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Frequency Dependence of Pulsed EPR Experiments

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Abstract

The frequency dependence of the signal-to-noise ratio (S/N) that is theoretically possible for pulsed EPR experiments is the same as for continuous wave experiments. To select the optimum resonance frequency or frequencies for pulsed EPR experiments it is important to consider not only S/N, but also orientation selection, depth of spin echo modulation, and intensities of forbidden transitions. Evaluation of factors involved in selecting the optimum frequency for pulsed EPR measurements of distances between spins is discussed.

Keywords

double electron electron resonance; frequency dependence; interspin distance; pulsed EPR; sensitivity

1. Introduction

There have been dramatic improvements in the sensitivity of continuous wave (CW) X-band (ca. 9-10 GHz) electron paramagnetic resonance (EPR) spectrometers in recent years, and increased availability of spectrometers at frequencies other than X-band. Both trends have stimulated new applications of EPR, and especially consideration of the use of multiple frequencies. The theoretical dependence of signal-to-noise ratio (S/N) on resonance frequency (ω) varies with characteristics of the sample: does the signal saturate at the available power, is the sample size limited, and does the sample exhibit dielectric loss? (1) Predictions for various combinations of these characteristics have been derived (2-5) and are the same for CW and pulsed experiments. For constant sample size and a loop gap resonator with dimensions that scale as $1/\omega$, spectrometer S/N is predicted to improve as $\omega^{11/4}$. A review of actual spectrometer performance indicates that for a variety of technical reasons, high frequency spectrometers have not yet achieved the predicted enhancement in performance (6) so S/N may not be an incentive to use a microwave frequency other than X-band. In addition to the spectrometer S/N, there are other factors that impact the information provided by an experiment, which should be taken into account in selecting operating frequency.

Reviews have focused on the advantages of low-frequency (7), high frequency (8,9), or multi-frequency (10) EPR for particular types of experiments. An increasingly important application of EPR is the measurement of distances between paramagnetic centers in biomolecules and polymers (11-13). The focus of this article is on the information that is available from multiple-frequency pulse measurements of electron-nuclear interactions by electron spin echo envelope modulation (ESEEM) and from electron-electron interspin distances measured by pulsed dipolar spectroscopy (PDS), which includes double electron-

electron resonance (DEER, also called PELDOR) and double quantum coherence (DQC) spectroscopy.

2. Electron-Nuclear Coupling

In a 2-pulse or 3-pulse electron spin echo (ESE) measurement, the microwave frequency is chosen to be resonant with the electron spin Larmor splitting. If the microwave B_1 is large enough to encompass the nuclear hyperfine splitting of the electron spin transition, both allowed and forbidden transitions will be excited, and there will be modulation of the amplitude of the ESE signal with a period that is determined by the nuclear Zeeman frequency (14,15). For example, at X-band, the resonant frequency of protons is ca. 14-15 MHz. Hence, a time-trace of echo intensity exhibits oscillations in the amplitude with a spacing of ca. 72 to 67 ns, due to weakly-coupled ^1H nuclei. The oscillation is the ESEEM signal. If the nucleus has spin $> 1/2$, then quadrupolar energy splittings also contribute to the ESEEM. The depth of modulation increases at lower frequency, proportional to the inverse square of the magnetic field. A very common case is ^{14}N , which has nuclear spin $I = 1$. The ^{14}N ESEEM from ligands bound to paramagnetic metals in biomolecules is a powerful tool in characterizing coordinating ligands (16). When the nuclear Zeeman and hyperfine interactions cancel in the $m_s = +1/2$ spin state, the nuclear spin Hamiltonian reduces to the pure quadrupolar Hamiltonian (17) and ESEEM frequencies are well-defined. One of the most common applications of ESEEM has been the identification of imidazole ligands coordinating Cu(II), measured at X-band (16). In this case, the ^{14}N modulation is due to the distant N in the imidazole ring, not the N that is coordinated to the Cu(II). Although the directly-coordinated N has a coupling that is too large to detect at X-band, it can be observed at W-band (18) and high-field EPR can be used to accurately determine nitrogen quadrupole couplings (19).

ESEEM lines can be narrowed by optimal selection of the microwave frequency (20). For example, multifrequency ESEEM was important to identify the coordination environment of V^{4+} on silica-supported vanadium oxide with adsorbed ammonia. The nitrogen hyperfine and quadrupole parameters were obtained by tracking their frequency dependence (21).

Both CW and pulsed electron nuclear double resonance (ENDOR) provide much-enhanced information at higher microwave frequencies, because the nuclear Zeeman frequencies become separated by more than the hyperfine couplings, and transitions due to ^1H are more easily separated from those due to heavier nuclei (22-25).

