Published in final edited form as:
J Phys Chem B. 2011 September 8; 115(35): 10462–10469. doi:10.1021/jp2016243.

2D-ELDOR Study of Heterogeneity and Domain Structure Changes in Plasma Membrane Vesicles upon Crosslinking of Receptors

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Abstract

2D electron-electron double resonance (2D-ELDOR) with the "full Sc-" method of analysis is applied to the study of plasma membrane vesicles. Membrane structural changes upon antigen crosslinking of IgE receptors (IgE-FcεRI) in plasma membrane vesicles (PMV) isolated from RBL-2H3 mast cells are investigated, for the first time, by means of these 2D-ELDOR techniques. Spectra of 1-palmitoyl-2-(16-doxyl stearoyl) phosphatidylcholine (16-PC) from PMV's before and after this stimulation at several temperatures are reported. The results demonstrate a coexistence of liquid-ordered (L_o) and liquid-disordered (L_d) components. We find that upon crosslinking, the membrane environment is remodeled to become more disordered, as shown by a moderate increase in the population of the L_d component. This change in the relative amount of the L_o versus L_d components upon crosslinking is consistent with a model wherein the IgE receptors, which when clustered by antigen to cause cell stimulation, lead to more disordered lipids, and their dynamic and structural properties are slightly altered. This study demonstrates that 2D-ELDOR, analyzed by the full Sc- method, is a powerful approach for capturing the molecular dynamics in biological membranes. This is a particular case showing how 2D-ELDOR can be applied to study physical processes in complex systems that yield subtle changes.

Keywords

2D-ELDOR; ESR; Phase coexistence; Plasma membrane; IgE crosslinking; liquid-ordered phase; liquid-disordered phase

INTRODUCTION

Over 16,000 missense mutations, which lead to single amino acid changes in protein sequences, have been identified and linked to human disease. Most of them occur in integral membrane proteins which have dynamic interactions with lipid molecules to form various microdomains on the surface of a cell[1]. In recent years the participation of plasma

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SUPPORTING INFORMATION

Extensive examples of detailed fittings, performed using the full Sc- method, are given in the Supplementary Materials (Figs. S1 - S6). This Supporting Information is available free of charge via the Internet at http://pubs.acs.org.

membrane lipids in regulating protein interactions and targeting early signaling events has become increasingly evident. The concept of “lipid rafts” emerged to explain a variety of experimental data that pointed to the possibility that phase-like behavior of membrane lipids provide a means for selective compartmentalization in cellular processes. A simplistic view of this concept is that the fluid plasma membrane has distinctive regions characterized by disordered lipids with mostly unsaturated acyl chains (L_d-like) *vs* ordered lipids with mostly saturated acyl chains, and enriched in sphingomyelin, and cholesterol (L_o-like) regions. Plasma membrane rafts correspond to the L_o-like regions that co-exist with L_d-like regions, and available evidence supports the view that these domains are small (10-200 nm), heterogeneous, and highly dynamic. In a functionally relevant manner, small L_o-like domains are sometimes stabilized as larger compartments through stimulated protein-protein and protein-lipid interactions.[2]

There is abundant evidence that domains exist/coexist in membranes and that small changes in lipid/protein composition can have dramatic effects on several signal transduction pathways.[2-5] A prominent example of a cell surface receptor that interacts differentially with L_o-like domains to initiate a transmembrane signal is FcεRI, the high affinity receptor for IgE on mast cells, which plays a central role in the allergic immune response. Antigen crosslinking of IgE-FcεRI causes its coupling with Lyn kinase and resulting phosphorylation within a stabilized L_o-like membrane environment to initiate assembly of the signaling complexes. Although considerable biochemical data support this view, and these are further corroborated with fluorescence measurements, knowledge of molecular dynamic properties of the domains before and after cell stimulation remains inadequate. Overall, the basic concept of co-existing domains in plasma membranes remains murky. Moreover, the nature and composition of these membrane structures very likely change with initiating and progressing events that accompany cell activation. Thus, although there is broad recognition of the importance of L_o-like membrane compartments in cell function, progress is currently limited by lack of adequate tools for their dynamic characterization.

Physical methods that have been found useful in investigating domains in membranes include fluorescence techniques (e.g. fluorescence resonance energy transfer (FRET)[6-11], fluorescence anisotropy[4,12], and fluorescence microscopy), and electron spin resonance (ESR). FRET and fluorescence anisotropy provides information about membrane heterogeneity and lipid order but much less about molecular dynamics of lipids than can be obtained ESR with spectral simulation[13-17]. Confocal, multi-photon and super-resolution microscopy[18-20] and single particle tracking techniques[13,21,22] have provided compelling, but still limited, visualization of heterogeneity of cellular membranes.

ESR offers a unique view of lipid dynamics in a complex membrane environment, and considerable effort has been devoted toward a comprehensive evaluation of membrane phase coexistence. In model membranes, continuous wave (cw) ESR can been used to map out the dynamic structure of the dipalmitoyl-sn-glycero-phosphatidylcholine/dialauryl-sn-glycero-phophatydlycholine/cholesterol (DPPC/DLPC/chol) ternary phase diagram[14], wherein liquid-disordered (L_d), liquid-ordered (L_o), and gel phases exist or coexist. Also, a thermodynamic tie-line in the two-phase coexistence region of the ternary lipid mixtures was determined using cw-ESR[15], and this approach has been extended to provide the complete tie-line field[23]. Studies of cellular membranes have also been carried out. Ge et al demonstrated the coexistence of (L_o-like) and (L_d-like) components in plasma membrane vesicles (PMVs[24]) isolated from rat basophilic leukemia (RBL-2H3) mast cells, using cw ESR with several different acyl chain spin-labeled phospholipids and a spin-labeled cholestane (CSL)[16,17]. More recently, Swamy et al[25] provided direct evidence for the co-existance of two different types of lipid populations (i.e., L_o-like vs. L_d-like) in the plasma membranes of live cells from four different cell lines by cw-ESR.

