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Spin-label W-band EPR with seven-loop–six-gap resonator: Application to lens membranes derived from eyes of a single donor

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Abstract

Spin-label W-band (94 GHz) EPR with a five-loop–four-gap resonator (LGR) was successfully applied to study membrane properties (L. Mainali, J.S. Hyde, W.K. Subczynski, Using spin-label W-band EPR to study membrane fluidity in samples of small volume, *J. Magn. Reson.* 226 (2013) 35–44). In that study, samples were equilibrated with the selected gas mixture outside the resonator in a sample volume ~100 times larger than the sensitive volume of the LGR and transferred to the resonator in a quartz capillary. A seven-loop–six-gap W-band resonator has been developed. This resonator permits measurements on aqueous samples of 150 nL volume positioned in a polytetrafluoroethylene (PTFE) gas permeable sample tube. Samples can be promptly deoxygenated or equilibrated with an air/nitrogen mixture inside the resonator, which is significant in saturation-recovery measurements and in spin-label oximetry. This approach was tested for lens lipid membranes derived from lipids extracted from two porcine lenses (single donor). Profiles of membrane fluidity and the oxygen transport parameter were obtained from saturation-recovery EPR using phospholipid analog spin-labels. Cholesterol analog spin-labels allowed discrimination of the cholesterol bilayer domain and acquisition of oxygen transport parameter profiles across this domain. Results were compared with those obtained previously for membranes derived from a pool of 100 lenses. Results demonstrate that EPR at W-band can be successfully used to study aqueous biological samples of small volume under controlled oxygen concentration.

Keywords

W-band; Loop-gap resonator; Saturation recovery; Spin-label; Membrane; Eye lens

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1. Introduction

Nitroxide spin-labels or spin probes are used in many aqueous biological and biomedical studies [1–4]. This work focuses on samples in which control of the dissolved molecular oxygen concentration is required, and sample availability is low. After the first description of the broadening of EPR spectra in liquid samples by dissolved oxygen [5, 6], it became customary to remove oxygen using freeze-pump-thaw cycles. The easier method of bubbling nitrogen gas through the sample was also employed. A third alternative was introduced by Plachy and Windrem, who proposed using a gas-permeable polytetrafluoroethylene (PTFE) capillary as an EPR sample tube [7]. Flowing dry nitrogen gas over the PTFE sample tube resulted in gas exchange and removal of dissolved molecular oxygen. Solving the deoxygenation problem allowed recording of the CW spectra without oxygen-induced line broadening.

Although the effect of dissolved molecular oxygen on spin-label spectra in aqueous samples is small and often neglected, the effect on the electron spin-lattice relaxation time, T_1 , is profound. Popp and Hyde [8] used the method of Plachy and Windrem with a gas permeable sample tube made from a methylpentene plastic known as TPX to remove dissolved molecular oxygen in electron-electron double resonance (ELDOR). This experiment depends on the value of T_1 . In addition, removal of molecular oxygen is necessary to obtain the correct value of the spin-lattice relaxation time in saturation-recovery (SR) measurements.

It may be necessary in some experiments to control the partial pressure of molecular oxygen over the sample. Kusumi et al [9] used a gas blender to control the relative amounts of oxygen and nitrogen that flowed over the sample. There was a double benefit in this approach: the temperature of the sample and the partial pressure of oxygen could both be controlled. Adjustment of the ratio of oxygen to nitrogen concentration in the sample was used for oxygen accessibility studies [9–11]. This field was reviewed in 2005 [12].

Early spin-label oximetry studies were carried out using conventional microwave cavities. The use of a gas permeable sample tube was greatly advanced by the introduction of the loop-gap resonator (LGR). See Fig. 1a. The development of new LGRs [13–15] has allowed transfer of the T_1 -sensitive SR-oximetry method to higher microwave frequencies. The three-loop–two-gap resonator, developed for 35 GHz Q-band measurements with aqueous samples, has a sample loop diameter of 0.65 mm [16]. A PTFE sample tube from Zeus (Orangeburg, SC) with an ID of 0.25 mm and a wall thickness of 0.075 mm was used. Sample holding is performed with a ball and collet system that compresses the PTFE sample tube at the bottom. It is then pulled tight and affixed at the top.

