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Research Article

Regarding the measurement of microviscosity in lipid bilayers by EPR

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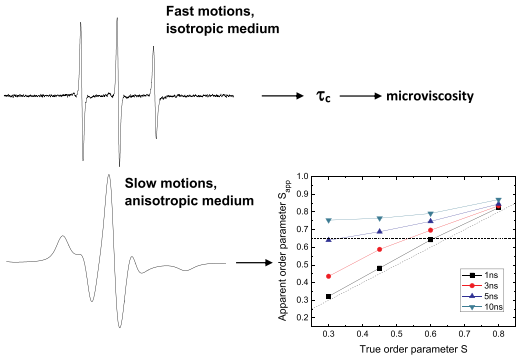
HIGHLIGHTS

- EPR spectra of stearic acid spin labels in slow motion regime are simulated.
- Differences between apparent and true order parameters are discussed.
- Erroneous procedures to evaluate membrane microviscosity are pointed out.
- Apparent order parameter is not reliable to evaluate membrane microviscosity.
- Apparent order parameter is useful to detect trends in membrane fluidity.

GRAPHICAL ABSTRACT



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ABSTRACT

Simulations of electron paramagnetic resonance spectra of stearic acid spin labels in lipid bilayers are used to illustrate the fact that the apparent order parameter S_{app} calculated from the spectral shape does not coincide with the true order parameter S in the case of slow motions. While S reflects a static property as the degree of order of the lipid chains, S_{app} depends on both order and dynamics. Thus, calibration procedures intended to obtain bilayer microviscosity values from S_{app} in the slow motion regime are not reliable. However, S_{app} is a useful tool to describe trends in membrane fluidity.

1. Introduction

Continuous wave X-band electron paramagnetic resonance (EPR) spectra of nitroxides are mainly sensitive to the orientation of the nitroxide z axis (which coincides with the direction of the p-orbital of the unpaired electron) relative to the magnetic field. This is because at X-band EPR frequency (i) anisotropic contributions to the spectra are dominated by a hyperfine interaction of an unpaired electron with the ^{14}N magnetic nucleus ($I = 1$) that has an approximately axial character and (ii) g-factor anisotropy could be neglected for all practical reasons. Molecular reorientation at adequate rates can average out the anisotropic part of this interaction causing noticeable changes in the EPR spectra [1,2]. When the spin label is dissolved in an isotropic medium of low viscosity, the spectrum consists of three narrow lines, and rotational correlation times τ_c in the range 10^{-12} s $< \tau_c < 10^{-9}$ s can be estimated using a simple equation involving the measured linewidths and peak-to-peak heights [3] (See Supplementary Material for an example).

The concept of microviscosity, introduced by Shinitzky [4] refers to the friction effects between solvent and label that oppose its rotational reorientation. Rotational correlation time can be related to the solvent microviscosity η through the Stokes-Einstein-Debye (SDE) equation

$$\eta = \frac{kT}{V}\tau_c \quad (1)$$

where V is the hydrodynamic volume of the rotating molecule. An

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adequate calibration with spectra of a small spin label in media of known viscosities allows one to measure the microviscosity of the solvent with a very good resolution in the range 1–10 mPa·s [5]. In the case of slower dynamics (higher viscosities), spectral simulation is needed to obtain a correct evaluation of rotational correlation times. However, the calibration approach should still be valid, provided the unknown medium is isotropic. Conventional X-band CW EPR spectra become insensitive to changes in rotational correlation time above ca. 5×10^{-7} s [2].

Lipid bilayers, either natural (biological membranes), or synthetic (liposomes) are anisotropic media due to the structure of the membrane forming lipids, consisting of polar heads and long hydrophobic chains. Lipid chains can be more or less packed and ordered depending on temperature and composition [6]. The fluid state allows for a high degree of mobility at the bilayer center, but preserves lipid ordering near the polar heads, while in gel or liquid ordered states the lipid chains have mobility restrictions at all depths [6,7]. As order and mobility are interdependent, the mere definition of membrane microviscosity should be a matter of discussion. For this reason, the more comprehensive concept of "membrane fluidity", involving both dynamics and order, is widely used [7,8]. Although no precise numbers can be given for membrane fluidity, it is a valuable concept to detect qualitative changes in model and biological membranes.