The advanced ESR technique of 2D-ELDOR (two-dimensional electron-electron double resonance) has been used to study the phase structure of membranes and lipid-protein interactions in several studies[26-29]. Earlier work showed that 2D-ELDOR provides unusually clear spectral distinctions between the L_o and L_d phases in model membranes[27], and more recently it has been used to explore the phase properties of a model membrane and even deconvolute the spectra in terms of coexisting L_o and L_d phases using the "full Sc-" format[30] of spectral analysis (Chiang, Costa-Filho and Freed[28] hereafter referred to as CCF). In the present work, we extend this approach to plasma membranes, which are inherently more complex systems than model membranes. In particular, the CCF-based deconvolution of the 2D-ELDOR spectra in the two phase region into the L_o and L_d components in equilibrium was greatly aided by the pre-knowledge of the pure L_o and L_d spectra at the phase boundaries, such that only their linear combination in the two phase region needed to be determined. For PMV no such prior knowledge is available. In cw ESR studies on PMV and live cells, two-components were successfully resolved[16,25], but fitting with the many parameters that are needed is typically accompanied by substantial ambiguity. Thus the challenge addressed in the present study was to show how 2D-ELDOR with "full Sc-" analysis could 1) clearly and unambiguously resolve the two spectral components in biomembranes, and 2) provide accurate dynamics and ordering parameters to characterize these phases. Our driving interest in the present work was to show how 2D-ELDOR can be successfully utilized to study functionally relevant but subtle changes in cell membranes, to which cw-ESR with its poorer resolution is insensitive[16]. We chose for our first biological test case, ligand-mediated activation of cell surface receptors that leads to assembly of transmembrane signaling structures. In particular, we show how the molecular structure and dynamics of membrane domains change from before to after crosslinking IgE receptors on the surface of PMV derived from RBL-2H3 cells using 2D-ELDOR[26-40]. Thereby, we demonstrate how 2D-ELDOR can be applied to the study of subtle physical processes in complex systems.

MATERIALS AND METHODS

Materials and sample preparations

The spin label 1-palmitoyl-2-(16 doxyl stearoyl) phosphatidylcholine (16PC) was purchased from Avanti Polar Lipids (Alabaster, AL). Plasma membrane vesicles (PMV) were isolated from RBL-2H3 mast cells by chemically induced "cell blebbing" according to the procedure described previously[12,16]. This preparative method results in the recovery of 20−25% of the plasma membrane IgE receptors, with little or no contamination of intracellular membranes[24]. For these studies the RBL cells had first been sensitized by binding IgE specific for 2,4 dinitrophenyl(DNP) groups. The PMV preparation was divided into two aliquots (2.5×10^7 cell equivalents/0.5 ml phosphate-buffered saline pH 7.0, PBS). The antigen $DNP_{16}BSA$ (100 ng/ml) was added to the "crosslinked" (C) sample, and the other sample served as the "uncrosslinked" (U) control. After 10 min incubation at room temperature, 25 μl of 0.07 mM spin label in methanol was added to each PMV sample. The labeled PMV were vortexed for 1 min and centrifuged in a Sorvall SL050T rotor at 27,0003 g for 45 min at 4 °C. After decanting of supernatant, the pellet was resuspended in the PBS solution, repelleted, and then transferred to a capillary tube for pulsed ESR measurement at 17 GHz.

2D-ELDOR experiment and the full Sc- method

The 2D-ELDOR experiment is closely analogous to the 2D-exchange experiments of NMR[31-35,40] and is particularly sensitive to the dynamics and ordering of spin labels in complex systems such as membranes[34,36,37]. Examples of 2D-ELDOR spectra from the present study are shown in Fig. 1 as plots with respect to the frequencies f_1 and f_2. They are

for an “Uncrosslinked” PMV sample and one that is “Crosslinked” as discussed in the Results section. Details of this technique have been reported extensively elsewhere. Details of the methodology that are particularly relevant to the present study can be found elsewhere[26-30].

The spectra in this study (including Fig.1), were obtained using the end-chain label 16PC, which was found to be the most favorable acyl-chain label for high-quality 2D-ELDOR spectra[26-30,33,37]. This is because it is more motionally dynamic resulting in much sharper spectral lines (especially compared to labels closer to the headgroup), which translates into much better signal strength, and it provides excellent distinguishability of the different phases. In addition, 16PC partitions nearly equally between the L_o and L_d phases, whereas this is not the case for lipids labeled closer to the headgroup, (e.g., 5PC partitions with a 3:1 ratio favoring the L_d phase).

Three-pulse 2D-ELDOR[40] experiments at 17 GHz were performed (cf. Fig. 2). One collects the FID signals during the time-interval t_2 (after the spectrometer dead time, t_d) for fixed values of the preparation time, t_1 and the mixing time, T_m (cf. Fig. 2A). The FID signal is Fourier transformed in t_2 and then in t_1 to construct a frequency-domain spectrum as a function of frequencies f_1 and f_2 (cf. Fig 2). The FID signal is in a “hypercomplex” format, containing two ordinary complex signals that we call Sc+ and Sc-. We only use the Sc- signal[30], because the Sc+ signal typically decays rapidly within the finite spectrometer dead-time. Rigorous analyses of these two signals in different motional regimes are given elsewhere[34-37].

