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High-field ESR on aligned membranes: A simple method to record spectra from different membrane orientations in the magnetic field

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Abstract

A combination of isopotential spin-dry ultracentrifugation (ISDU) and microtome techniques was used to facilitate the collection of high field/high frequency (170 GHz) ESR spectra corresponding to different orientations of the membrane normal relative to the magnetic field. This technique is particularly valuable for aligned biological samples *in vitro*. At 170 GHz, conventional sample preparation techniques based solely on ISDU constrained the sample to be oriented so that the membrane normal was parallel to the applied magnetic field due to the geometry and the millimeter wave field distribution of the Fabry–Pérot resonator used in these experiments. This orientational constraint limited the information that could be obtained from aligned membranes at high field. The combined ISDU/microtome technique overcame this limitation. Spectra from spin-labeled Gramicidin A and the spin label cholestane in aligned DPPC membranes provide a demonstration of the technique. We also discuss some virtues of high field/high frequency ESR on aligned membranes compared to X-band.

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Keywords: High field/high frequency ESR; Aligned membranes; Gramicidin A

1. Introduction

Multifrequency spin-label ESR is a valuable and well-proven approach to study the structure and molecular dynamics of biological membranes and membrane proteins [1]. Recording and analyzing ESR spectra of the same system over a range of ESR frequencies can effectively separate different modes of molecular motion, such as the overall tumbling of the entire molecule vs. the relative motion of its parts or the specific mobility of nitroxide tethers, and can facilitate unambiguous assignments of ordering and dynamical parameters via spectral analysis.

Utilization of well-aligned membrane samples not only dramatically improves ESR spectral resolution, but also provides particularly valuable information on the orientation of the nitroxide moiety relative to the membrane normal. Such information, particularly in the slow-tumbling regime, is difficult to extract from spectra obtained from vesicles. Because all orientations of the membrane normal relative to the magnetic field are averaged in vesicles, the orientation of the nitroxide moiety manifests itself only as a result of anisotropic molecular motion around the principal axes of the molecular frame. As one approaches the rigid limit, the vesicle spectrum converges to a “powder” spectrum, which is not sensitive to any properties of molecular structure, except for the magnetic tensors of the nitroxide.

The vesicle spectrum is a superposition of spectra corresponding to different orientations of the membrane normal relative to the external magnetic field (MOMD model [2,3]). Ambiguity in model parameters derived from spec-

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tral fitting is not unusual in vesicles. On the other hand, simultaneous fitting of ESR spectra of an aligned membrane sample in different orientations is in general less susceptible to such ambiguity [3,4].

There is a variety of methods to obtain well-aligned membrane samples. For the purpose of ESR spectroscopy, the best alignment on model membranes [4] can be achieved by ISDU or the isopotential spin dry ultracentrifugation method, introduced by Clark et al. [5]. The method is based on a simultaneous application of ultracentrifugation and drying and gives significantly improved membrane alignment compared to that achievable by either method alone. Another important advantage for the method is its ability to keep good membrane alignment in relatively thick membrane layers. Samples of DPPC membrane prepared by ISDU containing various spin labels with subsequent hydration, showed virtually identical ESR spectra in the whole range of membrane thicknesses 100–2000 μm . Thus, unlike other alignment methods, which are appropriate for samples only a few molecular layers thick, ISDU can be used to generate aligned samples consisting of many molecular layers. This feature is of special value for ESR, because one can work with an absolute number of spins large enough to facilitate spectral acquisition without increasing the spin concentration to levels that would cause spectral broadening. In the present work, cutting such thick discs of ISDU aligned membranes at a desired orientation yields flat thin slices, large enough to handle and study by ESR spectroscopy.

At X-band (~ 9.5 GHz) one can get ESR spectra corresponding to different orientations of the membrane normal relative to the magnetic field direction merely by rotating the sample in the resonator. Higher frequencies, however, require very thin (<100 μm) flat samples with B_0 directed perpendicular to the plane of the sample in order to minimize dielectric losses in the resonant structure.

