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PELDOR Spectroscopy

Broadband Inversion PELDOR Spectroscopy with Partially Adiabatic Shaped Pulses**

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Pulsed electron electron double-resonance spectroscopy (PELDOR[1], also called DEER (double electron electron resonance[2])) is a unique method to determine distance distributions between two or more paramagnetic centers ranging from 2–10 nm. It is widely employed in structural biology and materials science.[3] Nitroxides, usually covalently attached to specific sites of the macromolecule, are an important application, used mostly as spin labels.[4] Such spin labels, which do not occur in the natural form of the biomolecule, exhibit additional degrees of conformational flexibility, which have to be modeled by molecular dynamics or rotamer libraries.[5] However, their linewidth is sufficiently narrow that good inversion efficiency can be achieved with rectangular microwave pulses. A second category of paramagnetic centers, interesting in their own right, are naturally occurring transition-metal ions and iron–sulfur clusters. Unfortunately, PELDOR experiments on this group are much more demanding, owing to their large EPR linewidth. In most cases, this inhomogeneous EPR linewidth far exceeds the excitation and, hence, inversion bandwidth of rectangular microwave pulses. This leads to a poor inversion efficiency of the pump pulse in the PELDOR experiment, causing low modulation depth and therefore reduced signal quality. Nevertheless PELDOR has been successfully applied to specific cases, such as copper complexes,[6] iron–sulfur centers,[7] gadolinium complexes,[8] and manganese ions.[9] In all of these cases, the insufficient inversion of the EPR line by monochromatic rectangular pulses limits the quality of the data. Polychromatic pulses created by five microwave sources have been used to increase the bandwidth of the pump-pulse excitation on the manganese–tyrosine spin pair in photosystem II.[9b]

It has been shown in NMR and EPR spectroscopy that amplitude and phase modulation can increase the excitation bandwidth under the condition of limited peak excitation power.[10] Recently, we implemented a fast microwave pulse shaping unit for amplitude and phase modulation on a commercial pulsed EPR spectrometer and demonstrated significantly increased excitation bandwidth for Fourier transform (FT)-EPR.[10g] Here, we demonstrate such concepts for broadband inversion pulses, which strongly increase the modulation depth and therefore the signal-to-noise ratio of PELDOR time traces. The PELDOR experiments have been carried out on a nitroxide–nitroxide and a cobalt–nitroxide biradical (shown in Figure 2 and Figure 3), both synthesized in our group. The Co^{2+}–nitroxide biradical was chosen to demonstrate the effect of improved pump-pulse bandwidth on a paramagnetic center with a broad spectral width.

The Co^{2+}–nitroxide system was studied as a 0.2 mM solution in toluene with three equivalents of pyridine as axial ligands. The bisnitroxide system was immobilized in *o*-terphenyl at a concentration of 0.2 mM. The PELDOR measurements on the bisnitroxide were carried out at 298 K, whereas the measurements of the Co^{2+}–nitroxide biradical were performed at 20 K.

A custom-made pulse-shaping unit was implemented on a Bruker ELEXSYS E580 pulsed EPR spectrometer, which is described elsewhere.[10g] The spectrometer operates with two microwave sources that enable pump and probe pulses to be applied at different microwave frequencies. A fully overcoupled Bruker MD5 probehead, which allows large differences in pump and probe frequency, was used. It generated a B_1 strength of approximately 10 G at 1 kW pulse power. The samples were placed in 4 mm tubes and used the full active volume of the resonator. The refocused echo pulse sequence at the probe frequency of the four-pulse sequence was not modified (i.e., standard rectangular pulses of 32 ns length were used). Instead we investigated the advantages of using broadband, shaped inversion pulses for the pump pulse at the second frequency. In addition to the larger bandwidth it is desirable for PELDOR applications to minimize the frequency overlap between pump and probe pulses.[11] This imposes rather stringent requirements on the pulse, which must not only invert spins over a desired broad range of passband frequencies, but leave spins untouched in the stopband outside this frequency range.

The family of adiabatic pulses is known for providing robust wideband inversion. The amplitude and frequency of

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the pulse is modulated in such a way that in a frame rotating with the instantaneous frequency of the pulse, the resulting applied field starts along the z-axis (parallel to the external static magnetic field B_0) and rotates to the $-z$-axis. If the rotation is sufficiently slow (adiabatic), the spins are locked to the applied field, resulting in inversion of the magnetization. In addition, the sech/tanh functions for modulating amplitude/frequency are unique among known adiabatic functions for also providing sharply defined frequency selectivity.[12] The difficulty for our application, which we address below, is that to be sufficiently adiabatic and give the desired inversion performance, the pulse can be impractically long.