Although we show the 2D-ELDOR spectra in Figs. 1 and 3 in the magnitude mode for convenience in display, we employed the full Sc- method[30] to analyze them. In this method, the real and imaginary parts of the Sc- spectrum are simultaneously fit, instead of fitting the absolute magnitude of the complex Sc- signal, which is less resolved, and therefore less informative. The Sc- spectrum obtained in experiments is beset with phase distortions, which had previously hindered their use in fitting to theory, whereas the magnitude mode (cf. Fig.1) is not, so it had previously been used at the expense of poorer resolution. The problem of phase distortions due to imperfect pulses, finite spectrometer dead times, and other instrumental effects[38] is solved in the full Sc-method by simultaneously extracting the dynamic parameters and the phase corrections from the spectral analysis. The full Sc- results and their comparison with the theoretical fits are rather complex to display[28,30]. (In fact, it was shown[30] that the rigorous analysis requires that the theoretical spectra be phase-adjusted to correspond to the experiments.) For this reason the complex full Sc- results are shown in the Supplemental Materials. As we have previously found[28,30], the accuracy in fitting the dynamic parameters is significantly enhanced by employing the full Sc- approach. It is important to note that once the phase corrections are obtained they may be used in an approximate (although non-rigorous) fashion to convert the experimental Sc- spectra into an absorption spectrum displayed in the Spin-Echo Correlation Spectroscopy (SECSY) format (cf. Fig. 2b and below). It provides much higher resolution than spectra in the magnitude mode. True pure absorption spectra are, of course, obtainable from the (best-fit) theoretical spectra.

A virtue of displaying the absorption spectra in the SECSY format is that it provides a clear separation of inhomogeneous vs. homogeneous broadenings as discussed by Chiang, et al[28,30].

Spectral simulations were performed using the modified nonlinear least squares fitting program, called NLSPMC-fullscm, which is modified from Budil et al[39] to include the full Sc- fitting. The definitions for the dynamic parameters and the homogeneous linewidths are

given in reference[28]. The spectral simulations for membrane vesicles used in the NLSPMC-fullscm program were obtained with the microscopic order with macroscopic disorder (MOMD) model[41,42].

RESULTS

The spectra of the PMV from RBL-2H3 mast cells in the present study include those from the uncrosslinked (U) vs. crosslinked (C) samples at three temperatures: 15, 20 and 30 °C. Figure 1 shows a comparison between the magnitude 2D-ELDOR spectra for the U and C cases at these temperatures for a common mixing time, T_m = 50 ns. The U and C spectra have been normalized to a common value of the double integral (over f_1 and f_2). Their difference is also shown on the same scale. This comparison demonstrates small but significant changes in the spectrum as a result of crosslinking IgE-FcεRI with antigen on the PMV. Figure 3 shows magnitude spectra at 30 °C as a function of T_m for both U (Fig. 3A) and C (Fig.3B) cases. The spectra for all the T_m are fit simultaneously, so that the spectral changes, which provide additional information, are included. Figs. 3A and B also show the best fit spectra, as well as the residual between experiment and fit. The fits are clearly very good. Also, the residuals are much smaller and of different character than the differences displayed between U and C spectra in Fig. 1. The quality of fits we obtain provides substantial justification for interpreting the observed differences due to crosslinking in Fig. 1 as significant. As stated above, the detailed fitting was performed using the full Sc- method, and extensive examples of this fitting are given in the Supplementary Materials (Figs. S1 - S6). Fig. 3 represents an easily visualized display in the magnitude mode.

Fluorescence techniques can measure structural changes among membrane lipids upon crosslinking membrane proteins[7]. Our ESR spectroscopic results, such as in Fig. 1, not only demonstrate lipid structural changes in the PMV upon crosslinking IgE-FcεRI, but they can provide a more detailed quantitative assessment. That is, the “full Sc-” analysis of the 2D-ELDOR data unequivocally demonstrate that the spectra are the superposition of two distinctly different components, which can be characterized as L_o-like and L_d-like regions in the PMV. The relative amounts of the two and how they change with crosslinking is shown in Fig. 4, and the respective dynamic and ordering parameters for these components are given in Fig. 5. The primary parameters, which are shown in Fig. 5, are the rotational diffusion constant, $R_\perp$, and the order parameter, S_o[16,17,25,39,41,42]. These values for U-PMV are comparable to those previously obtained by cw-ESR[16]. The L_d-like phase is characterized by a small S_o for the 16 PC label and the L_o-like phase by a larger ordering (e.g., 0.10 vs. 0.27 at 30 °C). It is clear from Fig. 4 that the L_o-like phase is the predominant component for both U and C cases. The most important change upon crosslinking IgE-FcεRI on the PMV is the increase of the relative amount of the L_d component. Overall, the ordering and dynamic properties of both the L_o and L_d components do not change very substantially upon crosslinking (cf. Fig. 5): S_o, increases slightly for both components, but the value of $R_\perp$ does decrease substantially for the L_d component and less so for the L_o component.

The fitting and spectral analysis provide the spectral phase corrections, and it is then possible to obtain (approximate) absorption spectra in the SECSY mode. In Fig. 6, examples are shown for the U and C cases at the three temperatures for T_m = 50 ns. The 1D-ESR spectrum is along the f_2 axis and the homogeneous linewidths is along the f_1 axis. Their respective differences are shown, as in Fig. 1, and these again reveal that significant spectral changes occur upon antigen crosslinking of IgE-FcεRI in the PMV. From the best fit theoretical spectra, it is also possible to obtain the component L_o and L_d spectra. We show an example in the pure absorption SECSY mode in Fig. 7 for the U case at 30 °C and T_m = 50 ns. It is clear that the L_o and L_d spectra are distinctly different, as expected[27,28]: the L_o

spectrum with its low ordering is much sharper with narrower linewidths than the L_d spectrum with its greater ordering. These results underscore the capacity of 2D-ELDOR full Sc- analysis to discriminate these two lipid phase components and to assess quantitatively their respective properties[27,28].