Previously, Barnes and Freed [6,7] designed Fabry–Pérot resonators for a 250 GHz ESR spectrometer to accommodate a thin sample that must rest with its flat surface perpendicular to the optical axis of the incident FIR beam. This geometry makes it possible to obtain high field/high frequency (HFHF) ESR spectra of macroscopically ordered samples at director tilts of 0° and 90° . However, this elegant solution is difficult to implement for the most common types of HF ESR spectrometers based on conventional resonators [8].

To obtain high field spectra in an orientation different from 0° (where the membrane normal corresponds to the sample normal), we apply a novel microtome technique to ISDU aligned samples. This simple technique allows samples to be prepared at any director tilt value, does not need special instrumentation, and can be used on ESR spectrometers at any frequency. The microtome, a mechanical device for cutting thin slices to be examined under microscope, has a long history in biomedical applications [9]. We have found that cryosection on fully hydrated membranes, where water-rich tissues are hardened by freez-

ing and cut frozen, is the most suitable and convenient for our purposes.

2. Materials and methods

2.1. Materials

DPPC was obtained from Avanti polar Lipids (Birmingham, AL), CSL (3β -DOXYL- 5α -cholestane) was bought from Aldrich, GAsl (spin labeled gramicidin A) was synthesized as described in [10].

2.2. Preparation of aligned membranes

ISDU—aligned lipid membranes were prepared as described in [4]. The technique utilizes sedimentation of the membrane fragments (in the gel phase) with simultaneous evaporation of the water phase in a vacuum ultracentrifuge. Measured amounts of DPPC and spin labeled compounds in a molar ratio 200:1 were dissolved in chloroform/methanol 3:1 v/v. The solvent was evaporated by nitrogen gas flow. To ensure complete removal of the solvent the sample was evacuated for 10–12 h. One milliliter of deionized water per 5 mg of lipid was added for the preparation of thin aligned samples which were studied at the 0° orientation. A higher concentration of 30 mg lipid in 1 ml water was used to produce thicker aligned samples which were later cut via the microtome technique. The mixture was sonicated 12 min above the chain melting temperature at a frequency of 20 kHz, and an incident power of 20 W/cm². For the ISDU procedure, a Beckman L8M-70 centrifuge with standard SW27 rotors at 15,000 rpm was used. The procedure took 24 h for DPPC at 20 $^\circ\text{C}$. After ultracentrifugation, the alignment of the dry membranes was checked by a polarizing microscope (Nikon, Instrument Division, Garden City, NJ). Only those samples that exhibited the distinctive interference pattern indicative of an aligned membrane were used in the ESR experiments. The samples were initially kept at 100% relative humidity for 24 h and then transferred into water for 12 more hours. Typically, 25 mg of the dry lipid mixture per centrifuge bucket (volume 0.9 ml) yield a final ~ 300 μm thick hydrated aligned sample with area ~ 1.1 cm².

2.3. Microtome procedure

Thin cuts (~ 80 μm) of aligned membranes were made on a cryostat microtome (International Equipment, Model CTD), with an American Optical steel microtome knife. Initially, a lump of ice was frozen on the microtome cold plate (-15 $^\circ\text{C}$) and shaped in the form of a rectangle (perpendicular cut) or a wedge, with one side resting on the cold plate and another side forming a smooth plane. The wedge angle corresponds to the desired director tilt angle. A flat piece of hydrated aligned membrane was put on the vertical or inclined plane and embedded in ice in this fixed position by dripping water onto the sample mounted

on the wedge at -15°C . After the cutting procedure, 3–4 slices of the membrane were put with a small amount of water into the space ($\sim 100\text{ }\mu\text{m}$ clearance) between two etched round quartz slips ($100\text{ }\mu\text{m}$ thickness, 12 mm diameter, diameter of the etched area 10 mm) and hermetically sealed there with vacuum grease. The ESR spectrum of the sample was then measured on the HF spectrometer. A brief description of the technique has been given in [11].

2.4. X-band spectroscopy

ESR spectra were recorded on a Bruker EMX spectrometer at a frequency of 9.55 GHz under standard conditions. The field sweeps were calibrated with a Bruker ER 035 gaussmeter. The microwave frequency was monitored with a frequency counter. Dry nitrogen gas flow under the control of a Varian temperature controller was used to stabilize the temperature.