3. Electron-Electron Coupling

Measuring distances between unpaired electron spins is important in many areas of science. For background information, see ref. (26). This article provides a synopsis of selected features of distance measurements that depend on the microwave frequency or the magnetic field position in the spectrum.

3.1 Intensity of half-field transitions

Dipolar coupling between unpaired electrons causes mixing of wave functions and an otherwise forbidden double quantum transition becomes partially allowed. At constant microwave frequency this transition occurs at half the magnetic field that is required for the allowed transitions and therefore is called a half-field transition. For the half-field transition in a randomly-oriented powder sample with the customary $B_1 \perp B_0$ detection configuration,

$$\text{relative intensity} = \frac{(19.5 \pm 0.5)(9.1)^2}{r^6 \nu^2} \quad (1)$$

where the relative intensity is the ratio of the integrated intensity of the half-field transition to the integrated intensity of the allowed transitions, r is the interspin distance in Angstroms, and ν is the microwave frequency in GHz. The relative intensity of the half-field transition is independent of the magnitude of the through-bond exchange interaction, and is particularly useful in systems with relatively short interspin distances. The relative intensity of the half-field transitions is predicted to be greater at lower frequency, which was confirmed with S-band and X-band CW spectra (27).

3.2 Saturation Recovery Measurements of Relaxation Enhancement

Analysis of recovery curves at X-band (9.5 GHz) and S-band (3 GHz) demonstrated the frequency dependence of the contribution to spin lattice relaxation from processes that are described by spectral density functions. Simulations predict that longer distances between low-spin Fe(III) and nitroxyl can be determined at 2.5 GHz than at 9.2 GHz. For example, at S-band there were substantial differences in the saturation recovery curves calculated at 30 and 40 Å, which indicates that the S-band measurements will extend the distance range for low-spin heme by about 10 Å (28). The calculations predict that measurements at S-band will permit greater precision in distances at short distances and will extend the range to longer distances. The underlying basis for these predictions is spectral density functions in the relaxation enhancement equation with the form $T_{2f} / (1 + (\omega_f - \omega_s)^2 T_{2f}^2)$ (29) where ω_f and ω_s are the resonance frequencies for the faster and more slowly relaxing center, respectively. The maximum impact on spin label relaxation occurs when $T_{2f} \sim 1/(\omega_f - \omega_s)$. For low-spin Fe(III) and spin labels, the dominant contribution to the difference in resonance frequency is g value so $\omega_f - \omega_s$ scales with operating frequency. As $\omega_f - \omega_s$ decreases, the value of $T_{1f} \sim T_{2f}$ required for maximum impact increases, which increases the denominator of the term $T_{2f} / (1 + (\omega_f - \omega_s)^2 T_{2f}^2)$. The longer values of T_{2f} occur at lower temperatures, where T_{1s} is also longer, so smaller increments can be detected.

3.3 DEER

The 4-pulse DEER measurement of distances includes a 90°, 180°, 180°-spin echo pulse sequence at one microwave frequency and a single 180° pulse at a second microwave frequency. The pulse at the second frequency is stepped in time between the 2nd and 3rd pulses at the first frequency (30). The two frequencies are separated such that they excite different parts of the spectrum. Commonly, the microwave frequency for the echo-forming sequence is on the low-field side of the spectrum, and the second frequency is placed at the intensity maximum in the center of the spectrum. One wants B_1 for each pulse to be big enough to excite a large number of spins, but not so big that the excitation regions overlap. With ideal pulses, the pulse at the second frequency will have no effect on the echo formed by the pulses at the first frequency if the spins excited by the two frequencies do not interact; the result of this measurement is a spin echo of constant amplitude. However, if the spins excited by the two frequencies are dipole-coupled, the echo amplitude varies with a modulation period that is proportional to the distance between the spins. There is nothing in the DEER method per se that is frequency-dependent. However, there are aspects of the way that the experiment is performed that make the result dependent on the frequency and on the characteristics of the spectrometer and resonator. For conciseness, the following discussion is in terms of nitroxyl spin labels, since this is the most common application of DEER and DQC, but the principles apply to any spin systems.