The five-loop–four-gap resonator that was developed for 94 GHz W-band for measurements with aqueous samples was designed with a sample loop diameter of 0.65 mm, the same as the Q-band two-loop–one-gap resonator [14]. Quartz capillaries with an ID of 0.15 mm and a wall thickness of 0.05 mm were used. Samples were equilibrated either with nitrogen or an air/nitrogen mixture outside the resonator, transferred to a quartz capillary, sealed with Baxter Miniseal wax B4425.1 (Scientific Products), and positioned in the resonator of the

W-band spectrometer. A special gas-exchange chamber was designed to equilibrate samples with nitrogen or air/nitrogen mixture at 1 atm pressure and to transfer them without contact with air and without changing the partial pressure of oxygen in the quartz capillary (Fig. 1b). External equilibration is not as convenient and precise as samples equilibrated within the resonator with gas used for temperature control. In addition, the volume of the sample in the gas exchange chamber was more than one hundred times larger than the sensitive volume of the W-band LGR, which placed limitations on the possible experiments.

This problem was overcome using a recently designed seven-loop–six-gap W-band LGR, Fig. 2, which has a sample loop diameter of 0.825 mm. The ID of the PTFE tube is 0.25 mm, the same as previously used at Q-band, but the wall thickness was increased from 0.075 to 0.125 mm, resulting in an OD of 0.50 mm. The tubing is more rigid and easier to use than the thin-walled tubing used previously, and easily fits the sample loop. Samples are deoxygenated or equilibrated with the desired air/nitrogen mixture as shown in Fig. 1c. This LGR allows the combination of small sample volume of the W-band LGR with the convenience of equilibrating the sample directly in the resonator with the gas that is also used for temperature control. This approach to making measurements for limited biological samples was tested on lens lipid membranes derived from the cortex and nucleus of just two porcine lenses (single donor) from a two-year-old animal. Results were then compared with those previously obtained for membranes derived from a pool of 100 lenses [17].

We used EPR spin-labeling methods to study the organization and dynamics of eye lens lipid membranes. Lipids were derived from eyes of different species [18–20], from donors of different age [18–22], and from different regions of the eye lens, namely the cortex and nucleus [18–20]. These studies were summarized in a review [23]. Recently, we moved our studies to intact lens membranes isolated from human [24] and porcine [19] eye lenses. Most of these measurements were performed at X-band using both conventional and SR EPR spectrometers with an LGR that has a sample volume of approximately 3 μ L. To complete all measurements and obtain detailed profiles of membrane properties, membranes were extracted from 50 to 100 lenses. This created a significant problem for the interpretation of results, especially for human lenses, which can be different not only because of age, but also because of the varying health histories of the donors. Pooling samples from 50 lenses eliminates the health history of a donor from data analysis. As such, the ability to perform experiments on one or two eye lenses from a single donor is an advantage. Because of this, we focused on using the W-band spectrometer, which has both conventional and SR capabilities. We showed that all profiles of major membrane properties obtained at X-band can also be obtained at W-band. Conventional spectra of phospholipid spin-labels at W-band allow more detailed analysis of rotational motion (parallel and perpendicular) than spectra at X-band [25], which leads to a more detailed analysis of membrane fluidity that could be used to study differences due to disease or age. Results demonstrate that EPR at W-band with the seven-loop–six-gap resonator can be successfully used to study aqueous biological samples of small volume, particularly for studies of lens membranes obtained from eyes of a single donor.

2. Materials and Methods

2.1. Materials

One-palmitoyl-2-(n-doxylstearoyl) phosphatidylcholine spin-label (n-PC, n = 5, 7, 10, 12, 14, or 16) and tempocholine-1-palmitoyl-2-oleoylphosphatidic acid ester (T-PC) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). 9-doxylstearic acid spin-label (9-SASL) and cholesterol analogues, androstane spin-label (ASL), and cholestane spin-label (CSL) were purchased from Molecular Probes (Eugene, OR) (see Fig. 3 for spin label structures). Other chemicals, of at least reagent grade, were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Isolation of the total lipids from the cortical and nuclear regions of the porcine eye lenses

Fresh porcine eyes from two-year-old animals were obtained on the day of slaughter from Johnsonville Sausage, LLC (Watertown, WI). The cortical and nuclear regions of the lenses were separated based on differences in tissue consistency by the methods described in Refs. [26] and [27]. The total lipids from the cortical or nuclear samples were extracted separately based on minor modifications of the Folch procedure [28]. Details of these procedures were described earlier in Ref. [17]. The resultant (~1.5 mg for cortex and ~1.0 mg for nucleus) lipid samples were soft, white solids. They were stored at -20°C .

