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Multiple-quantum ESR and distance measurements

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Abstract

It is shown that allowed double-quantum coherences (DQC) can now be routinely generated in disordered and oriented solids containing nitroxide biradicals and random distributions of stable radicals. The Pake doublets obtained from DQC pathways can be effectively used to determine long (~ 30 Å) distances in the former case, and concentrations in the latter. The DQC signals are strong and often comparable to standard single-quantum signals. In the limit of non-selective pulses their interpretation becomes independent of complicating features which affect other ESR distance methods. © 1999 Elsevier Science B.V. All rights reserved.

1. Introduction

Multiple-quantum coherence (MQC) is a wellknown phenomenon in modern NMR, with many useful applications [1]. On the other hand, it has been a considerable challenge to generate MQC in pulsed ESR. In recent work, Saxena and Freed have shown how 'forbidden' coherence (FC) pathways can be utilized to generate double-quantum coherence (DQC) spectra from a doubly labeled nitroxide [2,3]. These FC pathways could be generated even under spectrometer conditions that were not favorable for producing DQC in nitroxides by allowed pathways (e.g., insufficiently large microwave magnetic (B_1) fields in resonators with wide band-widths). A useful feature of the FC pathways is that they are able to provide orientational selectivity with respect to the angle between the inter-spin vector, \mathbf{r} , and the static magnetic field \mathbf{B}_0 . This enhances their utility for measuring the distance between electron spins. A disadvantage is in weaker signals, and the precise adjustment of the B_1 field to its optimum magnitude is a complicating factor.

Given recent developments in pulsed ESR capabilities in our laboratory [4] consisting of shorter dead-times, increased B_1 's without a loss in resonator bandwidth, and increased signal-to-noise ratio (SNR) at higher working frequency, it seemed appropriate to address the challenge of directly producing MQC by allowed pathways. We report here our success in this endeavor using several pulse sequences similar to those employed in NMR, but specially tailored for the ESR challenges. These challenges include short relaxation times, T_2 . They also include the typically broad spectral extent arising from electron–nuclear dipolar and g-tensor interactions that requires very strong B_1 -fields in order to irradiate all the electron spins. Our successful results

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show, that by allowed coherence pathways, DQC signals can be achieved that are 10–100 times greater than for FC pathways. They also indicate some of the potentially rich uses to which MQC ESR can be employed.

In the present Letter we shall emphasize the use of DOC for measurements of large distances, which is important in solid-state studies [5-7] and in biophysics. In the latter context one can use nitroxide bilabeled macromolecules to study aggregation and conformations in peptides and proteins as well as movement of their fragments [8,9]. However, these biophysical applications based upon cw-ESR are limited by an upper range of distance of ~ 20 Å [10]. Whereas fluorescence energy transfer is capable of measuring distances greater than 20 Å, it requires two different types of fluorescent probes, and it is often limited by the uncertainty in the κ^2 parameter. which depends on the relative orientation of the transition-moments of the two chromophores. In the case of ESR, electron spins are oriented along B_0 .

Pulsed ESR methods have been used in the past to vield spatial distributions in solids [5-7]. Several methods of practical importance have emerged. They are based on modifying the single-quantum (SQ) echo produced by a 2- or 3-pulse sequence with a pulse at either the same frequency [11] or at a different frequency [12–15]. The latter, based on selective pulses, which is known as DEER (double electron-electron resonance) or PELDOR, [12], originated from a solid-state NMR concept [16]. It has proven effective in a number of applications, including bilabeled biomolecules. Unlike DQC, it does not directly involve coherence transfer. While there are some similarities, we find that DQC based upon allowed pathways offers several advantages compared to DEER. For example, DOC can vield signals that are at least an order of magnitude stronger, in fact comparable to those of allowed SO signals from non-selective pulses. This is a very desirable feature when working with small amounts of bilabeled biomolecule and/or when greater sensitivity is needed for measuring longer distances, up to ~ 60 Å.

Finally, we note that MQC was predicted [17] and observed [18] in spin-correlated radical pairs formed by laser-induced dissociation. The relevant zero quantum (ZQ) coherence arises naturally from the initial non-equilibrium state. This important special case is sufficiently different from the present study that we do not consider it further.