2.5. High field spectroscopy

The spectrometer we used to collect spectra at 170 GHz (6 T) was designed and built at ACERT at Cornell University. A brief description of the spectrometer has been recently published [11]. The data collection routine is based on LabView virtual instrument drivers written at ACERT. It provides options for phase correction, signal averaging, control of field sweeps and field modulation control. A Lakeshore 340 temperature controller was used to stabilize the temperature.

2.6. Spectral analysis

Nonlinear least-squares (NLLS) analyses of the CW-ESR spectra based on the stochastic Liouville equation [12,13] were performed using the fitting program by Budil et al. [3]. The magnetic A -tensor and g -tensor components needed for simulations were obtained from fitting rigid limit spectra obtained in vesicles at 77 K . The value of the A_{zz} component was taken as half of the outer splitting at the rigid limit spectrum at 9 GHz . Initial values for ($A_{xx} + A_{yy}$) were then estimated from the isotropic splittings $a_{\text{iso}} = \frac{1}{3}(A_{xx} + A_{yy} + A_{zz})$ at or above room temperature in low-viscosity solvents, e.g., ethanol/water, which at 77 K attested A_{zz} values close to the value observed in the membrane under study. The g -tensor values were determined from fitting rigid limit spectra at 170 GHz . For GAsl in the L_{β} phase of DPPC we obtained the following values of the A - and g -tensors: $A_{xx}, A_{yy}, A_{zz} = 5.5, 5.5, 35.4$, and $g_{xx}, g_{yy}, g_{zz} = 2.0090, 2.0061, 2.0022$. For the P_{β} phase of DPPC we inferred the following values for the hyperfine tensor: $A_{xx}, A_{yy}, A_{zz} = 5.3, 5.3, 34.0$, where the A_{zz} value is determined from the X-band spectrum for a sample quenched from 39 to $\sim -140^{\circ}\text{C}$ (the freezing point of pentane) thereby trapping the metastable conformation. The corresponding g -tensor components are assumed to be $g_{xx}, g_{yy}, g_{zz} = 2.0092, 2.0061, 2.0022$ as determined from

measurements on DMPC, where GAsl attests a similar polarity for the local environment [10].

In the P_{β} phase, we see an increase in the rate of molecular motion. At X-band the separation between outer spectral extrema decreases, but the spectra still retain some features of the rigid limit spectra. These spectra can be approximately simulated with the fast internal motion model (FIM) [14,15], where the internal dynamics of the C-terminus leads to partial averaging of the magnetic tensors, and the global motion is described in terms of a diffusion tensor.

The least-squares fits for the aligned samples were performed simultaneously for the $0^{\circ}, 45^{\circ}$, and 90° orientations using the same set of parameters [3].

3. Results and discussion

A schematic, graphic representation of the microtome cuts of aligned membrane samples at 90° and 45° is given in Fig. 1. A microscope view of a 90° cut under polarized light is shown in Fig. 2. The foliated structure is apparently caused by slight nonuniformities in the layer-by-layer formation of the sample during the ISDU procedure. The visible lines are parallel to the sample surface and are perpendicular to the membrane normal. As seen in Fig. 3, from the X-band spectra (spin-labeled GA in DPPC, $0.5\text{ mol } \%$), the microtome cuts at 90° and 45° match well with corresponding spectra obtained by rotating a thin (" 0° angle") ISDU aligned sample in the resonator. Residual differences at the 45° orientation are most likely due to a small deviation of the cut angle from 45° as well as the much stronger dependence of the spectrum on the precise director tilt angle in this angular range compared to the 0° and 90° orientations, which correspond to turning points in the angular variation. As a future development of the method we plan high-precision angular positioning of the membrane in the microtome. Note also a



Fig. 1. A schematic representation of 90° and 45° microtome cuts of an ISDU aligned DPPC membrane.