2.3. Preparation of lipid bilayer membranes

The membranes used in this work were multilamellar dispersions (multilamellar liposomes) made either from the lipid extract from the eye-lens cortex or the nucleus to which 1 mol% spin label was added. The membranes were prepared using the rapid solvent-exchange method [29–31] with apparatus that was constructed in our laboratory and described in detail in Ref. [29]. Details of our preparations are described in Ref. [18]. This method is preferable to the film-deposition method previously used. At a high cholesterol (Chol) content in the lipid mixture (which is the case for lipid extracts from the lens cortex and nucleus), and during membrane preparation using the film deposition method, the lipid mixture passes through the solid-state intermediate at which point solid-state demixing of Chol can occur (see Ref. [30] for further detail).

The amount of lipids from cortex and nucleus was sufficient to prepare a minimum of ten samples, with all ten spin labels used in this work (see Fig. 3). Membranes were prepared in 300 μL of buffer and then were centrifuged briefly (16000g, 15 min, 4°C). The volume of the loose pellet (approximately 20% lipid, wt/wt) on the bottom of the Eppendorf tube was too small for convenient sample handling and was increased to 2–3 μL . Even with that dilution the final spin label concentration was about 0.5 mM.

2.4. Loop-gap resonator

The resonator body of the seven-loop–six-gap resonator is shown in Fig. 2. This LGR implements a number of advanced microwave features that have been developed in our laboratory, including enhanced microwave field uniformity end sections [32], and long-slot iris coupling [33]. The resonator has a Q_0 -value of 150 with aqueous sample and a resonator

efficiency of $5.1 \text{ G/W}^{1/2}$. The reduction of the magnetic field at the sample is due to the increase of the inner sample loop diameter to 0.825 mm (Fig. 2), compared to 0.65 mm in the previous W-band LGR design [34].

A new sample-holding technique using a slot and clamp structure has been implemented to hold up to 0.81 mm outer diameter PTFE tubing, illustrated in Fig. 2b. This clamp system replaces the ball and collet method, which proved to be challenging with small sample sizes and resulted in an unstable tune. Here, the PTFE sample tube is easily inserted into a slip fit sample loop and held in place by a clip that compresses the sample tube. The resonator and tuning structure held exceptionally well for hours. The new resonator structure has a gas-exchange port in the narrow face of the waveguide. This port, along with the ability to use PTFE tubing, allows the sample to be immersed in air/nitrogen mixtures, as opposed to full air or nitrogen [17].

2.5. EPR measurements

The membrane suspensions prepared as described in Sect. 2.4 were transferred to a gas permeable PTFE capillary (ID 0.25 mm) and used for EPR measurements. Samples were equilibrated directly in the resonator with nitrogen or air/nitrogen mixture adjusted with a flowmeter (Matheson Gas Products, model No. 7631 H-604) [35]. The same gas was used for temperature control. The spectrometer used for W-band measurements, including SR capabilities, was described earlier [14, 34].

The spin-lattice relaxation times, T_1 , 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 long-pulse SR EPR. At W-band, the low-field hyperfine line is most intense, shown in Fig. 4. For lipid spin-labels in membrane suspensions, the pulse duration was 0.3–1 μs . The motion of spin-labels for these samples was slow enough 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 Sect. 2.3 in Ref. [16]).

For each sample, the following measurements were made: first, a low power EPR spectrum was acquired (without oxygen), which provides two values of rotational diffusion (parallel and perpendicular) as well as the order parameter. Second, the T_1 value without oxygen was obtained, which provides an additional measure of membrane fluidity. And third, measurements of T_1 in the presence of oxygen allow calculation of the oxygen diffusion-concentration product around the nitroxide moiety. Thus, three complementary measurements of membrane fluidity were made.