2. Experimental

The pulsed multi-frequency two-dimensional FT-ESR spectrometer described elsewhere [4] was used for the experiments. Initial experiments were performed at both 9.2 and 17.3 GHz, but we report on results obtained at the higher frequency, since they provided the maximum $B_1 \approx 30$ G, least spectrometer dead-times, $\tau_d \approx 30$ ns, and higher SNR. Minimum $\pi/2$ and π pulse widths were 3–3.5 and 5–6 ns, respectively. It was necessary to use extensive phase cycling of the pulse sequences to suppress the numerous SQ signals as well as unwanted multiplequantum signals [2]. We could achieve suppression by a factor of 300–3000 depending on the sharpness of the SQ echoes (e.g., the smaller figure applies for nitroxides, which have sharper echoes).

We first studied, as a model system, E' centers in y-irradiated silica. They have a narrow ESR spectral extent (~ 5 G) thereby posing few instrumental challenges. Then we employed several nitroxide biradicals. These included the linear biradical: piperidinyl- CO_2 -(phenyl)₄- O_2C -piperidinyl (R-I) which was a gift from A. Rassat (École Normale Supérieure, Paris). It could be aligned in the liquid-crystal mixture: phase V [19], by slowly cooling the 1.0 mM solution into the nematic phase in an orienting magnetic field of 13.5 kG. The nematic phase persists to low temperatures, and the sample typically remained fully aligned to -57° C, where the experiments were performed. For comparison, the sample was also quickly frozen in zero magnetic field to yield an isotropic powder (glass). In addition, we studied the spin-labeled synthetic peptide described in [2] which has the structure: MTSL-CPPPPC-MTSL (R-II), where MTSL is the (1-oxyl-2,2,5,5,-tetramethyl-pyrroline-3-methyl) methanethiosulphonate spin label, and C and P are amino acids cysteine and proline, respectively. The concentration was ~ 1.5 mM in glycerol/water/trifluoroethanol mixture, which was buffered with MOPS (3-[N-morphilino] propane sulphonic acid). It was studied at about -82° C. All samples were $\sim 20 \ \mu l$ in volume.

We considered a variety of pulse sequences, but we found the three illustrated in Fig. 1 to be the most useful. Fig. 1a shows the basic 4-pulse sequence leading to an echo at $t_2 = t_p$ from the coherence pathways illustrated. Fig. 1b shows a 5-pulse variant wherein a π pulse is added to the 4-pulse sequence to refocus the signal. This yields an echo at $t_2 = t_m$ for the coherence pathways shown. Fig. 1c shows the 6-pulse sequence and associated relevant coherence pathways. It yields an echo for $t_2 = t_m - t_p$. The latter two cases allow for 'zero dead-time' signals.

For example, for Fig. 1c, one can define a $t_{\xi} = t_{\rm m} - 2t_{\rm p}$; then one steps out $t_{\rm p}$ in the range $0 \le t_{\rm p} \le t_{\rm m}$ keeping $t_{\rm m}$ fixed; this yields a signal vs. t_{ξ} which ranges from $-t_{\rm m} \le t_{\xi} \le t_{\rm m}$. This also has the effect of canceling out the role of phase (or T_2) relaxation on the echo at $t_2 = t_{\rm m} - t_{\rm p}$, since the total duration of the pulse sequence is independent of $t_{\rm p}$. Similar comments apply to Fig. 1b.

Note that for each pulse sequence, the $\pi/2-t_1 - \pi - t_1 - \pi/2$ 3-pulse 'sandwich' plays the identical role of producing the DQC, refocusing it, and then



Fig. 1. Pulse sequences used in DQ distance measurements and the associated coherence pathways with coherences, p that are selected by the phase cycling. (a) 4-pulse sequence used for DQM. The 2-pulse sequence $(\pi/2)_x - t_p - (\pi/2)_x$ generates DQ $(S_1^+ S_2^+, S_1^- S_2^-)$ and ZQ $(S_1^+ S_2^-, S_1^- S_2^+)$ coherences. These coherences are refocused by the sequence $(t_1 - \pi - t_1)$, and the fourth pulse converts them into antiphase coherences $(S_1^- S_{2,z}, S_1^- S_{2,z})$ which then evolve into observable SQ coherences S_1^- and S_2^- . DQM is detected as the envelope of the DQ echo as a function of t_p . (b) 5-pulse sequence employs an additional refocusing π pulse. (This pulse can instead be inserted before the pulse sandwich refocusing DQ coherences.) In this sequence, t_m is kept constant and t_p is varied. This eliminates decay of the signal due to T_2 relaxation. (c) 6-pulse sequence contains the 3-pulse sandwich $(\pi/2)_x - t_p - (\pi)_x - t_p - (\pi/2)_x$, which generates DQ coherences and the sixth pulse refocuses them to form an echo. This sequence is used with constant t_m , and t_p is varied. The signal envelope is recorded as a function of $t_{\xi} = t_m - 2t_p$ which has a range of $(-t_m, t_m)$. This sequence is essentially dead-time free and eliminates T_2 relaxation.