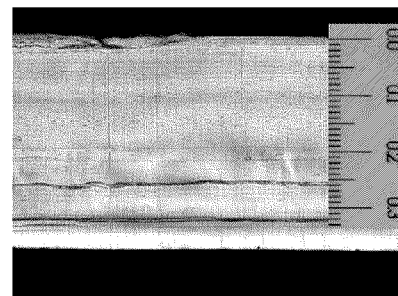


Fig. 2. An $80\text{ }\mu\text{m}$ perpendicular cut made of a $300\text{ }\mu\text{m}$ thick layer of ISDU aligned membrane in polarized light.

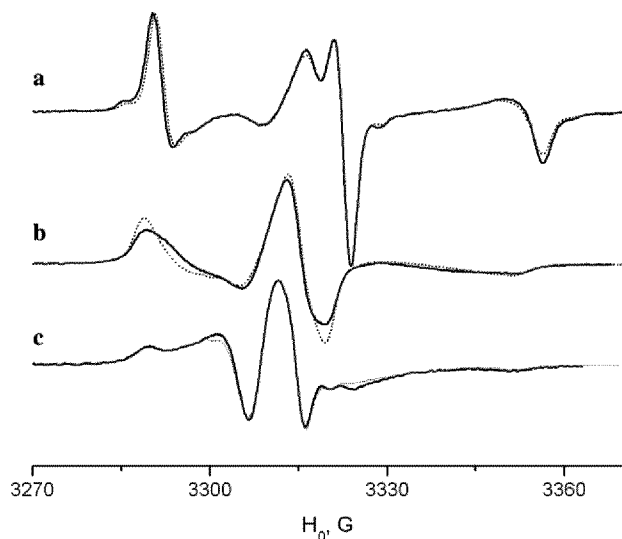


Fig. 3. X-band spectra of GAsl in microtome cuts of an aligned DPPC membrane (solid) with equivalent orientations of the membrane director relative to the magnetic field obtained by rotation of an uncut flat ISDU membrane sample in the resonator (dots). (a) “90° cut” positioned parallel to the B_0 vector vs. uncut membrane positioned perpendicular to B_0 . (b) “45° cut” positioned perpendicular to the B_0 vector vs. uncut membrane positioned at a 45° tilt to B_0 . (c) “90° cut” positioned perpendicular to the B_0 vector vs. uncut membrane positioned parallel to B_0 . The molar ratio GAsl/DPPC=0.6%. $T = 20^\circ\text{C}$.

good “0° orientation” spectrum obtained by positioning a 90° cut sample parallel to the magnetic field. These examples provide compelling evidence of the excellent alignment achievable in the thick ISDU samples prepared for the microtome procedure and that the alignment is not destroyed by either the cutting or the accompanying freezing and thawing imposed by the microtome technique. Furthermore, the cut sections of DPPC membrane in excess water showed surprising stability. They retained their alignment unchanged, as detected by ESR, for at least 24 h at room temperature, throughout multiple freezing/thawing cycles and ~ 1 h exposure at 37°C (above the L_β - P_β pretransition point of 35°C), enough to record a HFHF ESR spectrum at that temperature. Longer exposure in the P_β phase, however, eventually degrades the ordering. Handling microtome slices of DMPC membranes is also feasible, although the stability of the sample is not as high as in DPPC.

Spectra (170 GHz) of spin labeled gramicidin A in the DPPC membrane, a system that was previously studied at 9 GHz and analyzed in detail [10], are shown in Fig. 4. Fig. 5 gives another example of the microtome technique for cholestane spin label (CSL) in DPPC at 170 GHz.

Several inferences can be drawn from a comparison of the ESR spectra of the system at 9 and 170 GHz. At 170 GHz, the superior orientational selectivity provided by the rhombic g -tensor dominates the nearly axial A -tensor splitting, and there are three well-resolved spectral regions corresponding to g_{xx} , g_{yy} , and g_{zz} . In aligned membranes this affords a conceptually simple visualization of