A sech/tanh pulse of duration t_{pump} can be defined by the following amplitude and frequency modulation functions. The amplitude modulation can be written as Equation (1),

$$B_1(t) = B_1^{\max} \sec h(\beta t) \tag{1}$$

where $B_1^{\max}$ is the maximum B_1 field and β is a parameter chosen to truncate the waveform at a desired minimum amplitude, since the hyperbolic secant does not go to zero for finite time t. The instantaneous frequency $\Delta\omega(t)$ at time $t \in \left[-t_{pump}/2, t_{pump}/2\right]$ is given by Equation (2),

$$\Delta\omega(t) = BW_{\infty}\tanh(\beta t) \tag{2}$$

where BW_{∞} is the bandwidth of the pulse for an infinite value of βt (i.e., $\tanh(\beta t) = 1$). In this ideal case, the pulse sweeps in frequency over the range $\pm BW_{\infty}$.

In practice, the instrumentation available requires phase modulation, which is the integral of frequency modulation, giving a phase offset (in radians) described by Equation (3).

$$\Delta p = (BW_{\infty}/\beta)\log[\cosh\ (\beta t)] \tag{3}$$

A common value for β is $10.6/t_{\mathrm{pump}}$ which truncates the amplitude at 1% of the peak B_1 value and gives $\tanh(\beta t) = \pm 0.099995$ at the endpoints of the time interval.

The limiting value of $B_1^{\max}$ for the probe was 25 MHz, corresponding to a 20 ns rectangular inversion pulse. This requires $t_{\mathrm{pump}} > 400$ ns to achieve 90% inversion over a desired bandwidth of 150 MHz using the standard sech/tanh waveform. In order to keep the pulses as short as possible, more severe truncation was introduced here. The resulting pulse is no longer strictly adiabatic,[13] having been defined by parameters outside the range of those known to give good adiabatic performance.[14] However, we found that the values of BW_{∞} and β could be optimized by simple trial-and-error to achieve tolerable tradeoffs in inversion uniformity within the passband and the steepness of the transition from passband to stopband. The resulting pulse (Figure S1 in the Supporting Information) was calculated with $t_{\mathrm{pump}} = 200$ ns, $\beta = 4/t_{\mathrm{pump}}$, $\mathrm{BW}_{\infty} = 2\pi\ 90$ MHz, and $B_{1_{\max}} = 25$ MHz. Although the pulse may not be truly adiabatic, its final performance is similar to that of an adiabatic sech/tanh pulse. The inversion bandwidth of this pulse, seen as the fraction of inversion in the desired bandwidth, is more than three times larger than that of the 20 ns rectangular-shaped π-pulse utilizing the same peak B_1 field (Figure 1).

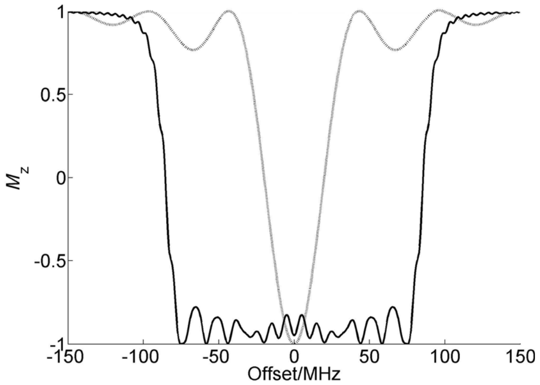

Figure 1. Inversion profile of the pulse shown in Figure S1 in the Supporting Information (which was calculated with $t_{\mathrm{pump}} = 200$ ns, $\beta = 4/t_{\mathrm{pump}}$, $\mathrm{BW}_{\infty} = 2\pi$ 90 MHz, and $B_{1_{\max}} = 25$ MHz; black) and that of the rectangular-shaped pulse utilizing the same peak B_1 (gray).