returning it to SQC. The phase cycling associated with this 3-pulse 'sandwich' preserves the DQC pathway while suppressing the other coherence pathways.

The repetition rate for these pulse sequences was set to 1 kHz for the γ -irradiated silica sample and to 6–8 kHz for the nitroxide samples. At these repetition rates there were no detectable effects from refocussing of residual coherences from the previous pulse sequence.

3. Results

We show in Figs. 2-5 a series of DQ signals obtained with the pulse sequences in Fig. 1 and with the various samples. In Fig. 2 (curves (i)–(ii)) we show the DQ signal from the 4-pulse sequence of Fig. 1a for the biradical R-I for the cases of 0 and



Fig. 2. DQ signal detected by 4-pulse sequence of Fig. 1a (i, ii), and 5-pulse sequence of Fig. 1b (iii, iv), for the biradical R-I dissolved in the nematic phase V for the aligned sample at 0 (i, iii) and 90° (ii, iv) tilt angles. The spectrometer frequency was 17.358 GHz; the sample temperature was -57° C. In both experiments pulse widths were set to 3.6 and 6.2 ns for $\pi/2$ and π pulse, respectively, t_1 was set to 26.5 ns, and the step size in t_p was 8 ns. A total of 200 points yielding a t_p maximum of 1.6 μ s were sampled in 9 min for the 4-pulse sequence (114 points shown), and 114 points were sampled in 49 min for the 5-pulse sequence. The signal for the 5-pulse sequence is plotted as a function of $t_m - t_p$, so as to correspond to the graph of the 4-pulse sequence.



Fig. 3. DQ signals for the biradical R-I dissolved in nematic phase V. The 6-pulse sequence of Fig. 1c was used at 17.35 GHz. Pulse widths were set to 3.6 and 6.2 ns for $\pi/2$ and π pulse, respectively. Here, $t_1 = 28$ ns, $t_m = 1200$ ns, and the step size in $t_{\rm p}$ is 8 ns. Data collection times were 8, 24, and 45 min for the 90, 0° orientations and the disordered sample, respectively. The signal is plotted as a function of $t_{\xi} = t_{\rm m} - 2t_{\rm p}$. (A) Signals for the disordered sample (circles) and simulation (solid line) with average distance r of 28.8 ± 0.5 Å and a gaussian distribution of distances with $\Delta r_{1/2} \leq 1$ Å. The same value of r was obtained from the aligned sample for all the pulse sequences. Additionally, the inset shows the FT of the experimental (solid curve) and simulated (dashed) data. The zero frequency peak is largely due to the finite sampling time. (B) Signals for the ordered sample at (i) 0° and (ii) 90° tilt. The insets show the Pake patterns obtained from the real part of FT of the signals (in (B), the dashed curve is for 0° tilt and the solid curve is for 90° tilt). Modeling of the biradical geometry with PCMODEL v7 software yielded 28.06 Å for the H-H distance between NH fragments which had to be used instead of NO ' (see insert to (A)).

90° alignment. The t_p minimum here was 35 ns and one observes that for 0° alignment a significant build-up of signal has occurred during this 'deadtime'. The experiments are affected by a T_2 decay of ~ 0.9 µs. In addition, there is some ESEEM, due to matrix protons, which does not pose a problem, since it occurs at 26.2 MHz. The modulation of the DQ signals due to dipolar interaction (DQM) [3] is strong for both orientations, and Fourier transformation (FT) produces frequencies of 2.13 ± 0.15 and 4.2 ± 0.15 MHz for 90 and 0° tilt, respectively. The decay of the oscillations is presumably due to a small distribution of interspin distances, as well as to an order parameter close to, but not exactly unity.

The lower traces (iii, iv) show the signal obtained with the 5-pulse sequence of Fig. 1b. These signals have the characteristic features of no decay due to T_2 , a baseline offset, and they are a sum of two components (cf. Section 4).