3. Results and discussion

The most abundant phospholipids in porcine lens membranes are phosphatidylcholines (PCs), ~35%; sphingomyelins (SMs), ~26%; phosphatidylserines (PSs), ~21%; phosphatidylethanolamines (PEs), ~12%; and dihydrosphingomyelins (DHSMs), ~3% (see Fig. 3 for their structures). Fatty acids are mainly saturated or monounsaturated, and phospholipids of young animals contain ~33% palmitate, ~33% oleate, and ~10% nervonate

[36]. These membranes contain extremely high amounts of cholesterol, which not only saturate the phospholipid bilayer but also induce formation of pure cholesterol bilayer domains (CBDs) within the phospholipid bilayer (PCD) [19, 20]. Co-existence of these domains creates the possibility for partitioning of lipid spin-labels. The phospholipid-type spin-labels (n-PCs and T-PC) partition mainly into the PCD, while the cholesterol analogues (ASL and CSL) partition into both domains. Thus, only ASL and CSL can discriminate between both co-existing domains. Partitioning of n-PCs and T-PC mainly into the PCD ensures that information about the structure and dynamics obtained with these spin-labels describes properties of the bulk PCD without “contamination” from the CBD. These partitioning properties form the basis for interpretation of our data (see [22] for more details).

3.1. Conventional EPR spectra

W-band spectra of all spin-labels used in this work for cortical and nuclear lens lipid membranes are shown in Fig. 4. All spectra were obtained for the same cortical and nuclear membrane preparations from two porcine eye lenses, which we call single-donor samples. Spectra presented in parallel panels for cortical (Fig. 4a, b, and c) and nuclear lens lipid membranes (Fig. 4d, e, and f) are practically identical. They are also similar to W-band spectra previously obtained for cortical and nuclear lens lipid membranes from the samples of 100 pooled porcine eye lenses [17]. Moreover, the signal-to-noise ratio is even better for single donor preparation than for pooled samples. This is most likely due to the use of PTFE capillaries with ID greater than that of the quartz capillaries used for pooled samples. It also indicates that W-band spectra obtained from single donor samples can be successfully analyzed using Freed’s MOMD (microscopic-order macroscopic-disorder) model [37, 38], which provides rotational diffusion coefficients ($R_{\perp}$ and $R_{\parallel}$) and order parameters (S_0) [25]. Spectral analysis at X-band provides only one rotational diffusion coefficient, $R_{\perp}$.

At W-band the separation of the g_{xx} , g_{yy} , and g_{zz} components of EPR spectra along with good signal-to-noise ratio allow a few significant conclusions to be drawn from these spectra. Panels of n-PC spectra (Fig. 4a and d) indicate that x - and y -components start to be averaged out close to the membrane surface (5- and 7-PC). At deeper locations (12-, 14-, and 16-PC), the rotational motion about the long axes (z -axis) is fast enough to average x - and y -components. Spectra become more isotropic toward the membrane center, indicating that the z -component starts to be averaged because of the increase in wobbling motion of the z -axis. For T-PC we expected fast rotational motion about the x -axis which should average y and z components, leaving the x -component separated. However, spectra in T-PC panels (Fig. 4b and e) indicate nearly complete averaging of all components. Without cholesterol in the lipid bilayer membranes, the x -component is clearly separated from averaged y - and z -components [25]. We can conclude that saturating amounts of cholesterol in lens lipid membranes greatly separate headgroups, increasing the amplitude and the rate of wobbling motion. In the absence of cholesterol, headgroups are tightly packed, which somewhat restricts wobbling [25]. Similarly for cholesterol analog spin-labels, ASL and CSL, we expected fast rotational motion about the y -axis which should have averaged x - and z -components. In lipid bilayers without cholesterol, all components of W-band spectra for ASL and CSL are averaged (unpublished results). In the presence of a saturating amount of

cholesterol, seen in lens lipid membranes, the rotational motion about the long axes is much slower, which induces the rise of x - and z -components (ASL and CSL panels in Fig. 4c and f). These conclusions would be strengthened if W-band spectra obtained for lens lipid membranes saturated with cholesterol were compared with spectra from membranes without cholesterol. These spectra were, however, not obtained in these studies because of difficulty with the depletion of cholesterol from lens lipid membranes. These problems for DMPC membranes with and without cholesterol are discussed in more detail in paper [25].