Stearic acid spin labels (n-DSA, *aka* n-SASL) are derivatives of the 18-carbon saturated fatty acid, with a nitroxide doxyl ring replacing the n-carbon (Fig. 1). They are useful labels for lipid bilayers, as they readily incorporate to phospholipid assemblies or cell membranes placing the carboxyl near the polar headgroups and mimicking the orientation and dynamics of the lipid hydrocarbon chains [7,8]. The z axis of the nitroxide is along the normal to the lipid bilayer when the stearic acid chain is in the extended *trans* conformation, but *trans-gauche* isomerizations, cumulative down the stearic acid chain, cause fluctuations of the orientation of the nitroxide z axis. The amplitude of these fluctuations is dependent on the degree of ordering of the neighbor lipid chains. The rotational reorientation of the nitroxide is no longer isotropic, as rotation is hindered, because the explored angles are confined to a cone around the bilayer normal [9]. The amplitude of this cone is characterized by the order parameter S, related to a restoring potential [10]. S varies in the range $0 < S < 1$, the larger S implying smaller cone amplitude, i.e., higher order. The dynamics of the fluctuations, governed by the dynamics of the lipid chains, is still characterized by the rotational correlation time [10].

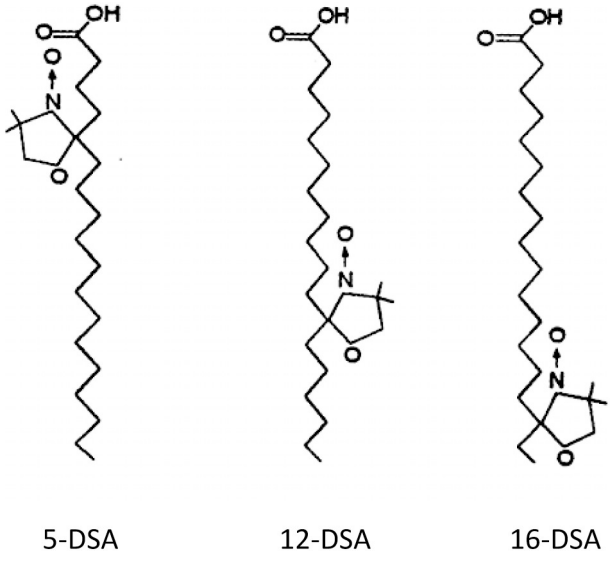

Fig. 1. Structure of 5-, 12-, and 16-stearic acid spin labels (n-DSA or n- SASL).

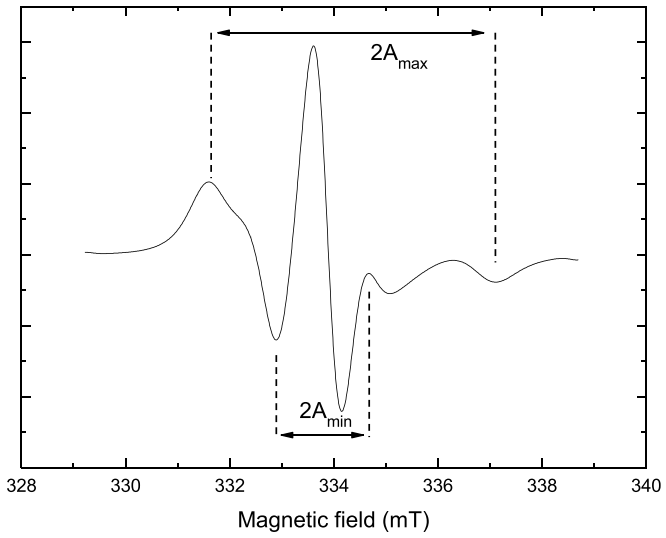

Fig. 2. X-band EPR spectrum of n-DSA in a lipid bilayer simulated with *Chili* using order parameter S = 0.33, rotational correlation time τ_c = 5 ns, and the fixed parameters mentioned in the text. The hyperfine splittings used in the calculation of S_{app} (Eq. (2)) are shown.