We show in Fig. 3 the DQ signal obtained from the 6-pulse sequence of Fig. 1c for the same sample. Fig. 3A and B correspond to the disordered and aligned case, respectively. Here we see the DQ modulation pattern plotted vs. t_{ϵ} . This signal has



Fig. 4. DQM signal, obtained at -82° C for the rigid biradical R-II dissolved in a glycerol/H₂O/trifluoroethanol mixture. The 6-pulse sequence of Fig. 1c was used at 17.35 GHz. Pulse widths were 3.2 and 6.0 ns for $\pi/2$ and π pulse, respectively. The Pake doublet from the real part of FT of the signal is shown in the inset. No components are well resolved, suggesting a distribution of distances.



Fig. 5. DQ, SQ, and ZQ signals observed for E' centers in γ -irradiated fused silica with: (A) the 4-pulse sequence of Fig. 1a; and (B), (C) the 6-pulse sequence of Fig. 1c. The $\pi/2$ and π pulses were set to 6.8 and 13.2 ns, respectively. In (A) $t_p = 475$ and $t_1 = 50$ ns with t_2 varied to give the echo shape: case (i) the SQ signal; (ii) the algebraic sum of the SQ and ZQ signals; (iii) the algebraic sum of the SQ and ZQ signals; (iii) the algebraic sum of the SQ, and DQ signals; (iv) the DQ signal; and (v) the 3-pulse stimulated echo. In (C) $t_m = 1200$ and $t_1 = 50$ ns, and the DQ signal is shown vs. t_{ξ} for a step size in t_p of 20 ns. In (B) are echo shapes vs. t_2 for: (i) the 2-pulse COSY echo; (ii) the DQ echo at $t_p = 200$ ns. Cases (iii) and (iv) are the COSY and DQ echoes, respectively, for a more dilute sample. All other cases in (A), (B) and (C) are for the same more concentrated sample.

zero dead-time, and it is symmetric about $t_{\xi} = 0$. We also show the real part of the FT of the time domain signals. For the aligned sample we obtain basically the same result as in the 4-pulse sequence. The Pake doublet for the disordered sample shows the same characteristic frequencies as does the aligned sample, for 0 and 90° tilt. This Pake doublet may readily be interpreted (cf. below) to yield a distance between nitroxides of 28.8 ± 0.5 Å. Molecular modeling by means of the PCMODEL v.7 software package yielded values of 28.06 and 27.81 Å for the H–H and N–N distances, respectively, between diamagnetic NH fragments, which had to be used in the modeling instead of NO⁺, cf. Fig. 3.

In Fig. 4 we show the DQ signal, arising from the 6-pulse sequence, from the polyproline biradical R-II at -82° C, where T_2 is 340 ns, as well as the associated Pake doublet. There are no clear Pake components of the type seen in Fig. 3 for R-I, which

indicates that there is a range of distances between the two ends in this synthetic peptide. One can interpret the half-width of the doublet (9 MHz) and the weak splitting frequency of 4.5 MHz to yield an average distance of 22.6 Å (cf. below) and with a distribution of distances with halfwidth of 2 Å This is consistent with the value previously found for R-II [2,3].

In Fig. 5 we show results from samples of E'centers in γ -irradiated fused silica: Fig. 5A and B are from the 4- and 8-pulse sequence, respectively. Uniform coverage was obtained for these samples since they have a spectral bandwidth of ~ 5 G. By varying t_2 we obtained the actual shapes of the echoes. By phase cycling we can separate the DQ signal from the sum of ZQ and SQ signals. It was possible to suppress the ZO signal vielding just the SO one. by 'detuning' the former. This is achieved by setting the two t_1 periods in Fig. 1 to be unequal. In Fig. 5A we show the 4-pulse allowed SQ echo (signal (i)) and the 3-pulse stimulated echo (signal (v)) (arising from eliminating the π pulse of Fig. 1a). It is equal to the SO echo but opposite in sign. The DO signal (iv) is close to 25% of that for the stimulated echo at this $t_{\rm p}$. By 'retuning' we get the algebraic sum of the SO and ZO signals (cf. ii). Finally with a modified phase cycling, we get the algebraic sum of SO + ZO+ DO (cf. iii). One sees that the ZO and DO signals are equal and opposite in sign to the SO signal. These relationships are consistent with the theory given in Section 4.