DISCUSSION

A biologically significant observation in the present work is that, as a result of antigen crosslinking of the receptor IgE-FcεRI in PMV derived from RBL-2H3 mast cells, the majority L_o component is reduced relative to the L_d component for the spin-label 16PC. Our ESR analysis is the first demonstration of such behavior of the lipid domains in response to receptor activation in plasma membrane preparations. We previously showed for U-PMV that the L_o component is the primary component by utilizing cw-ESR, but this technique was not sensitive enough to distinguish any changes upon receptor crosslinking[16]. The use of the more powerful 2D-ELDOR technique with the improved method of data processing and analysis has enabled us to clearly distinguish the subtle changes occurring. The previous cw-ESR work[16] showed that when the lipids were extracted from the PMV, only the L_o component remained. These results suggested that the L_d component is associated with lipids in the vicinity of the proteins. This interpretation is supported by the more recent study on the plasma membranes of live cells[25]. Substantially more L_d component was observed for the live cells than for the PMV, (although the L_o component remained the major one). This is consistent with the fact that the live cell plasma membranes have additional, cytoskeleton linked, proteins and thereby a higher concentration of proteins; consequently their disordering effect on the lipid structure is enhanced. Thus our new results imply that antigen crosslinking of IgE receptors on the plasma membrane leads to increased disordering of the lipid structure. We suggest that the stimulating event of receptor crosslinking may mediate changes in membrane lipid organization that contribute to transmembrane signal initiation.

A previous study that is consistent with the implications of our 2D-ELDOR results is that of the phospholipid composition of detergent resistant membrane (DRM) vesicles isolated from RBL cells that had been antigen crosslinked (C) or not (U) using mass spectroscopy. Fridriksson et al[43] found a large increase (12-22%) in the abundance of polyunsaturated phospholipids for the DRM from C cells vs. U cells. They proposed that this large increase in the abundance of polyunsaturated phospholipids in DRMs corresponds to substantial lipid remodeling due to crosslinking of IgE-FcεRI, possibly including recruitment of intracellular membrane material into the plasma membrane as a result of stimulation. Furthermore, the increase in polyunsaturated lipids should lead to greater lipid disordering, corresponding to an L_d-like phase. These observations regarding the increased isolation of polyunsaturated phospholipids with DRMs after IgE-FcεRI crosslinking and cell activation were recently been extended in a systematic study carried out by Han et al[44]. Our results on the PMV indicating increased lipid disordering due to receptor crosslinking may be related to the results of these mass spectrometry studies.

In our previous cw-ESR studies we have discussed the fact that the actual relative amounts of L_o-like and L_d-like domains in biological membranes depends also on the partition coefficient of the probe, K_p. We found a K_p of approximately unity for 16 PC in a model three-component membrane system at 22 °C[25]. Thus we estimate that the fractions of the spectral components are approximately those of the L_o-like and L_d-like domains in the PMV. The increase in L_o component with temperature in the U PMV samples, cf. Fig. 4, was not observed with the other acyl chain labels used in previous work[16]. [In that work, the cw-ESR spectra from 16PC did not permit a separation of the L_o and L_d components, but labels further up the acyl chain did]. It is possible that the K_p of 16PC is increasing with

temperature over the range of 15 to 30 °C, which would imply improved solubility in the L_o-like phase as its ordering decreases with increasing temperature. Alternatively, there may be restructuring of the lipids with temperature in the U PMVs such that fewer disordered lipids are required to support the individual IgE receptors, and this temperature effect is significantly reduced upon crosslinking as observed for the C PMVs.

In conclusion, we emphasize the power of 2D-ELDOR spectroscopy in association with the full Sc- method of analysis to uncover subtle details of lipid structure and dynamics in membranes derived from cells. Our examination of effects on membrane lipids of crosslinking IgE-FcεRI in PMV demonstrates how 2D-ELDOR can be applied to study physical processes in complex systems that yield subtle changes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We wish to thank Arun Gidwani for preparing the PMV samples for these experiments, and David Holowka for his encouragement, advice, and critical reading of the manuscript. Work was supported by grants from the National Institutes of Health/National Center for Research Resources (NIH/NCRR) Grant P41RR016292 and National Institute of Biomedical Imaging and Bioengineering (NIH/NIBIB) Grant 2R01EB003150 to J.H.F. and NIH grant AI018306 to B.B. Computations were implemented at the Cornell Center for Materials Research Computing Facility.