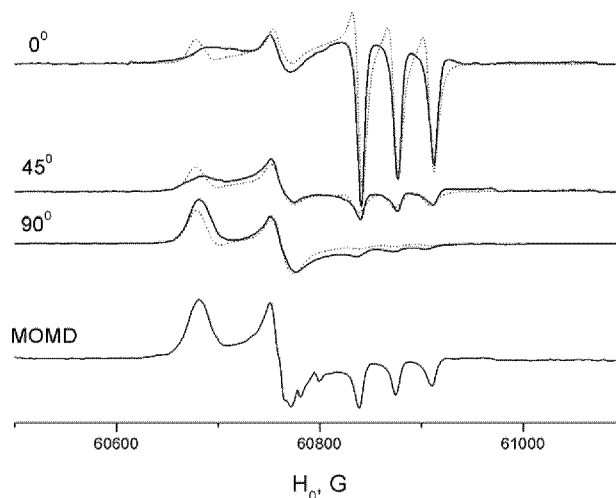


Fig. 4. ESR (170 GHz) spectra of GAsl (0.6% mol) in aligned DPPC membrane and vesicles at 16°C . The angular dependent spectra are obtained using microtome cuts. The spectrum in vesicles contains a small admixture of free spin label. Dotted lines show simulations. Simulation parameters (see [13]) are: $g_{xx} = 2.0090$, $g_{yy} = 2.0061$, $g_{zz} = 2.0022$, $A_{xx} = 5.5$, $A_{yy} = 5.5$, $A_{zz} = 35.4$, axial symmetric rotational diffusion, $R_\perp = 8 \times 10^6 \text{ s}^{-1}$ (which corresponds approximately to the rigid limit for this ESR frequency), $R_\parallel = 2.5 \times 10^7 \text{ s}^{-1}$, the order parameter S is 0.41.

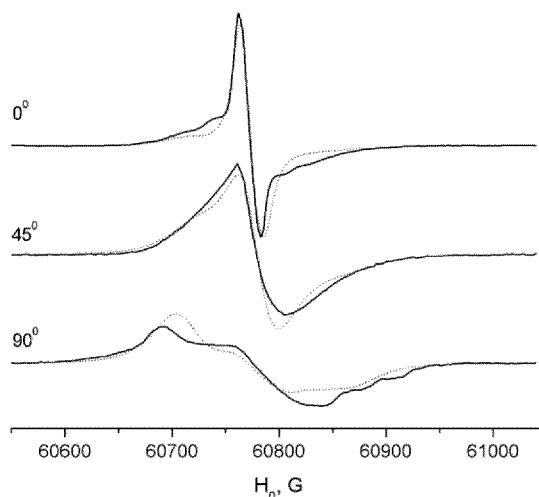


Fig. 5. ESR (170 GHz) spectra of 0.4% mol. CSL in aligned DPPC membrane at 22°C . The spectra are obtained using microtome cuts. Dotted lines show simulations for a simplified model of ideal Y -ordering and axial symmetric diffusion. Simulation parameters are: $g_{xx} = 2.0082$, $g_{yy} = 2.0053$, $g_{zz} = 2.0018$, $A_{xx} = 5.6$, $A_{yy} = 5.6$, $A_{zz} = 34.1$ [18] $R_\perp = 5.6 \times 10^7 \text{ s}^{-1}$, $R_\parallel = 1.8 \times 10^8 \text{ s}^{-1}$, the order parameter S for the Y -orientation is 0.36.

the orientation of the magnetic axis of the nitroxide moiety relative to the membrane normal and substantially simplifies the spectral analysis.

For example, Z -ordering for GAsl and Y -ordering for CSL in DPPC are very clear at 170 GHz just by inspection (Figs. 4 and 5).

At 35°C in DPPC, at the L_β - P_β transition point, an abrupt change in the GAsl spectrum occurs (Figs. 6 and 7). The observation that this change is very pronounced

