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Journal of Magnetic Resonance 175 (2005) 317–329

JMR
Journal of
Magnetic Resonance

www.elsevier.com/locate/jmr

# Application of the out-of-phase absorption mode to separating overlapping EPR signals with different $T_1$ values

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Received 21 December 2004; revised 27 April 2005
Available online 8 June 2005

## Abstract

The use of 90°-out-of-phase first-harmonic absorption ($V_1'$-) EPR to resolve the spectra from nitroxide spin labels with differing $T_1$-relaxation times is described. Non-linear $V_1'$-EPR spectra recorded under moderate saturation have sharper lines compared with the in-phase $V_1$-EPR spectra, and amplitudes that preferentially enhance components with longer $T_1$-relaxation. Discrimination between $V_1'$-spectral components can be increased further by means of selective paramagnetic relaxation enhancement agents. Examples are given of biophysical applications to double labelling in single-component membranes and phase separation in two-component membranes, to lipid–peptide complexes, and to binding of spin-labelled reagents. It is concluded that optimal resolution in $V_1'$-EPR spectroscopy is obtained at relatively low Zeeman modulation frequencies (20–30 kHz) and moderate saturation ($H_1 \sim 0.2$–$0.3$ G).

*Keywords:* Non-linear EPR; First-harmonic 90°-out-of-phase; Spin label; Lateral phase separation; Lipid–peptide interaction

## 1. Introduction

One of the important problems in spin-label EPR spectroscopy of microheterogeneous systems, in particular of lipid membranes, concerns the presence of two (or more) overlapping EPR signals that correspond either to different paramagnetic species or to the localization of the same spin label in different environments. Examples frequently encountered are: paramagnetic ions that have strong background EPR signals at fields close to that for nitroxide spin labels, overlapping EPR spectra of a spin-labelled lipid or of two different lipid spin labels, partition of a spin label between different environments (e.g., aqueous phase and membrane, lipid bilayer and the boundary layer at the protein/lipid interface, or different lipid phases in two- or three-component lipid mixtures), and covalently bound spin labels in different conformational states or binding sites.

Conventionally, analysis of such systems has been performed by difference spectroscopy in the linear EPR regime. For its success, this requires a reasonable degree of resolution of the component spectra and knowledge of the lineshape of at least one of the components. This is, however, not always the case. We suggest that non-linear out-of-phase first-harmonic absorption EPR signals ($V_1'$) may be used to solve this problem, in several cases, and thus to increase the range of multi-component systems that can be analyzed. Earlier, first-harmonic EPR signals detected in phase-quadrature with the Zeeman modulation, under conditions of partial microwave saturation, were used to determine spin–lattice relaxation times [1]. Sensitivity of the $V_1'$-EPR spectra also to molecular motion was investigated by spectral simulations for nitroxide spin labels, over the entire range of rotational correlation times

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doi:10.1016/j.jmr.2005.05.004

[2]. It was shown that determination of the effective spin–lattice relaxation times from $V_1'$-EPR spectra is less dependent on rotational mobility than for other non-linear CW-EPR methods (see also [3]), especially at lower Zeeman modulation frequencies. Experimental measurements on spin labels in the slow, intermediate, and fast motional regimes of molecular rotation were used to test and validate the method. Measurement of the spin–lattice relaxation enhancements of bound spin-labelled fatty acids by using $V_1'$-EPR spectroscopy allowed us to obtain information on the microtopography of the fatty acid binding sites in human serum albumin [4]. The $V_1'$-EPR approach was further generalized to anisotropic rotation of spin labels [5], and the validity of the method was tested by using oxygen-induced relaxation enhancements of spin labels in membranes that were measured simultaneously by this and the progressive CW-saturation method [6].

As regards the problem of spectral separation of overlapping EPR signals, the potential of the $V_1'$-approach is based on two properties of the $V_1'$-EPR signals that will be deduced below: (i) spectral resolution in the $V_1'$-spectra is higher than for the corresponding in-phase signals, and (ii) there is a rather strong dependence of the intensity of the $V_1'$-spectrum on spin–lattice relaxation time [7]. The intensity of one of two overlapping signals will be relatively much higher in the $V_1'$-EPR spectrum than in the conventional $V_1$-EPR spectrum, if $T_1$ of one signal is significantly longer than for the other.