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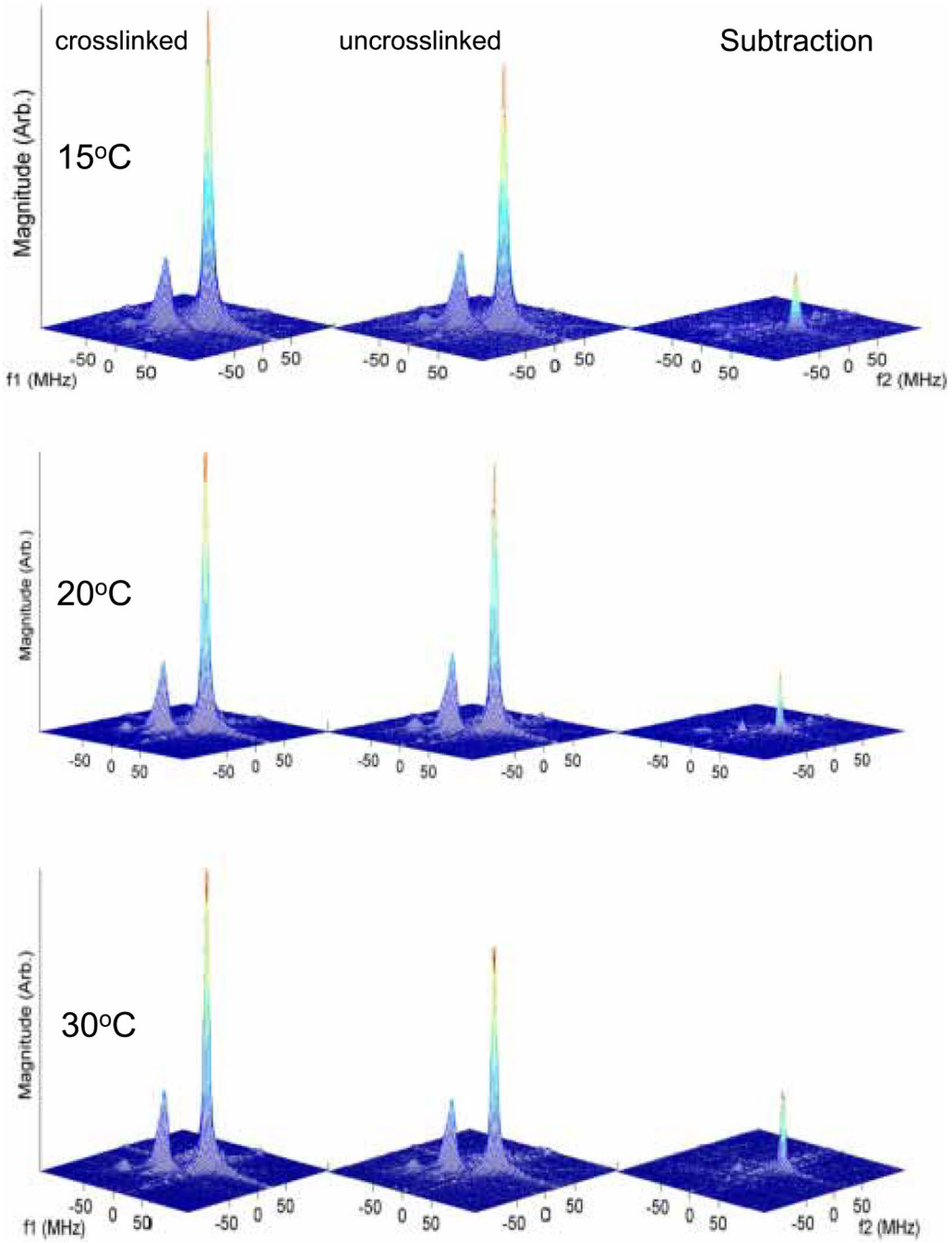

FIGURE 1.

Experimental 2D-ELDOR spectra for 16PC in crosslinked and uncrosslinked PMV and their respective difference spectra at temperatures of 15 °C, 20 °C, and 30 °C, shown in the standard magnitude mode for convenience of display. (The real and imaginary parts of the full Sc- spectra are shown in the Supplementary Materials). Here T_m=50 ns. (The spectra were first normalized to a common double integral value before taking their difference.)

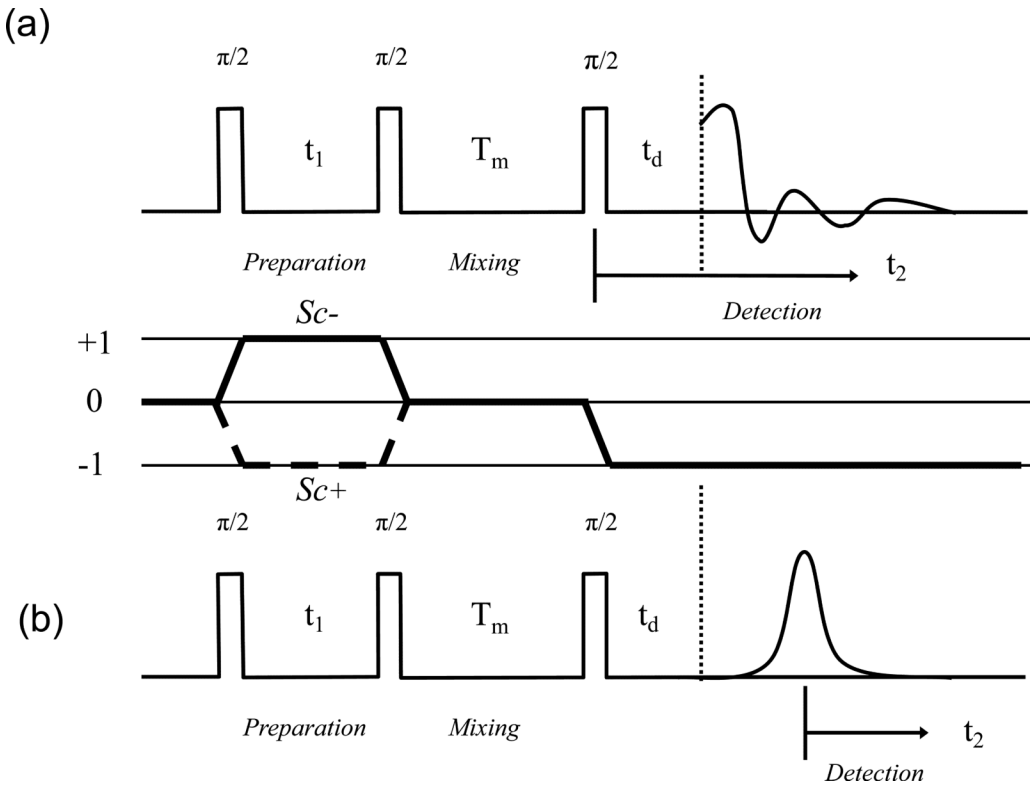

FIGURE 2.
The pulse sequences for (a) the standard 2D-ELDOR experiment and (b) SECSY format 2D-ELDOR experiments.