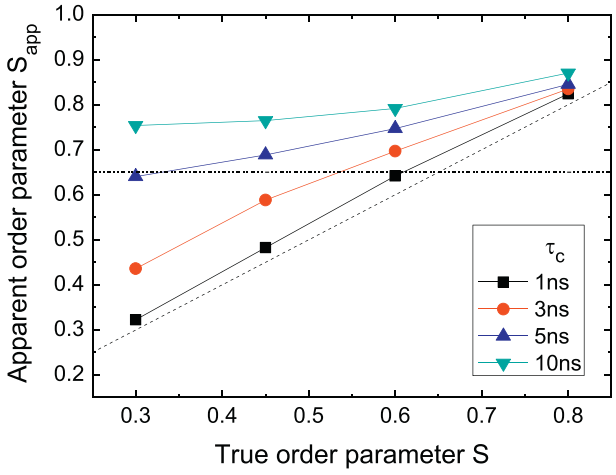

Fig. 3. Values of apparent order parameter calculated applying Eq. (2) to spectra simulated with *Chili* [18] for different combinations of true order parameter and rotational correlation time τ_c. The insert shows the different values of τ_c used in the simulations. The diagonal dashed line corresponds to the case $S_{app} = S$, and the horizontal dash-dotted line corresponds to $S_{app} = 0.65$.

When it is enough to have a qualitative description of membrane properties, an empirical "apparent order parameter" S_{app} can be calculated from the spectral hyperfine features [1,2,8,11]. This parameter is related to membrane fluidity: higher/lower Sapp corresponds to lower/higher membrane fluidity. Changes in membrane fluidity detected through changes in S_{app} can give useful information about lipid-lipid or lipid-peptide interactions in cell membranes and model systems [9,12–16]. However, S_{app} cannot be related separately to membrane order or microviscosity, as will be shown in the following sections.

The motivation of this paper is to point out the incorrect use of S_{app} values to calculate the microviscosity of lipid bilayers [17]. The wrong hypothesis states that if the same value of S_{app} is obtained for a n-DSA in a lipid bilayer and in an isotropic liquid, the microviscosity is the same in the two systems. Spectral simulations allow us to show that this hypothesis is wrong.