3.2. Saturation-recovery measurements

Figure 5 shows representative SR signals of 5-PC (a,b) and ASL (c,d) in nuclear (b,d) and cortical (a,c) lens lipid membranes recorded at 25°C for single donor samples. Measurements were performed for deoxygenated samples and samples equilibrated with a selected air/nitrogen mixture. All saturation-recovery signals shown in Fig. 5 were satisfactorily fit to a single exponential function except for ASL in nuclear membranes in the presence of 20% air (Fig. 5d). Similar experiments were performed using CSL (data not shown). Single exponentials were seen in all cases. The double exponential of Fig. 5b was interpreted as being indicative of the presence of two domains, namely PCD and CBD [22]. Formation of these domains in lens lipid membranes was investigated in detail [18, 19, 22]. The data also indicate that the environment in which phospholipid analog spin-labels are located is homogeneous in terms of rotational motion of the nitroxide moiety, which determines the spin-lattice relaxation time measured in the absence of oxygen [25, 39].

Two points should be emphasized. First, the seven-loop–six-gap W-band LGR allows equilibration of the sample with the desired gas inside the resonator (Fig. 1c); thus, equilibration and measurement are performed at the same temperature and the same pressure (partial pressure of oxygen). This is extremely significant, because both the partitioning of oxygen into the membrane [40] and the diffusion coefficient of oxygen are affected by temperature. Using this method, the partial pressure of oxygen in the gas mixture can be better related to the atmospheric pressure at which equilibration is performed. Second, this design also ensures complete and rigorous deoxygenation. Fully deoxygenated SR data, presented here, also confirm that previous measurements with the use of the gas-exchange chamber (Fig. 1b) [17] were performed correctly.

As indicated in Sect. 3.1, the available observing power is sufficient to record CW spectra with a good signal-to-noise ratio. However, pump power available inside the resonator is less than that available in the previous LGR [17]. Increase in sample volume improved our ability to handle small PTFE sample tubes, which was critical to the use of the gas-exchange method. However, the larger sample volume led to the reduction in the efficiency parameter. The lower efficiency parameter creates challenges in SR signal amplitude, especially for samples with short spin-lattice relaxation times. To overcome this challenge, SR signals were accumulated longer, and were time averaged. We also used lower partial pressures of oxygen with which samples were equilibrated for oximetry measurements in order to avoid large reductions of the spin-lattice relaxation times. Yin and Hyde [41] showed that the use of high observing power in SR EPR experiments does not affect the ability to extract bimolecular collisions of spin-labels with oxygen and can increase the signal-to-noise ratio

up to ten times. This approach, in combination with the use of low oxygen levels, can be used if the signal-to-noise ratio in biological samples is too low.

For spin-labeled biological aqueous samples including intact membrane suspensions or protein solutions, which can be obtained only in limited quantities, the sensitivity of SR measurements is a significant issue. SR “concentration sensitivity” has not previously been discussed in the literature. The general conclusion of our papers, [15–17, 25] as well as unpublished data, is that the SR concentration sensitivity is experimentally found to be independent of microwave frequency. We find this point particularly significant when higher frequency SR EPR is applied to limited biological samples. Based on the measured number of spins, there is a significant increase in sensitivity as the microwave frequency increases because of the Boltzmann equilibrium and the improved filling factor for small samples. In our study, the volume of the sample was 150 nL at W-band, compared with 3 μ L at X-band. However, the SR signal-to-noise ratio at a fixed accumulation time remained about the same.

3.3. Profiles of the spin-lattice relaxation rate and the oxygen transport parameter

Finally, we show typical profiles of membrane properties that can be obtained using the SR capabilities of our W-band spectrometer for single donor samples. In Fig. 6a, the fluidity profiles (T_1^{-1} versus depth in the membrane) are presented for cortical and nuclear membranes obtained for single donor samples. These profiles were compared with those obtained for samples pooled from 100 lenses (Fig. 6b). Profiles obtained for single donor samples deoxygenated inside the LGR (Fig. 1c) are nearly identical to those obtained for pooled samples and deoxygenated in the gas exchange chamber (Fig. 1b). Profiles characterize dynamic properties of the alkyl chain that change gradually with membrane depth [23, 25].