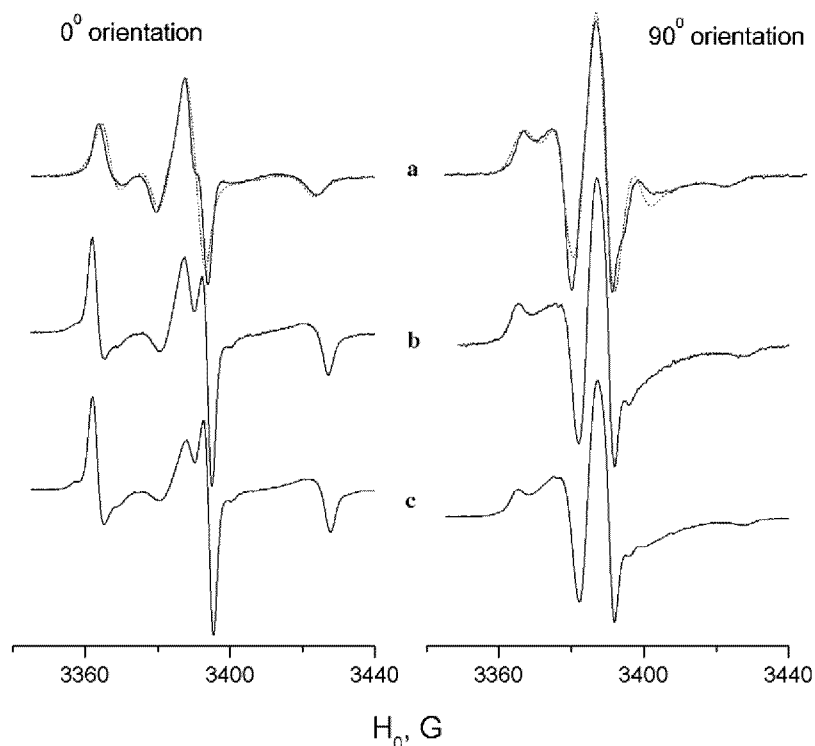


Fig. 6. Channel formation and dissociation, as concluded in [10], of the gramicidin channel in the DPPC membrane (GAsI/DPPC = 0.6%) detected by 9 GHz ESR for two orientations of the membrane normal relative to H_0 . (a) 36 °C, dotted line shows simulation using FIM model (see text and [10]); (b) at 16 °C, 20 min after cooling from 36 °C; (c) at 16 °C, 24 h after cooling from 36 °C.