Fig. 5B shows DQ echo shapes vs. t_2 for the 6-pulse sequence, as well as echoes from the 2-pulse COSY signal, for both a low and high concentration of E' centers. (Results from the latter sample were shown in Fig. 5A.) For the high concentration sample the DQ signal reached $\sim 50\%$ of its maximum value at this t_p (200 ns) by comparison with the COSY, whereas it is relatively much smaller for the low concentration sample due to considerably slower signal growth. The DQ signal vs. t_{ξ} is shown for the more concentrated sample in Fig. 5C. An analysis of this signal (cf. below) in terms of a random distribution of interacting spins, yields a sample concentration of 1.7×10^{18} spins/cm³. This value is close to the value 2.0×10^{18} spins/cm³ obtained from a standard instantaneous diffusion measurement [5] for this sample.

4. Theory

A rigorous treatment of DO-ESR was developed by Saxena and Freed [3]. Detailed expressions, suitable for computer simulations, were provided for the cases of 2- and 5-pulse 'forbidden' echo sequences. as well as for the 6-pulse 'allowed' DO sequence. This approach can be adapted to the other 'allowed' pulse sequences used in the present study. However, this necessitates a numerical solution, and we chose for the present work an approximate approach, suitable when dipolar and exchange couplings are weak in comparison with electron spin Zeeman and hf interactions. We shall also ignore small nuclear Zeeman and quadrupolar terms, as is appropriate for the systems under study [3.5.6]. Thus we adopt a simplified form of the biradical spin Hamiltonian, as is commonly done in analyses of weak dipolar and exchange interactions in pulsed ESR [20.21]:

$$H \approx \hbar \,\Omega_1 S_{1z} + \hbar \,\Omega_2 S_{2z} + \hbar a S_{1z} S_{2z}, \qquad (1)$$

where a = d + J, representing both the dipolar interaction $d = \gamma_{e}^{2} \hbar (1 - 3\cos^{2}\theta)/r^{3}$ and exchange interaction J for a radical pair separated by a distance r. Here Ω_1 and Ω_2 represent the Larmor frequencies of electron spins 1 and 2 due to their respective g and hf tensors and their respective orientations, the details of which we suppress for compactness. The angle θ is the angle between r and the direction of the static magnetic field, B_0 . Given the reasonable approximation that in most cases $|\Omega_1 - \Omega_2| \gg |a|$ as noted above, the pseudo-secular terms (i.e., terms in $S_1^{\pm}S_2^{\mp}$) are not included in Eq. (1) [20,21], since they would only enter into the eigenenergies to order $a^2/(\Omega_1 - \Omega_2)$. We shall also assume that the microwave pulses are intense enough that $|\gamma_e B_1/2| \gg$ a, so that during the pulse we have in the frame rotating at ω , the applied microwave frequency:

$$H_{r} \approx \Delta \Omega_{1} S_{1z} + \Delta \Omega_{2} S_{2z} + \frac{1}{2} \gamma_{e} B_{1} \Big[(S_{1}^{+} + S_{2}^{+}) e^{-i\phi} \\ + (S_{1}^{-} + S_{2}^{-}) e^{i\phi} \Big], \qquad (2)$$

where $(\Delta \Omega_i \equiv \omega - \Omega_i)$. Here ϕ is the phase of the pulse such that for $\phi = 0$ and $\pi/2$, B_1 is along the rotating x and y axis, respectively.

Whereas the standard product operator method [22] can now be applied to obtain the solution for the various pulse sequences for the case of non-selective

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pulses, we wished to utilize Eq. (2) which is suitable for pulses of arbitrary strength. It is possible to do this by writing the pulse propagator associated with Eq. (2) in terms of Caylev-Klein parameters [23] using a direct product representation $(E, S_{1,2}, S_1^+, S_1^-)$ $\otimes (E, S_{2,2}, S_2^+, S_2^-)$ for elements of the density operator. The evolution of the density operator between pulses is given by the evolution operator associated with Eq. (1), which includes dipolar and exchange effects. The pulse propagator superoperator is constructed as a direct product of pulse propagator superoperators for individual spins. For simplicity we did not treat relaxation explicitly in this work, but we refer the reader to Refs. [3.24.25]. The appropriate product operator formalism is then applied for each pulse sequence. We greatly reduce the computational effort by only tracking those coherence pathways of interest and which are selected by the phase-cycling. We have obtained general expressions for DQC and ZQC signals associated with the coherence pathways in Fig. 1. We show here only the results when the nominal pulse rotation angles were set to $\pi/2$ and π as shown in Fig. 1. The signal amplitudes for the 4-, 5-, and 6-pulse sequences are:

