Journal of Magnetic Resonance 226 (2013) 35–44

Contents lists available at SciVerse ScienceDirect

Journal of Magnetic Resonance

journal homepage: www.elsevier.com/locate/jmr

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

Laxman Mainali, James S. Hyde, Witold K. Subczynski *

Department of Biophysics, Medical College of Wisconsin, Milwaukee, WI, USA

ARTICLE INFO

Article history:
Received 13 July 2012
Revised 15 October 2012
Available online 12 November 2012

Keywords:
W-band
Spin label
Cholesterol
Membrane fluidity
Saturation-recovery
Spin-lattice relaxation time
Spin-lattice relaxation rate

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. Dimyristoylphosphatidylcholine (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 (2011) 418–425). 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 (1984) 4995–5004) provided rotational diffusion coefficients ($R_\perp$ and $R_\parallel$) and order parameters (S_0). Spectral analysis at X-band provided one rotational diffusion coefficient, $R_\perp$. 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 phospholipid 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 μL at X-band.

1. Introduction

An intensive development and a broad application of T_1-sensitive EPR spin-labeling 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, 1–10 μs) 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 va-

* Corresponding author. Address: Department of Biophysics, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226-0509, USA. Fax: +1 414 456 6512.
E-mail address: subczyn@mcw.edu (W.K. Subczynski).

1090-7807/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jmr.2012.11.001

lue 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 2-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 or T-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 0.6 mm 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_1s, 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 μs. 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