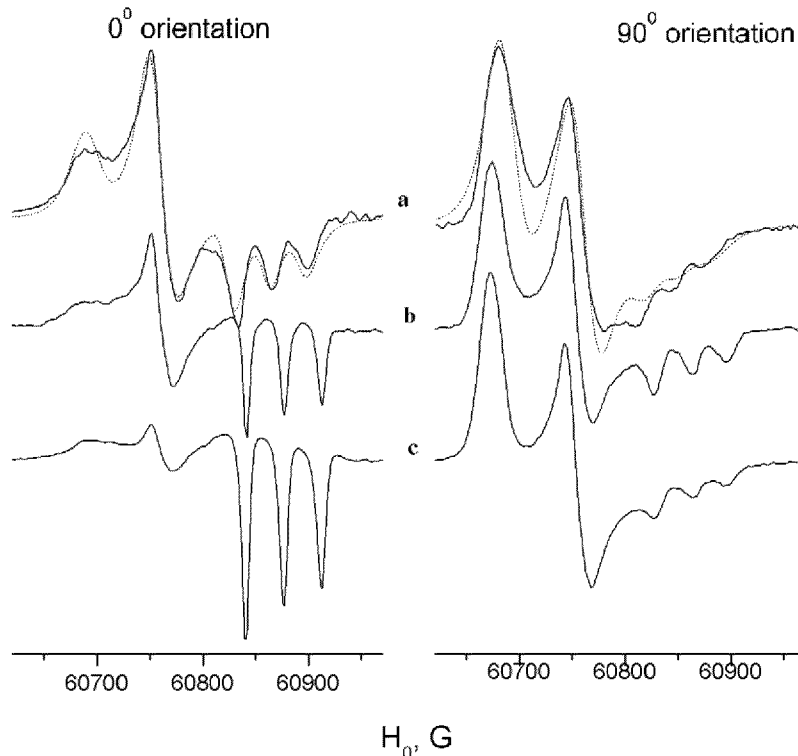


Fig. 7. Channel formation and dissociation of the gramicidin channel in the DPPC membrane (GAsI/DPPC = 0.6%) detected by 170 GHz ESR for two orientations of the membrane normal relative to H_0 . (a) 36 °C; (b) at 16 °C, 20 min after cooling from 36 °C; (c) at 16 °C, 24 h after exposure at 36 °C. Simulation parameters for (a) (dotted line) are: $g_{xx} = 2.0092$, $g_{yy} = 2.0061$, $g_{zz} = 2.0022$, $A_{xx} = 5.3$, $A_{yy} = 5.3$, $A_{zz} = 34.0$, axial symmetric rotational diffusion, $R_{\perp} = 2.5 \times 10^7 \text{ s}^{-1}$, $R_{\parallel} = 5.8 \times 10^7 \text{ s}^{-1}$, the most probable orientation of the long molecular axis relative to the membrane normal forms a cone. The cone angle $\varphi = 33^\circ$ is calculated as described in [10] from potential parameters $C_{20} = 0.8$ and $C_{40} = -0.5$ obtained from simulations [3]. The diffusion tilt angle (see [13]) is taken to be $\beta = 20^\circ$.

in aligned membrane samples and less obvious in vesicles hints at a change in the orientation of the nitroxide moiety. The transition at 35–36 °C has substantial hysteresis. After cooling down from 36 to 22 °C it sometimes takes hours until the spectrum regains its initial shape prior to heating above the L_β – P_β transition. It has been shown [10] that in DPPC in the P_β phase, GAsI starts to form channels. The channel formation manifests itself as a disruption of Z-ordering due to the tilt of the nitroxide moiety. The conclusion, which could be inferred from the 9 GHz spectra only after extensive spectral simulations [10], is very clear at 170 GHz even by inspection (Fig. 7). One can see the spectral intensity shifting from the Z-region to the XY-region and back upon performing a cooling/heating cycle. We attribute the considerable hysteresis time in the cycle to a slow dissociation of the channel form in the L_β phase. As seen from the Z-region in both 0° and 90° orientations (Fig. 7), the spectral intensity shifts towards ideal Z-ordering while the system is recovering from the hysteresis state.

As mentioned above, the orientation of the spin label relative to the membrane normal can only be reliably determined from simulations of the ESR spectra, at least in the slow motional regime, using aligned membranes. As we have shown in the case of gramicidin [10], one can form conclusions on the conformation and function of the membrane-embedded spin labeled peptides from changes in the orientation of the spin labeled moiety as experimental conditions are varied. However, at biologically relevant temperatures, simulation of the ESR spectra and extraction of physical parameters may be complicated due to the variety of motions existing in a spin labeled protein system. The motions that affect the ESR line shape of a spin labeled peptide include (i) the overall tumbling of the whole molecule, (ii) specific motions in the peptide side chains, and (iii) the particular reorientation of the nitroxide moiety and its tethers [14]. Motions due to the internal dynamics are fast, but global tumbling of the macromolecule occurs on a slower time scale. Generally speaking, the simulation of ESR spectra resulting from such a superposition of complex motional modes requires the so-called slowly relaxing local structure model (SRLS) [14] and a large-scale computational resource. However, there are two simple limiting cases of the SRLS model, which are very common and allow one to analyze ESR spectra using standard, convenient and efficient software [13,3]: the fast internal motion (FIM) model, wherein the internal motion is considered to be so rapid that one observes partial averaging of magnetic tensors [17], and a model which neglects the global tumbling of the macromolecule as if it were in the rigid limit. The latter model for macroscopically disordered systems (e.g., vesicles) is known as MOMD. The derivation of these limiting cases from the SRLS model is discussed elsewhere [14]. The overall tumbling in the FIM case and internal motion, for MOMD, are parameterized in terms of diffusion constants. It is shown that, in general [14], the MOMD model is a better approximation for ESR spectra obtained