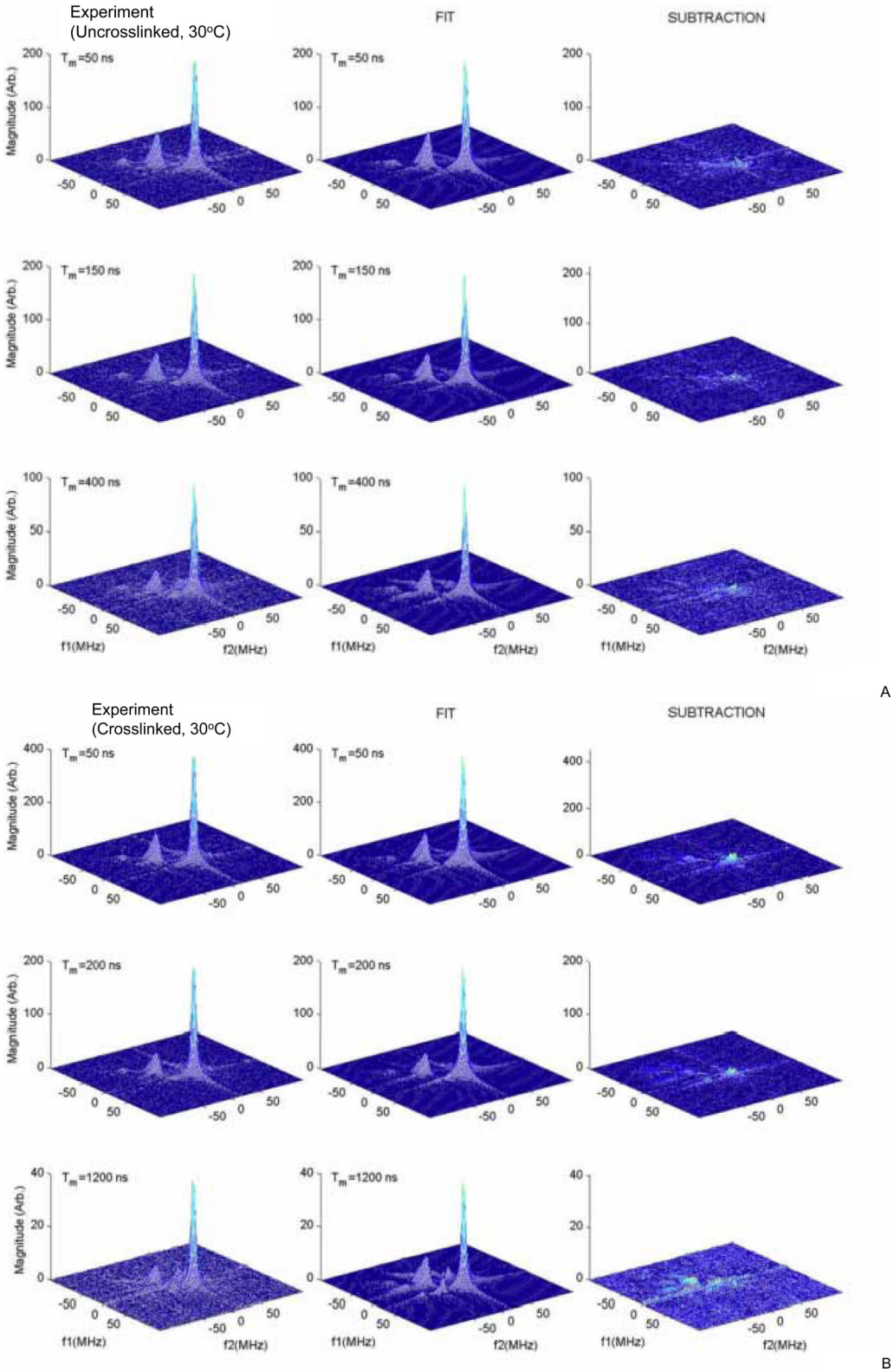

FIGURE 3.

2D-ELDOR spectra from 16PC for A) Uncrosslinked and B) Crosslinked PMV at 30 °C as a function of mixing time, T_m. The experimental spectra are shown in the left column, the best non-linear least squares fits in the middle column, and their difference in the right column. Spectra are shown in the magnitude mode for convenience. The T_m are noted on the Figure.