Profiles of the oxygen transport parameter (OTP) (OTP was introduced as a convenient quantitative measure of the rate of the collision between spin probe and molecular oxygen [9]) are presented in Fig. 7 for single donor samples (Fig. 7a) and for samples pooled from 100 eyes (Fig. 7b). Here, profiles obtained for single donor samples are substantially the same as for pooled samples. Additionally, profiles of the OTP obtained at W-band are nearly identical to those obtained at X-band [25], which is in agreement with our earlier conclusion that the measured collision rate between the nitroxide moiety and oxygen (OTP) is independent of microwave frequency up to 94 GHz [15, 34]. All profiles across the bulk PCD in both membrane preparations show abrupt change between the C9 and C10 positions; this is the depth at which the rigid steroid ring structure of the cholesterol is immersed into the bilayer (see Fig. 3). In these profiles, membrane organization and fluidity are characterized based on the diffusion of oxygen within the membrane, but not directly on the motion and organization of alkyl chains. Profiles obtained by these methods [42, 43] reveal more features than typical profiles of membrane fluidity reported by the alkyl chain molecular order parameter [44–46]. Values of the OTP obtained with ASL and CSL were included in the profiles presented in Fig. 7a. They allowed drawing approximate profiles of the OTP across the CBD in nuclear lens lipid membranes from single donor samples.

4. Concluding Remarks

This paper highlights major benefits for eye lens research that are offered by a new EPR approach at W-band. As is the case with many human age-related diseases, age-related cataract formation appears to be unavoidable. Thus, future strategies for slowing cataract formation may depend on a detailed examination of people with age-matched cataractous and normal lenses, as well as those who retain clear lenses into their eighth and ninth decades. For this examination, the individual (single donor) experimental approach is critical, as it will allow acquisition of results for the cortex and nucleus as a function of age and health history of donors.

With this approach, donors can be grouped into those with transparent or with cataractous lenses. The latter group can be sub-divided further into donors with nuclear cataracts, which primarily affect the center of the lens, and donors with two other, less common types of human cataracts: posterior subcapsular and cortical. Many agents such as mutations, diet, radiation, drugs, and toxic chemicals, as well as certain diseases, can induce lens opacification. The new approach allows us to include these agents in the donor health history and opens opportunities in eye lens research.

A major difficulty with animal experiments is that human lenses are different in many key ways from those of laboratory animals [36]. Most importantly, the lifespan of laboratory animals is significantly shorter than humans, and an age-related human cataract typically takes more than 50–60 years to develop. Currently ophthalmic researchers have little, or no, idea why some people develop cataracts at age 60, whereas other individuals retain transparent lenses well into their ninth decade of life. The single donor approach combined with EPR spin-labeling methods should help to elucidate the biophysical basis of lens transparency in the human eye at the cell membrane and molecular level, and to determine the causes and mechanisms of age-related changes in the lens that lead to cataract formation.

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Fig. 1.

Schematic drawings showing positioning of the aqueous sample in the LGR of X-band (a) and W-band spectrometer (b and c), and illustrating strategies used for equilibrating the sample with the appropriate air/nitrogen mixture when TPX (a), quartz (b), and PTFE capillaries (c) are used. At X-band (a), the sample in the gas permeable TPX capillary is positioned inside the LGR and equilibrated with the gas (nitrogen or air/nitrogen mixture) used for temperature control. The active length of the resonator (10 mm) is indicated. At W-band, when EPR measurements are done in the gas impermeable quartz capillary (b), the sample is first equilibrated with the gas (nitrogen or air/nitrogen mixture) inside the gas-exchange chamber (Left), then transferred to the quartz capillary and used for EPR measurements (Right). The gas exchange chamber consists of a glass tube, with a plastic holder, of approximately 1 cm diameter in which the gas permeable TPX capillary (the same as used for X-band measurements) is mounted. Gas flow from the bottom of the chamber equilibrates the aqueous sample after approximately 15 min. The top part of the glass tube is closed with a thin-walled rubber stopper (appropriate septum) with a hole, allowing a rather loose insertion of a quartz capillary. After inserting a new capillary, we allowed the gas to flow through it for about 1 min. When the equilibrium with the appropriate gas was completed, the bottom end of the quartz capillary was immersed in the sample. The capillary force and the positive pressure inside the chamber pushed the sample into the capillary almost filling the total length (10 cm). The bottom end of the capillary was sealed with the Miniseal wax and inserted into the LGR (Right) for EPR measurements. The active length of the resonator (approximately 3 mm) is indicated. In the seven-loop-six-gap W-band LGR (c), the sample in the gas permeable PTFE capillary can be positioned inside the resonator and equilibrated with the gas (nitrogen or air/nitrogen mixture). Equilibration is completed in less than 1 min. The active length of the resonator (approximately 3 mm) is indicated.