at high frequency, whereas, the FIM model is a better approximation for low frequency (9 GHz) ESR spectra. For macroscopically ordered systems, the practical outcome is that X-band spectra often require heuristic adjustments of the magnetic tensor parameters, justified by an appeal to the FIM model [17], but the corresponding HFHF spectrum will be well described by a fixed set of magnetic tensor parameters and a derived set of ordering and diffusion constants from the MOMD model. We used the FIM model, for example, to fit the X-band ESR spectrum of GAsI in the P_β phase (36 °C). To obtain the correct field positions of all spectral features we had to change the g_{xx} , g_{yy} , and g_{zz} values from the initial 2.0092, 2.0061, 2.0022 to 2.0082, 2.0061, 2.0032 and the corresponding A -tensor components to 6.7, 6.7, 31.4 from 5.3, 5.3, 34.0, in a way that keeps the trace of both tensors constant. By comparison, simulations of the same system at 170 GHz give the best results with the initial values of the tensors. At both frequencies the general conclusion about the disruption of Z-ordering and formation of a different conformation, which is characterized by a tilted position of the whole molecule relative to the membrane normal is similar (see caption to Fig. 7). However, the values of diffusion tensors inferred from the spectra are different, $R_\perp = 2.5 \times 10^7$ at 170 GHz vs. $8 \times 10^6 \text{ s}^{-1}$ at X-band [10] and $R_\parallel = 5.8 \times 10^7$ at 170 GHz vs. $4 \times 10^7 \text{ s}^{-1}$ at X-band [10] (see below about the precision in determination of $R_\parallel$ at different frequencies). We have observed similar differences in the values of diffusion tensor components determined from spectra at different frequencies in a variety of systems. The resolution of such differences requires a simultaneous fit of high and low frequency spectra to the SRLS model [16].

At 9 GHz, due to the relevant time scales in complex systems, one must often include more fitting parameters in order to accurately incorporate the various dynamical contributions to the spectral lineshape. Given that ordering and diffusion parameters can be strongly correlated at 9 GHz due in part to the spectral dominance by the (nearly) axial hyperfine tensor, there is less certainty in the determination of diffusion coefficients and tilts at the labeling position. Comparative studies using HFHF ESR, with its superior orientational resolution and enhanced sensitivity to internal dynamical modes in membrane systems, provides in many cases more reliable information on a smaller set of relevant parameters than that accessible to X-band alone.

In this connection we wish to emphasize two points which are relevant for extracting motional parameters for ESR spectra at different frequencies:

- (1) Since higher frequency spectra give a “faster snapshot” [1], the threshold where molecular motion becomes discernable from the rigid limit in the spectrum corresponds to faster tumbling rates at higher frequencies. For example, at 170 GHz the spectrum practically converges to the rigid limit spectrum at tumbling rates below 10^7 s^{-1} .

(2) X-band ESR, because of its low g -factor resolution and the nearly axial symmetry of the A -tensors of nitroxide spin labels, shows extremely low sensitivity to axial rotation about the direction of the magnetic Z -axis. For the common case in membrane ESR (PC labels, etc.), where the magnetic Z -axis is directed along the long molecular axis, reliable extraction of $R_{\parallel}$ from X-band spectra is a challenging proposition. On the other hand, high field spectra are very sensitive to this motional mode, which causes the otherwise well-resolved X - and Y -regions of the spectrum to become partially averaged in the 0° orientation. For such systems, analysis of the 90° orientation (see Fig. 4), provides very high sensitivity to the details of the parallel rotational diffusion mode.

4. Conclusions

A novel, easy to use method allowed us to record HFHF spectra in aligned membranes corresponding to different orientations of the membrane normal relative to the direction of the main magnetic field. ISDU aligned DPPC membranes were cut with a microtome in thin slices at different angles. The cuts retain their microscopic order throughout the sample preparation process and faithfully reproduce the equivalent orientation of the untreated membrane in the magnetic field. This method, as illustrated by spin labeled gramicidin A in DPPC, allowed us to reliably detect the orientation of the nitroxide moiety relative to the membrane normal and its temperature dependence, indicative of a conformational transition in the membrane-embedded molecule. Spectra from CSL in DPPC with its well-known ordering behavior provided a useful check on the reliability of the method.

Compared to the difficulties of identifying X - or Y -ordering symmetries at X-band, which must be inferred from a careful analysis of the congested central region of nitroxide spectra, where contributions from g_x , g_y , and g_z are overlapped, the general type of ordering (X -, Y - or Z -) is very clear even by casual inspection of the spectra at 170 GHz. Spectral simulations of oriented high frequency spectra thus help to clarify details of complex motions that are obscured by the lower resolution obtainable from lower frequency ESR and/or microscopically disordered samples. We discuss the implications of these effects elsewhere [11,14].

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