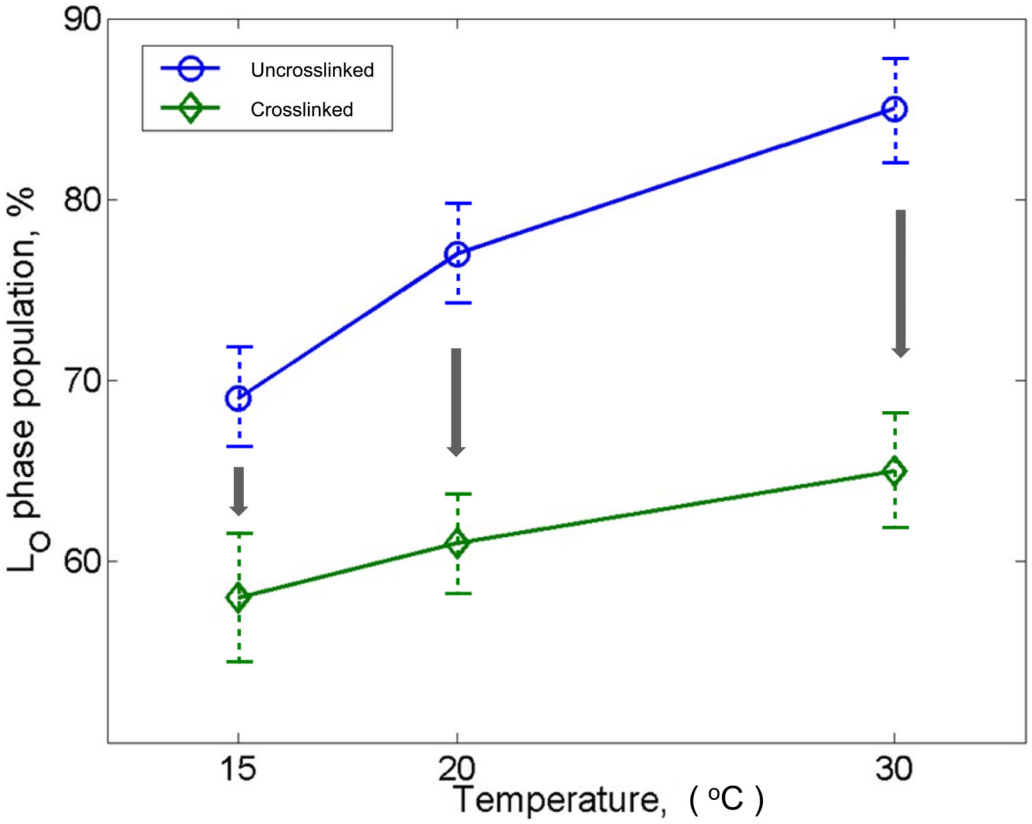

FIGURE 4.
The population of the L_o component, coexisting with the L_d, in the uncrosslinked vs. crosslinked samples with respect to temperature.

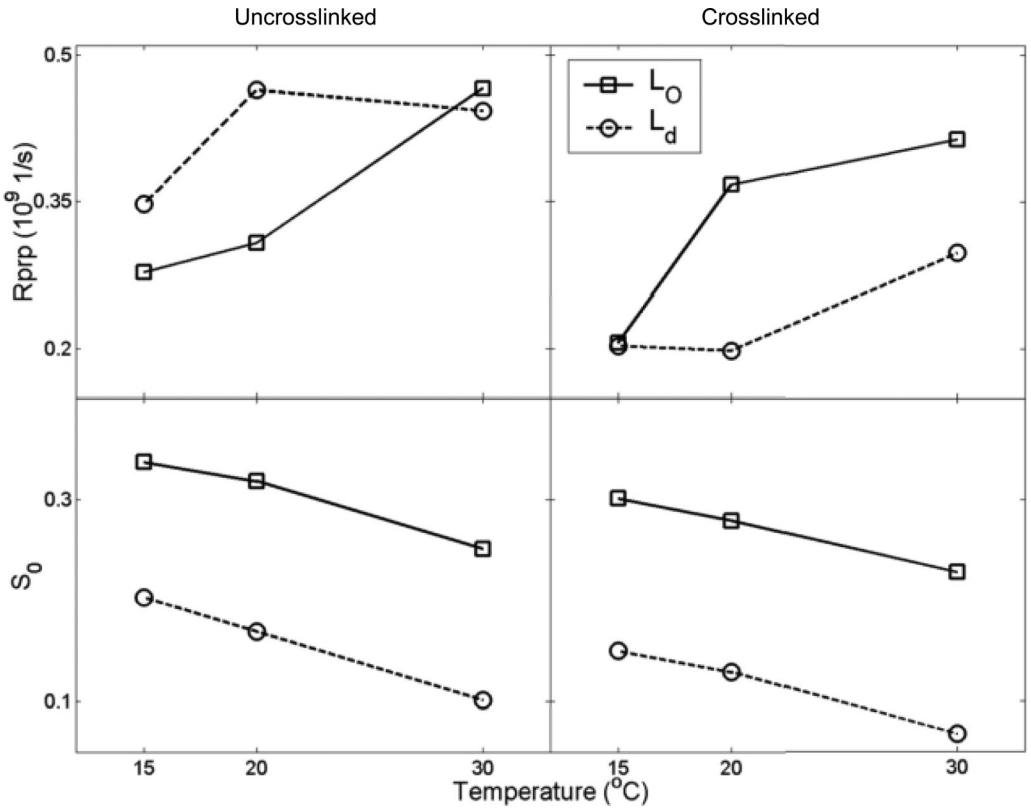

FIGURE 5.
The rotational diffusion rate, $R_\perp$ and the order parameter, S_o vs. temperature for uncrosslinked and crosslinked PMV.