$$V_{4,DQ}(\Delta \Omega_{1}, \Delta \Omega_{2}, \boldsymbol{r}, t_{p})$$

$$= V_{4,ZQ}(\Delta \Omega_{1}, \Delta \Omega_{2}, \boldsymbol{r}, t_{p})$$

$$= -\frac{1}{2} Im \{ S^{(1)}(\pi/2)^{*} \} S_{2}^{(1)}(\pi) S_{2}^{(2)}(\pi)$$

$$\times \left[|C_{2}^{(1)}(\pi/2)|^{2} + S_{2}^{(1)}(\pi/2)^{2} \right] |S^{(2)}(\pi/2)|^{2}$$

$$\times \sin^{2}(at_{p}/2) \equiv \overline{V}_{4}(\Delta \Omega_{1}, \Delta \Omega_{2}) \sin^{2}(at_{p}/2),$$
(3)

$$V_{5,DQ}(\Delta \Omega_{1}, \Delta \Omega_{2}, \mathbf{r}, t_{p})$$

$$= V_{5,ZQ}(\Delta \Omega_{1}, \Delta \Omega_{2}, \mathbf{r}, t_{p})$$

$$= \overline{V}_{4}(\Delta \Omega_{1}, \Delta \Omega_{2}) S_{2}^{(1)}(\pi) \left[S_{2}^{(1)}(\pi) \sin^{2}(at_{m}/2) - S_{2}^{(2)}(\pi) \sin^{2}\left\{ a(t_{m} - t_{p})/2 \right\} + \left\{ 1 - S_{2}^{(2)}(\pi) \sin^{2}(at_{p}/2) \right\} \right], \qquad (4)$$

$$V_{6,DQ}(\Delta \Omega_{1}, \Delta \Omega_{2}, \boldsymbol{r}, t_{p}) = -\operatorname{Im} \{S^{(1)}(\pi/2)^{*}\} [S_{2}^{(1)}(\pi) S_{2}^{(2)}(\pi)]^{3} \\ \times |S^{(2)}(\pi/2)|^{2} \sin(at_{p}) \sin[a(t_{m} - t_{p})], \quad (5)$$

In these equations we have for each electron spin: $C_2(\beta) = \tilde{\alpha}^2$, $S(\beta) = 2 \tilde{\alpha} \tilde{\beta}$, $S_2(\beta) = -\tilde{\beta}^2$. Here $\tilde{\alpha}$ and $\tilde{\beta}$ are the Cayley-Klein parameters associated with a nominal rotation by angle β about the rotating x-axis, and they are given by: $\tilde{\alpha} = \cos(\beta_{\alpha}/2) - \beta_{\alpha}/2$ $i\cos(\psi)\sin(\beta_0/2)$ and $\tilde{\beta} = -\sin(\psi)\sin(\beta_0/2)$, respectively, where $\beta_e = \beta [1 + (\Delta \Omega / \gamma_e B_1)^2]^{1/2}$ and $\tan(\psi) = \gamma_0 B_1 / \Delta \Omega$. Superscripts (i) for i = 1 or 2 are used in Eqs. (3)-(5) to label terms associated with the *i*th spin. We have set $\phi = 0$ for convenience. In deriving Eqs. (3)–(5) we have assumed that the spectrum is broad and inhomogeneous, so that any unrefocussed signals decay away rapidly. and any accidental refocussing is unlikely. Note that the dependence on $\Delta \Omega_1$ and $\Delta \Omega_2$ enters via the above definitions of β_{\circ} and ψ . Note also that for Eqs. (3)-(5) there is an equivalent expression obtained from permuting the superscripts (1) and (2). The two give identical results, thus we write out only one term. We can account for the time evolution with respect to t_2 (cf. Fig. 1) by simply multiplying the rhs of Eqs. (3)–(5) by $\exp(i\Delta \Omega_1 \delta t_2)$, where $\delta t_2 = t_2 - t_p$ for Eq. (3); $\delta t_2 = t_2 - t_m$ for Eq. (4), and $\delta t_2 = t_2 - (t_m - t_p)$ for Eq. (5). This corresponds to a neglect of the small term in a in Eq. (1) during period δt_2 . In the present study we are mainly interested in the case of $t_2 = 0$.