Fig. 2.

a. New Seven-Loop–Six-Gap LGR resonator with gas exchange port and sample holder. Highlighted here is the sample loop 0.825 mm ID, located in the center of the Seven-Loop–Six-Gap geometry. **b.** New sample holding mechanism for PTFE samples uses a clip that compresses the sample tube and holds it in place.

Fig. 3.

Chemical structures of phospholipid- (n-PCs, T-PC, 9-SASL) and cholesterol-type spin labels (ASL, CSL) together with structures of the most abundant phospholipids in porcine lens membranes (PC, SM, PS, PE, DHSM) and cholesterol (CHOL). Approximate locations of these molecules across the lipid bilayer membrane are indicated.

Fig. 4. Panels of W-band EPR spectra of lipid spin-labels in porcine cortical (**a**, **b**, and **c**) and nuclear (**d**, **e**, and **f**) lens lipid membranes. (**a** and **d**) n-PC, (**b** and **e**) T-PC, and (**c** and **f**) ASL and CSL. Spectra were recorded at 25°C.

Fig. 5.

Representative SR signals with fitted curves and the residuals (the experimental signal minus the fitted curve) for 5-PC (**a**, **b**) and ASL (**c**, **d**) in membranes made of cortical (**a**, **c**) and nuclear (**b**, **d**) porcine lens lipids. Signals were recorded at 25°C for samples equilibrated with 100% nitrogen gas or with a gas mixture of 30%/20% air and 70%/80% nitrogen. SR signals for 5-PC were satisfactorily fitted to a single exponential function in both the absence and presence of molecular oxygen with time constants, respectively $6.36 \pm 0.02 \mu\text{s}$ and $3.52 \pm 0.02 \mu\text{s}$ in cortical membranes (**a**) and $6.49 \pm 0.02 \mu\text{s}$ and $3.79 \pm 0.02 \mu\text{s}$ in nuclear membranes (**b**). SR signals for ASL in cortical membranes were satisfactorily fitted to a single exponential function in both the absence and presence of molecular oxygen with time constants, respectively $3.88 \pm 0.02 \mu\text{s}$ and $1.63 \pm 0.03 \mu\text{s}$ (**c**). However, in nuclear membranes SR signals for ASL were satisfactorily fitted to a single exponential function only in the absence of molecular oxygen with the time constant, $4.1 \pm 0.02 \mu\text{s}$ (**d**). The SR signal in the presence of molecular oxygen can be fitted satisfactorily with a double exponential curve with time constants of $3.09 \pm 0.30 \mu\text{s}$ and $1.52 \pm 0.11 \mu\text{s}$ (**d**) (compare the upper residual for single and lower residual for double exponential fit in **b** and **d**). Equilibration with 20% air shortened the T_1 value and the available pump power cannot saturate the signal. Both factors are the reason for the lower signal-to-noise ratio at **c** and **d** (accumulation time was kept the same). The major criterion for a good fit is the residual,

indicating that single exponential fits for all signals are excellent. Additional criteria for single and double-exponential fits are explained in Sect. 2.5 and Ref. [42].

Fig. 6. Fluidity profiles (T_1^{-1} versus depth) for n-PC spin-labels at 25°C across cortical and nuclear porcine lens lipid membranes obtained for single donor samples (a) and for samples pooled from 100 lenses (b). Approximate locations of nitroxide moieties of spin-labels are indicated by arrows.

Fig. 7.

Profiles of the OTP (oxygen diffusion-concentration product) at 25°C across domains in cortical and nuclear porcine lens lipid membranes obtained for single donor samples (a) and for samples pooled from 100 lenses (b). Dotted lines indicate the OTP in the aqueous phase. Approximate localizations of nitroxide moieties of spin-labels are indicated by arrows. Points obtained for phospholipid-type spin-labels are indicated as ■ and ●, and points obtained for cholesterol-type spin-labels are indicated as ▼, ▲, and ○. Two arrows for ASL and CSL indicate locations of these spin-labels in the PCD and the CBD.