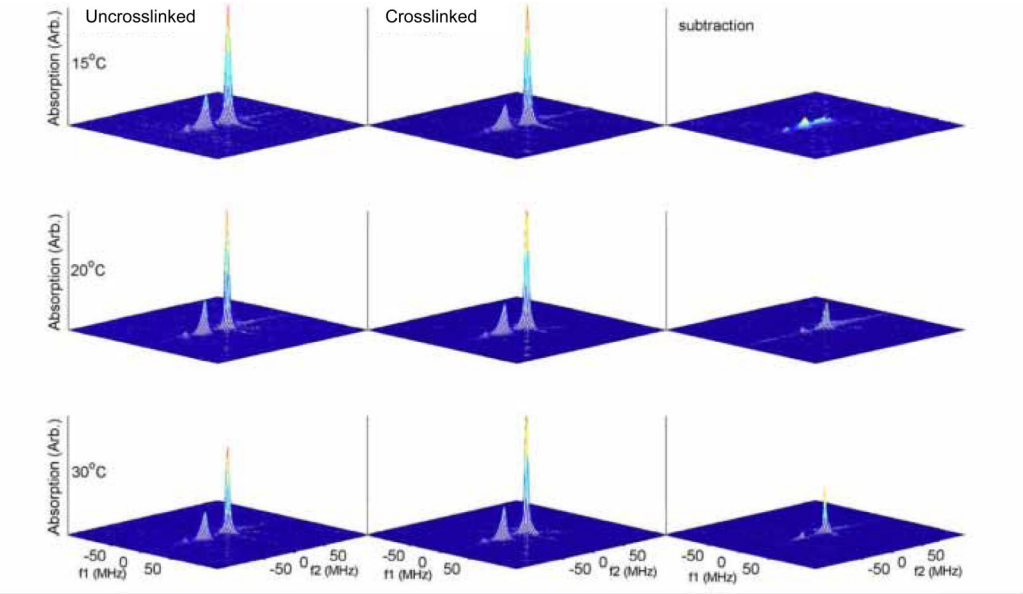

FIGURE 6.

Experimental 2D-ELDOR spectra for 16PC in uncrosslinked (left column), crosslinked (center column), and their respective difference spectra (right column) after converting to the (approximate) absorption in the SECSY format. Results are shown for 15 °C, 20 °C, and 30 °C and T_m= 50 ns.

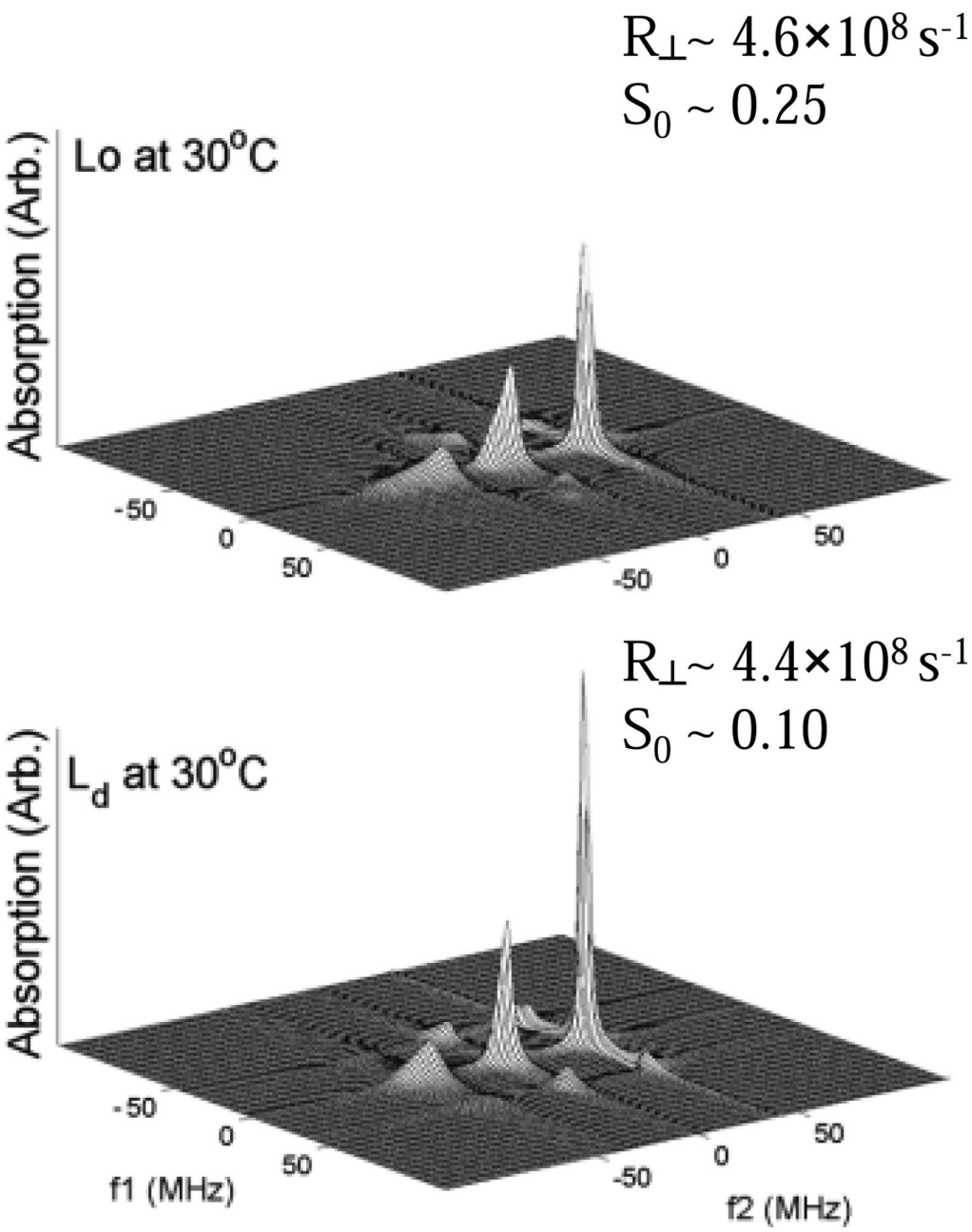

FIGURE 7.

The two spectral 2D-ELDOR pure absorption spectral components (in the SECSY mode) representing the coexisting L_o and L_d regions in the PMV. They were obtained from the best theoretical fit to the experimental spectrum for the uncrosslinked PMV at 30 °C for T_m=50 ns.