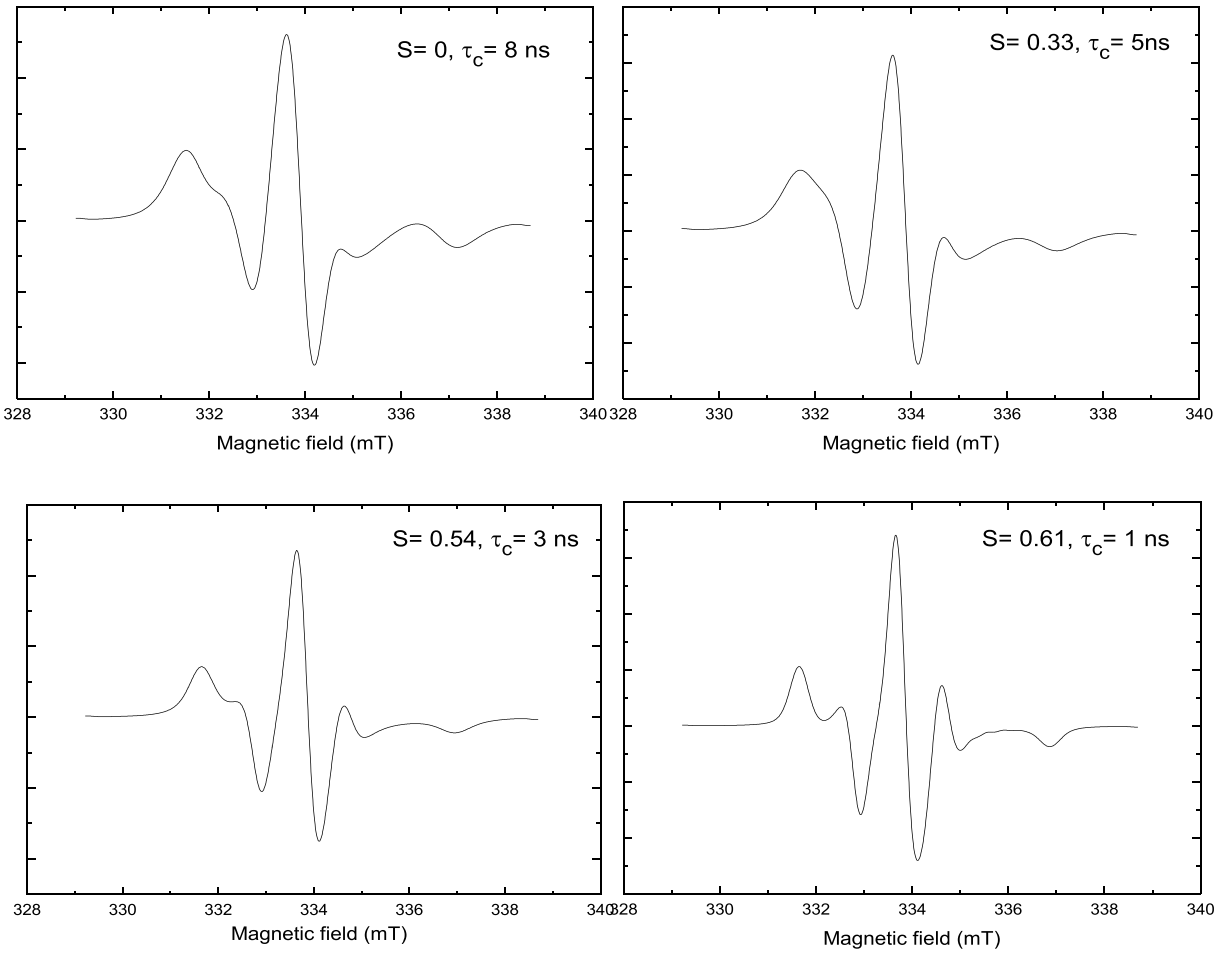

Fig. 4. An example showing that similar values of apparent order parameter S_{app} can correspond to different dynamics. EPR spectra of n-DSA in a bilayer simulated with *Chili*, a program of the EasySpin package [18], using the values of order parameter S and rotational correlation time τ_c shown in each case. All the spectra have an apparent order parameter $S_{app} = 0.65$, calculated with Eq. (2). Note the wide range of values of the rotational correlation time, which is the parameter related to microviscosity.

2. Methods

Schneider and Freed [10] developed computational methods based on solving the Stochastic Liouville equation that allow for simulation of EPR spectra of a nitroxide radical subjected to an ordering potential, like the one n-DSA is experiencing in a lipid bilayer. The methods are implemented in the routine *Chili* of the free software EasySpin [18]. We have used this routine to simulate EPR spectra in the slow motion regime [19] for different order parameters and rotational correlation times. The fixed parameters are the components of the g tensor (g_{xx}, g_{yy}, g_{zz}) = (2.0088, 2.0061, 2.0027), the components of the ^{14}N hyperfine tensor (A_{xx}, A_{yy}, A_{zz}) = (0.59, 0.54, 3.29) mT, and the peak to peak residual broadening, with a gaussian component of 0.3 mT and a lorentzian one of 0.03 mT. The microwave frequency was set at 9.375 GHz.

Dynamics can be described in the simplest way by two rotational correlation times, $\tau_{c//}$ characterizing molecular rotations around the main molecular axis, and $\tau_{c\perp}$, characterizing the rotation of the long molecular axis around an axis perpendicular to it, and used to model the wobbling dynamics. In all cases it is $\tau_{c//} < \tau_{c\perp}$, and it can be shown that the spectra are almost independent of the value of $\tau_{c//}$ [19]. Thus, only one correlation time τ_c (corresponding to $\tau_{c\perp}$) was used in the simulations. Order is described by the order parameter $S = \frac{1}{2} < (3cos^2(\theta) - 1) >$ where θ is the angle between the z axis of the nitroxide and the ordering director axis (the membrane normal), and the brackets indicate an ensemble average [8,19]. The restoring pseudopotential imposing molecular ordering is $U(\theta) = -kT\lambda\, cos^2(\theta)$. The proper potential coefficient λ giving the desired value of the order parameter S was calculated in each case using the results of the program D200 of Schneider and Freed [10]. Room temperature is assumed.

A set of spectra were simulated varying the rotational correlation time τ_c between 1 and 10 ns, and the order parameter S between 0.3 and 0.8. The apparent order parameter S_{app} was calculated from each spectrum as

$$S_{app} = \frac{A_{max} - A_{min}}{A_{zz} - \frac{1}{2}(A_{xx} + A_{yy})} \quad (2)$$

where A_{max} (A_{min}) is half the separation between the two outer (inner) hyperfine features of the spectrum, as shown for a typical spectrum in Fig. 2.

