additional anisotropic relaxation induced by molecular motions. It is important to note that the rate of the decay due to the mechanism of instantaneous diffusion is temperature independent. So this rate may be refined at low temperature, where motion is absent, and then subtracted from the all data at elevated temperatures.

In Ref. [18], an approach was suggested that provides a way to get information on the motion from a three-pulse stimulated ESE experiment. It was shown that this experiment evidences that in membranes, spin labels participate in two types of orientational motion: fast stochastic librations and slow rotations.

For fast stochastic librations, in the approximation of axial anisotropy of magnetic interactions, and for small excursion angles $\alpha(t) \ll 1$ of librational motion around the X molecular axis, echo decays as [18]

$$E(2\tau + T) \propto \exp(-2\langle\alpha(t)^2\rangle R_m^2(\theta,\varphi)\tau\tau_c), \tag{1}$$

where

$$R_m(\theta,\varphi) = \gamma\left[B(g_\perp - g_{||}) + \frac{m(A_\perp^2 - A_{||}^2)}{(A_\perp^2\sin^2\theta + A_{||}^2\cos^2\theta)^{1/2}}\right]\sin\theta\cos\theta\sin\varphi,$$

where τ_c is the correlation time of motion (fast motion means that $\tau_c < \tau$, T, i.e., τ_c lies on the nanosecond time scale), θ and φ are the angles determining the orientation of the magnetic field in the molecular framework, B is the magnetic field strength, $g_{||}$, $g_\perp$, $A_{||}$, $A_\perp$ are principal values of the g-tensor and of the tensor of hyperfine interaction, respectively, m is the nitrogen nucleus spin projection onto its

quantization axis. For small-amplitude motion, angles θ and φ in Eq. (1) may be considered as constants.

The m value, although it may fluctuate because of nuclear spin flips, also may be considered in Eq. (1) as a constant because of the following. The role of nuclear spin flips may be assessed from an inversion-recovery experiment [16]. From the known data on spin-labeled lipids in dipalmitoyl phosphatidylcholine membranes (see figure 4 in Ref. [16]), one may conclude that the influence of nuclear spin flips on stimulated ESE experiment is negligible on the microsecond time scale studied here.

For motion around the Y molecular axis, $\sin\varphi$ in Eq. (1) is to be replaced by $\cos\varphi$. Motion around the Z molecular axis under the used axial approximation does not produce echo decay.

Note that Eq. (1) does not depend on the time delay T. This is a property of fast motions, for which the magnetization evolutions during the two τ-intervals are statistically independent.

As an example of slow motions, $\tau_c > \tau$, we consider here a simple model of inertial rotations, in which the molecule freely rotates with an angular velocity Ω during the experimental time interval [18]:

$$E(2\tau + T) \propto \int \cos\{\Omega R_m(\theta, \varphi)(\tau^2 + \tau T)\} g(\Omega) d\Omega, \tag{2}$$

where $g(\Omega)$ is a distribution function of angular velocities ($\int g(\Omega)d\Omega = 1$). Let us expand Eq. (2) in terms of the parameter $|R_m(\theta, \varphi)|(\tau^2 + \tau T)$. When this parameter is zero, $E(2\tau + T)$ is unity. Conserving only the first term of this expansion, one obtains

$$E(2\tau + T) \propto 1 - |R_m(\theta, \varphi)|\Omega_0(\tau^2 + \tau T) \approx \exp(-|R_m(\theta, \varphi)|\Omega_0(\tau^2 + \tau T)), \tag{3}$$

where Ω_0 is a positive value determining the first term of the Taylor expansion of Eq. (2). In the case of Lorentzian $g(\Omega)$ distribution, the Ω_0 value is the half-width at the half-height of this distribution, and the exponential dependence in Eq. (3) presents an exact result. Note that the necessary condition for Ω_0 to have a non-zero value is the divergence of the second moment for the $g(\Omega)$ distribution.

The salient feature of Eq. (3) is its exponential dependence on the time delay T.

In previous studies of spin-labeled stearic acid in POPC bilayer [18], the multiplication of Eq. (1) and (3) was applied to explain experimental results. So spin labels were assumed to participate simultaneously in fast stochastic librations and in slow rotations. Data of the present work shown in Figs. 3 and 4 also may be interpreted in that way. Equation (1) determines the initial intercepting ($T = 0$), while Eq. (3) describes the linear T dependence.

Slight non-zero value of the line slopes in Fig. 4 most likely is not related to motion, because it does not depend on temperature. The possible explanation is destroying of instantaneous diffusion by mutual spin flips (spin diffusion in the system of electron spins, which is temperature independent).

The remarkable difference in the temperature dependence of the line slopes for cholesterol-free and cholesterol-containing samples seen from comparison of Figs. 3 and 4 (in the former case, the slope noticeably increases with temperature increase, while in the latter, it is almost temperature independent) implies that

rotational motion is suppressed in the presence of cholesterol. Meanwhile, the difference of intercepts at $T = 0$ between low-temperature and high-temperature data is somewhat larger for cholesterol-containing sample (cf. Figs. 3, 4). This fact means that fast stochastic librations in the presence of cholesterol become more pronounced.

Both these phenomena may be related to other known properties of cholesterol. Condensing [4, 5] and raft formation [6, 7] effects mean that "global" structure of the membrane becomes more stable, which may suppress rotational motions. Ordering effect [2–4] implies that lipid molecules are less mutually entangled, which may facilitate librations around the lipid axes.

Analysis developed in Ref. [18] has shown that for the excitations in positions 1 and 2 in the EPR spectra (see Fig. 1), $|R_m(\theta, \varphi)|$ may be replaced by a constant close to $\sim 3 \times 10^8\ s^{-1}$. So from comparison with experimental data in Figs. 3 and 4, one may obtain that $\Omega_0 \sim 10^3\ s^{-1}$. If we multiply this value by the time scale of the experiment (10^{-5} s), we obtain that the characteristic angle of reorientation in slow rotations is around 1° or smaller. This estimation a posteriori confirms the used model: any slow motion within such range of the angles may look like a free rotation.

5 Conclusions

Comparison of data presented in Figs. 3 and 4 shows that presence of cholesterol suppresses rotational motion and enhances fast stochastic librations. So in phospholipid membranes, cholesterol increases low-energetic barriers for orientational motions existing for lipid molecules in the membrane. Or, in other words, cholesterol increases stability of the "global" orientational structure of lipids in the bilayer. Meanwhile, the local surrounding of a lipid molecule becomes looser which induces more intensive librations.

The found properties may be related to other known functions of cholesterol. Condensing and raft formation effects of cholesterol mean that "global" structure of the membrane becomes more stable, which may suppress rotational motions. Ordering effect of cholesterol implies that lipid molecules are less mutually entangled, which may facilitate librations around the lipid axes. These issues are worthy of further investigation.

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