Note that in the limit of non-selective pulses (i.e., $B_1 \rightarrow \infty$): $V_{4,DQ} = -\frac{1}{4} \sin^2(at_p/2)$, $V_{5,DQ} = -\frac{1}{4} [\sin^2(at_m/2) - \sin^2\{a(t_m - t_p)/2\}]$, and $V_{6,DQ} = -\sin(at_p)\sin[a(t_m - t_p)]$. These represent the ideal cases, where the signal, as a function of t_p , just shows the coherent oscillations due to dipolar (plus exchange) interactions, independent of $\Delta \Omega_i$.

However, more generally, the $V_{n,DQ}$, n = 4, 5 or 6, signals will depend upon $\Delta \Omega_i$ via the spectral coverage factors associated with the finite pulses. It follows from Eqs. (3)–(5) that all the signals have terms of the following form (or else are a sum of terms of this form):

$$V_{n,\mathrm{DQ}}(\Delta \Omega_{1}, \Delta \Omega_{2}, \boldsymbol{r}, t_{\mathrm{p}})$$

= $G_{n}(\Delta \Omega_{1}) H_{n}(\Delta \Omega_{2}) F_{n}(\boldsymbol{r}, t_{\mathrm{p}}).$ (6)

We shall choose Euler angles $\lambda_i \equiv (\alpha_i, \beta_i, \gamma_i)$ to represent the transformation from the dipolar frame to the magnetic frame of the *i*th nitroxide fragment (i = 1 or 2) and Euler angles $\eta \equiv (0, \theta, \zeta)$ to represent the transformation from the lab frame to the dipolar frame [3]. The $G_n(\Delta \Omega_1)$ will depend upon λ_1 and η , while $H_n(\Delta \Omega_2)$ depends upon λ_2 and η , and $F(\mathbf{r}, t_p)$ just upon η as well as the magnitude r. It is then necessary to average the signal over an appropriate distribution in these variables to obtain:

$$D_n(t_p) = \langle G_n[\Delta \Omega_1(\lambda_1, \eta)] H_n[\Delta \Omega_2(\lambda_2, \eta)] \\ \times F_n(r, \eta, t_p) \rangle_{r, \lambda_1, \lambda_2, \eta}.$$
(7)

It is easily found that when there is no correlation between the λ_i and η , then Eq. (7) yields the expected simpler form, Eq. (8):

$$D_{n}(t_{p}) = \langle G_{n}\{\Delta \Omega_{1}(\lambda_{1},\eta)\}\rangle_{\lambda_{1}}$$
$$\times \langle H_{n}\{\Delta \Omega_{2}(\lambda_{2},\eta)\}\rangle_{\lambda_{2}}\langle F_{n}(r,\eta,t_{p})\rangle_{r,\eta}$$
$$\equiv G_{n}^{AV}h_{n}^{AV}\langle F_{n}(r,\eta,t_{p})\rangle_{r,\eta}.$$
(8)

Thus one can simply integrate over the distribution of orientations and magnitudes of r in the sample. A FT with respect to t_p will then yield familiar Pake doublets. Another simple limiting case occurs when the pulses can be regarded as non-selective, so that $G_n(\Delta \Omega_1) \rightarrow G_n^{\infty}$ and $H_n(\Delta \Omega_1) \rightarrow H_n^{\infty}$ independent of $\Delta \Omega_1$, $\Delta \Omega_2$, which follows immediately from the $B_1 \rightarrow \infty$ limiting forms of Eqs. (3)–(5) given above. Then Eq. (7) becomes:

$$D_n(t_p) = G_n^{\infty} H_n^{\infty} \langle F_n(r,\eta,t_p) \rangle_{r,\eta}.$$
(9)

This is the case when all the spins in the sample contribute to the signal. We can thus write as inequalities: $G_n^{\infty} \ge G_n^{\text{AV}}$ and $H_n^{\infty} \ge H_n^{\text{AV}}$.