At X-band the width of the nitroxyl spectrum is defined primarily by the A_z nitrogen hyperfine coupling, but at higher microwave frequencies the width of the spectrum increases proportional to g -anisotropy. The time constant for decay of the resonator response after a pulse is equal to Q/ω , where Q is the resonator quality factor and ω is the angular resonance frequency. The smaller the decay time constant, the shorter the instrumental deadtime, which is usually about twenty times the ring-down time, Q/ω . To reduce the dead time of the resonator for spin echo experiments, including DEER, the resonator Q is reduced by overcoupling. Since $Q = \omega/\Delta\omega$ the 3 dB bandwidth of the resonator ($\Delta\omega$) at a given Q is proportional to frequency. Thus, at the same Q , the dead time is inversely proportional to frequency and overcoupling becomes less necessary the higher the frequency. Typically, the frequency separation of the two pulses for DEER is ca. 75 MHz at X-band. If the resonator is overcoupled to ca. $Q = 100$ at X-band, the resonator 3 dB bandwidth is ca. 100 MHz. Typically, one sets the two DEER frequencies to be on opposite sides of the resonator frequency. If the two frequencies were 100 MHz apart, each would be at the point on the resonator response curve where twice as much power (3 dB) would be needed to achieve the same B_1 in the resonator. By reciprocity, the signal is also reduced from that achievable when the signal is at the resonator frequency. Since the resonator bandwidth, at the same Q , increases linearly with frequency but the extent of the spectrum increases more slowly with frequency (until Δg is larger than $A_z/2$), at higher frequency the DEER signal is not attenuated as much relative to that at the center of the resonator “Q-dip” as at X-band. This difference is significant even at Q-band.

The pulse sequence for DEER measurements also excites forbidden nuclear transitions, so there is potential for nuclear modulation effects in the DEER spectrum. When nuclear modulation interferes with electron-electron distance measurements, there is an advantage of using higher frequencies, because the depth of modulation is lower at higher magnetic field strengths. For example, ESEEM is less of a problem at K-band (17 - 18 GHz) than at X-band (31). One has to prove that the frequencies found in the Fourier transform of the DEER modulation are due to electron-electron interaction and not to electron-nuclear interaction. Milov et al. (32) showed that the exchange and dipolar interactions combine such that the frequencies of the turning points of the interaction spectra are given by:

$$\nu_{\text{dip}} = \frac{\nu(\theta_{\parallel}) - \nu(\theta_{\perp})}{3} \quad (2)$$

$$J = \frac{2\nu(\theta_{\perp}) + \nu(\theta_{\parallel})}{3} \quad (3)$$

The electron-electron interaction can be expressed as a sum of dipolar, ν_{Dip} , and isotropic exchange, J , contributions. Since the dipolar interaction depends on the angle θ between the interspin vector and the external magnetic field, the dipolar spectrum has extrema at the parallel and perpendicular turning points, from which ν_{Dip} and J can be calculated using Eq. (2) and (3). Weber et al. (33) used S-band and X-band PELDOR (DEER) to separate ESEEM from exchange and dipolar effects. Since the nuclear frequencies depend on magnetic field and the dipolar interaction does not, comparing S-band and X-band DEER clearly identifies the source of the modulation.

3.4 Orientation Dependence

The more rigid the spin label and the more rigidly it is incorporated into the protein, the greater the opportunity for obtaining orientation as well as distance information by PDS. For nitroxyl radicals at long distances, measuring the oscillation frequency yields the distance between the spins via the relation:

$$\omega_{dd}(\theta, r) = \frac{2\pi g_1 g_2}{g_e^2} (3\cos^2\theta - 1) \frac{52.04}{r^3} \quad (4)$$