3. Results and discussion

The calculated values of S_{app} are plotted in Fig. 3 as a function of the true order parameter S used in each simulation, for the different rotational correlation times. The oblique dashed line represents the ideal case when S_{app} coincides with the real order parameter. It can be seen in Fig. 3 that this coincidence is expected for the case of correlation times

lower than 1 ns (fast motion regime). Fig. 3 shows that the higher the correlation time, the larger the deviation of the calculated S_{app} from the real order parameter S, and this effect is especially important for the lowest values of S. Note that for a given value of S, representing a certain degree of order, a broad range of S_{app} values can be obtained depending on the dynamics of the spin label in the sample, i.e., depending on the microviscosity. This fact shows that trying to univocally associate a value of S_{app} with the microviscosity of the sample is completely unjustified in the slow motion regime. We obtained an estimation of the viscosity value delimiting fast and slow motion regimes for n-DSA at room temperature using Eq. (1) with $\tau_c = 1$ ns together with an estimation of the hydrodynamic volume of 12-DSA (see Supplementary Material). The result was 5 mPa·s, meaning that in a solvent with viscosity higher than this value, the spin labels n-DSA would be in the slow motion regime. However, Bahri et al. [17] use a "calibration" of S_{app} in isotropic solvents with viscosities up to 400 mPa·s to calculate (with 5 significant digits!) the microviscosity of DMPC bilayers at different depths, even in the gel phase. To make things worse, the calibration curves of Bahri et al. are still being cited to estimate microviscosity of lipid bilayers [20–22].

Additional graphical evidence is provided by the set of EPR spectra of Fig. 4. They correspond to conditions on the horizontal dash-dotted line of Fig. 3, i.e., they all have apparent order parameter $S_{app} = 0.65$. However, they were generated with a wide range of S and τ_c parameters, starting from S = 0 (isotropic environment) up to S = 0.61. Note that the corresponding rotational correlation times needed to generate a spectrum with $S_{app} = 0.65$ decrease from 8 to 1 ns, meaning that microviscosity, calculated from Eq. (1), is about 40 mPa·s in the case of spectrum (a) and 5 mPa·s in spectrum (d). However, if the calibration curve presented in Fig. 4a of Bahri et al.'s paper [17] were used, all these different spectra would correspond to a "bilayer microviscosity" of about 250 mPa·s. It is clear that microviscosity values calculated in this way are severely overestimated.

Despite this erroneous approach, it must be remarked that the correct use of simple estimations based on the positions of the hyperfine features of the nitroxide EPR spectra can provide useful information about lipid bilayer properties. First, calibration procedures of rotational correlation times as used by Bahri et al. [17] are still valid to estimate microviscosity values when the rotational reorientation of the nitroxide is almost isotropic and in the fast motional regime. This corresponds to the case of 16-DSA (which senses the bilayer center) in fluid membranes, and sometimes also to 12-DSA and even 5-DSA in micelles [23]. Second, for the case of anisotropic reorientation of the nitroxide (case of 5- and 12-DSA in bilayers, corresponding to the slow motional regime), calculation of the apparent order parameter S_{app}, or even the simple evaluation of the maximum hyperfine parameter A_{max}, give valuable clues of the changes of fluidity of a biomembrane upon changes in temperature, composition, interaction with surfactants, etc., [11–16]. However, detailed spectral simulations or fittings must be performed if one needs quantitative estimations of both the dynamic and order parameters. A recent paper by Marsh [19] gives empirical expressions to calculate the order parameter from the spectral hyperfine features, but a previous knowledge the rotational diffusion coefficient is needed.

Summarizing, the idea of calculating precise values of lipid bilayer microviscosity with spin label EPR using calibrations with solvents of known viscosity is based on the incorrect assumption that the label rotation is the same, in the solvent and in the bilayer. However, rotational reorientation of the spin label is hindered in lipid bilayers, and the label samples a restricted range of orientations. We have shown that this fact affects profoundly the spectral shape, rendering invalid the comparison with apparent order parameters calculated in isotropic media. The use of these calibration procedures results in a severe overestimation of bilayer viscosity.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bpc.2019.106223.

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