Eq. (9) readily applies to the random distribution of radicals in the experiments on E'-centers in γ irradiated fused silica shown in Fig. 5, due to the small spectral extent. To complete this case, we must sum over all the individual random radical-pair interactions. This summation has been performed previously by the Markov method [26] for SQ coherence [20], but it can be performed for DQC as well. It yields, for example, for the decay of the 6-pulse signal:

$$D_6(t_{\xi}) = \frac{1}{2} \left[\exp(-kt_{\mathrm{m}}) - \exp(-k|t_{\xi}|) \right], \qquad (10)$$

where $k = (4/3^{5/2})\pi^2 \gamma_e^2 \hbar C$ and *C* is the concentration of spins, which was used in Section 3.

However, we find that a $B_1 = 30$ G does not provide truly nonselective pulses for the ¹⁴N-labeled nitroxides. When there is spatial correlation between hf tensors of nitroxides and r. Eq. (7) needs to be used for simulations. In this case one can show that excitation selectivity results in modulation of the Pake doublet by a function $\mathcal{E}(\cos \theta)$ similar to that introduced in Ref. [21] to describe the effect of selective pulses used in DEER. It was shown there that in the case of typical inhomogeneous broadening, $\xi(\cos \theta)$ is a non-oscillating function of $\cos \theta$ which typically varies within a factor of 2. It distorts the Pake doublet, but does not change characteristic frequencies. A FT of our data for 4-, 5-, and 6-pulse sequences for the aligned sample of R-I shows that the amplitude ratio of the spectral peaks for the 90° orientation relatively to that for the 0° orientation are ~ 1.5, ~ 1, and ~ 0.65, respectively. This reflects the steady decline in spectral excitation with increasing number of pulses in the sequence. Our analysis indicated that the z-axes of the hf tensors of both nitroxides [3] were approximately perpendicular to the long axis of molecule. Based upon this observation, as well as Ref. [21], we found it to be sufficient for present purposes to simulate the time domain data for the disordered sample of R-I using for $\xi(\cos \theta)$, the empirical function $1/(1.2-\cos \theta)$. This led to the simulation shown in Fig. 3A. In the case of R-II the Pake doublet has weakly pronounced characteristic frequencies. This shape can be best reproduced by introducing a distribution of distances, as discussed in the Section 3.

5. Discussion and conclusions

Our results have shown that DQC experiments on bilabeled nitroxide molecules yield the Pake doublets from which one can readily extract distances. This objective is most readily achieved if there is no correlation between orientation of the nitroxide moieties relative to r (i.e., Eq. (8)). In general this need not be true, posing a complex analysis based upon Eq. (7). However in the DQC experiment it is possible, by using a large enough B_1 field relative to the spectral extent, to completely suppress any effects of the nitroxide moieties (i.e., Eq. (9)). Current experimental conditions should be close to this limit for

¹⁵N labeled nitroxides. Additional improvement should enable approaching this limit for ¹⁴N nitroxides. This 'decoupling' is a virtue of the DQC experiment not shared by DEER or 2 + 1 based upon selective pulses or by cw-ESR methods [27]. Correlation can probably be best addressed by 2D experiments wherein the echo shape as a function of t_2 is collected at each t_p and then FT'd. (Alternatively, the first pulse of the sequences of Fig. 1 could be made selective, and its field position stepped out.)

Another advantage of the DQC experiment is that the (nearly) non-selective pulses lead to (nearly) complete excitation of all the spins thereby enhancing signal strength, which is not the case for techniques based upon selective pulses. This should be of considerable value for spin-labeled biomolecules available only in small amounts.

We have seen how the 6-pulse sequence provides effective zero dead-time. This is analogous to an improved DEER pulse sequence [14]. The 4-pulse sequence lacks the zero dead-time feature, but may yield stronger signals when incomplete coverage is achieved due to finite B_1 . We do observe ESEEM in the DQC experiments due to surrounding nuclei, unlike in DEER. This posed no difficulty for protons at 17.3 GHz in our experiments, because the proton ESEEM at 26.2 MHZ is much higher in frequency than the dipolar modulations. Also, we have found a suppression effect on the proton modulation in the 6-pulse sequence.

We also remind the reader that the forbidden DQ pathways directly provides some orientational selectivity with respect to r [2,3], which is not achievable by any other ESR method. At present, however, FC signals are weaker, but future improvements could make them useful. Alternatively, as we have seen in this work, macroscopically aligned samples can be used.

All in all, we believe these results show how the development of multiple-quantum coherences in ESR can lead to a range of new and useful techniques, especially in applications to distance measurements.

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