The aim of this paper is to develop the first-harmonic, out-of-phase $V_1'$-approach to resolution enhancement in detail and to investigate its robustness for several spin-labelled systems of biological relevance. In particular, we investigate lateral phase separation in mixed lipid membranes composed of dimyristoyl phosphatidylcholine (DMPC) and distearoyl phosphatidylcholine (DSPC), and lipid–peptide interactions in mixtures of gramicidin A and DMPC. For the case of lateral phase separation, the non-linear relaxation-enhanced spectra reveal a spin-labelled lipid component that is masked in the conventional EPR spectra. For the GA/DMPC system, heterogeneity on the $T_1$-timescale is revealed in the lipid–peptide interactions.

## 2. Theoretical background

We consider here the reasons why first-harmonic out-of phase EPR signals can be useful for separation of overlapping EPR spectra. As in [1] we confine ourselves, for simplicity, to a single-line EPR spectrum in the absence of molecular motion. Expressions for the absorption lineshapes detected at the first-harmonic, both in-phase ($V_{1,1}$) and out-of-phase ($V_{1,1}'$) with respect to the field modulation, and under conditions of partial microwave power saturation, can be obtained by iterative solution of the Bloch equations (see [8,9]):

$$V_{1,1}(H) = -V_{0,0}(\gamma_e H - \omega)T_2 \times \frac{2(\gamma_e H - \omega)^2 T_2^2 + 2A - B\omega_m^2 T_2^2}{[(\gamma_e H - \omega)^2 T_2^2 + A]^2 + B^2\omega_m^2 T_2^2}, \tag{1}$$

$$V_{1,1}'(H) = V_{0,0}(\gamma_e H - \omega)\omega_m T_2^2 \times \frac{(\gamma_e H - \omega)^2 T_2^2 + A + 2B}{[(\gamma_e H - \omega)^2 T_2^2 + A]^2 + B^2\omega_m^2 T_2^2} \tag{2}$$

with

$$A = 1 - \omega_m^2 T_2^2 + \gamma_e^2 H_1^2 T_1 T_2 \frac{1 + \omega_m^2 T_1 T_2}{1 + \omega_m^2 T_1^2} \tag{3}$$

and

$$B = -2 + \gamma_e^2 H_1^2 T_1 \frac{T_1 - T_2}{1 + \omega_m^2 T_1^2}, \tag{4}$$

where $T_1$ and $T_2$ are the longitudinal and transverse relaxation times, respectively, $\omega$ and $\omega_m$ are the angular microwave and field modulation frequencies, respectively, and $H_1$ and $H$ are the microwave and static magnetic field strengths, respectively. In Eqs. (1) and (2), the zeroth harmonic solution, $V_{0,0}$, is given by the normal slow-passage absorption lineshape, i.e.,

$$V_{0,0}(H) = \frac{\gamma_e H_1 T_2}{1 + (\gamma_e H - \omega)^2 T_2^2 + \gamma_e^2 H_1^2 T_1 T_2}, \tag{5}$$

where the slow-passage saturation parameter in the denominator is $s = \gamma_e^2 H_1^2 T_1 T_2$.

We can simplify the analytical expressions for $V_{1,1}$ and $V_{1,1}'$ by using the reasonable approximation that $A^2 \gg B^2\omega_m^2 T_2^2$, which always holds for $\omega_m T_1 < 1$ (and also for $\omega_m T_1 > 1$, in the case of low saturation, i.e., $s \leqslant 1$) [1]. Additionally, we use the relations:

$$T_2/T_1 \ll 1, \omega_m T_2 \ll 1 \text{ and } \omega_m^2 T_1 T_2 \ll 1, \tag{6}$$

which hold for nitroxides in liquid solutions and in membranes, where the usual range of relaxation times is $5 \times 10^{-7}$–$10^{-5}$ s for $T_1$ and $10^{-8}$–$10^{-7}$ s for $T_2$ [10–13]. These inequalities lead to approximations for $A$ and $B$ that are given by:

$$A \approx 1 + \frac{\gamma_e^2 H_1^2 T_1 T_2}{1 + \omega_m^2 T_1^2}, \tag{7}$$

$$B \approx -2 + \frac{\gamma_e^2 H_1^2 T_1^2}{1 + \omega_m^2 T_1^2}. \tag{8}$$

We consider the case of low modulation frequency, $\omega_m/2\pi \leqslant 30$ kHz, which was concluded earlier to be optimal for spin–lattice relaxation measurements in the $V_{1,1}'$-mode [1,2]. For these frequencies, the condition: $\omega_m T_1 < 1$, $\omega_m^2 T_1^2 \ll 1$, holds for $T_1 < 5$ μs.