where θ is the angle between the interspin vector and the external magnetic field, r is the distance in nanometers and ω_{dd} is the dipolar oscillation frequency. The approximation in this expression is that the nitroxyl radicals have random orientations relative to one another. If they do not, it is potentially possible to determine the relative orientations as well as the distances. In some cases, the biological molecules could also be preferentially oriented in the magnetic field, thus selecting a particular range of θ . Experimentally, in the usual DEER experiment one selects pump and observe pulse frequencies for the best signal-to-noise ratio. The frozen solution nitroxyl spectrum inherently contains orientation information due to the g and hyperfine anisotropy. One can extract the orientation information by either stepping the frequency offsets between the two DEER frequencies or by stepping both frequencies through the spectrum with constant offset. Both methods have been used. Different field positions for DEER (with constant frequency separation) correspond to different orientations of the magnetic field relative to the g -matrix principal axis (34,35). Several groups have extracted orientation information from DEER spectra. W-band (95 GHz) DEER of radical pairs can yield simultaneously the relative dispositions of the g -tensors and the dipolar coupling (36). Margraf et al. (37) stepped the frequency offsets between the two DEER frequencies to explore the orientation selection, and found that they could better simulate their DEER data with a particular non-random geometric model. Gajula et al. (38) considered a semiquinone and a nitroxyl radical, whose g values are such that the quinone and the central portion of the nitroxyl are pumped, and the $g_z m_i = \pm 1$ components of the nitroxyl are observed. They found a small effect ($< 1 \text{ \AA}$) on the estimated distance by assuming a particular orientation of the nitroxyl relative to the vector between the nitroxyl and the semiquinone. The distance between two tyrosyl radicals in bacterial photosynthetic reaction center was measured at 95 GHz and changes in orientation of one of them upon light illumination was determined to be beyond the uncertainty in the measurements (39). Similarly, ribonucleotide reductase was measured at 180 GHz, in order to obtain mutual radical orientation under conditions where the spectrum is dominated by g anisotropy. The larger anisotropy of Cu(II) EPR spectra relative to nitroxyl radicals provided greater opportunity to test orientation selection. DEER measurements were made at several magnetic field locations in the spectrum of the Cu-Cu dimer. Simulations indicated that orientation dependence would be more important than was observed, possibly due to flexibility of the peptide linking the two Cu (40). The problem, or the opportunity, is greater the higher the microwave frequency.

3.5 Double Quantum Coherence (DQC)

The DQC experiment (12,41) involves exciting the electron spins such that both spins flip: $\beta\beta \rightarrow \alpha\alpha$. Ideally, the B_1 is large enough to excite all spins in the sample. The B_1 per square root of incident power is usually larger in the smaller resonators commonly used at higher microwave frequency, so if sufficient incident pulse power is available, more complete excitation of the spectrum will be achievable at higher frequency. This advantage of higher

frequency diminishes as the g-anisotropy causes the spectral width to increase at higher frequencies.

With a size-limited sample, the absolute spin sensitivity is expected to increase proportional to $\omega^{7/2}$ if the resonator size decreases inversely proportional to the frequency as summarized in Table 1 of Ref. (5). If the spectral width increases proportional to microwave frequency due to g anisotropy, which occurs at frequencies substantially higher than 35 GHz, the spreading out of the spectrum will cause the sensitivity improvement to be proportional to $\omega^{5/2}$ instead of $\omega^{7/2}$. Somewhat counteracting this trend is the fact that a higher resonator Q can be used at higher frequency to achieve the same dead time as was obtained a lower Q at lower frequency. Since sensitivity is proportional to Q, the ability to use higher Q at higher frequency can improve S/N for DQC (and DEER). However, lower Q is required to accommodate the shorter pulses required for DQC, so there are tradeoffs. Similarly, S/N is proportional to filling factor, η . For a size-limited sample, the filling factor would increase as the resonator size decreases, again favoring higher frequency, at least until the wall thickness of the sample tube prevents further increase in η at higher frequency.

Discussions of the frequency dependence of sensitivity often are expressed for a given number of spins. For biological samples, such as those that might be the subject of DEER and DQC measurements, the concentration of the sample often is limited. For concentration-limited samples it is important also to discuss trends in concentration sensitivity as distinct from absolute sensitivity in terms of number of spins. If the sample concentration is kept constant, and the sample fills the resonator at a low frequency, then as the resonator becomes smaller inversely with frequency, the volume of sample has to decrease by ω^3 . Thus, for a sample with negligible g anisotropy, the concentration sensitivity is proportional to $\omega^{1/2}$ and for a sample at high enough frequency that the spectral width is proportional to ω , the concentration sensitivity is proportional to $\omega^{-1/2}$ (13,41). In this case, concentration sensitivity will not improve at higher frequency.

At frequencies much above X-band, noise figures of the signal detection path may increase, limiting further increase in sensitivity at higher frequencies. The Freed lab has found that ca. 17 GHz works well for DQC (41). Comparison of sensitivity of DQC and DEER was discussed in detail by Borbat and Freed (13,41), taking into account differences in pulse sequences and the magnitude of B_1 for each type of experiment.

4. Summary

For any of these types of EPR spectroscopy if the sample is limited in size and the filling factor is high, sensitivity is improved at higher frequency. Orientation selection is improved by using higher microwave frequencies. The depth of echo envelope modulation for $I = 1/2$ is greater at lower frequency. However, for quadrupolar nuclei the width of lines and depth of modulation depends on the interplay for nuclear hyperfine and quadrupolar couplings.

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Abbreviations used

DQC	double quantum coherence
DEER	double electron electron resonance
ESEEM	electron spin echo envelope modulation
PELDOR	pulse electron double resonance
S/N	signal-to-noise