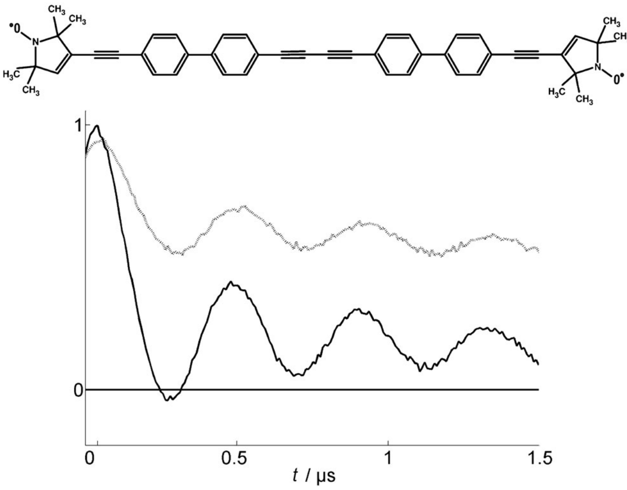

Figure 2. Comparison of PELDOR timetraces of the rigid nitroxide biradical (structure shown) generated with the adiabatic pump pulse (black) and with a rectangular-shaped inversion pulse of the same maximum power (gray).

PELDOR experiments on a bisnitroxide model system[15] were carried out to demonstrate the capabilities of this new pump pulse for PELDOR experiments involving two nitroxide radicals. In Figure 2 PELDOR timetraces generated with the sech/tanh pulse and a rectangular-shaped pump pulse are shown. All experimental parameters have been kept equal except the pump frequency. The pump frequency chosen for the rectangular pulse was 70 MHz below the probe frequency, which ensures pumping on the center of the nitroxide EPR resonance. For the sech/tanh pulse the pump frequency had to be shifted to the high-field edge of the nitroxide EPR spectrum (112 MHz below the probe frequency, see Figure S7 in the Supporting Information) due to the increased bandwidth of this pulse. This avoids substantial spectral overlap between pump and probe pulses. As can be seen, with the new pump pulse much higher modulation depth (crossing the zero line) of the PELDOR signal could be observed.

Whereas for nitroxide radicals good inversion efficiency can be achieved with rectangular microwave pulses, this does not hold for transition-metal radicals. Therefore, we used a cobalt–nitroxide biradical to demonstrate the improved performance of the sech/tanh pump pulse applied to paramagnetic centers with broad spectral width, such as the Co^{2+} radical ion. The echo-detected EPR spectrum in Figure 3 shows the large difference in the EPR linewidths of the nitroxide radical and the Co^{2+} ion. With the two additional axial pyridine ligands of the cobalt complex (not shown) the resulting cobalt spin state is $S = 1/2$. Nevertheless, its EPR linewidth exceeds 1.5 GHz, which is much greater than the EPR linewidth of the nitroxide radical (200 MHz).

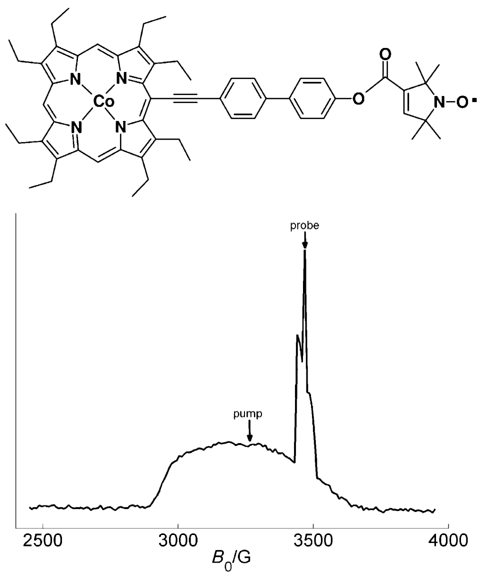

Figure 3. Echo-detected field sweep spectrum of the cobalt–nitroxide biradical (structure shown). The field-swept spectrum was recorded with a shot repetition time of 0.5 ms at 20 K, which causes reduction of the nitroxide signal intensity.

To investigate the improvement by the broadband inversion pulse the biradical was probed on the nitroxide spin and pumped on the broad cobalt paramagnetic center (600 MHz higher frequency), as indicated in Figure 3 by arrows. The pump-pulse frequency was set to the resonance frequency of the microwave resonator to not obscure the bandwidth of the pump pulse by the microwave resonator bandwidth. The shot repetition time for the PELDOR experiments was set to 25 ms to avoid saturation of the nitroxide spin.

In this case, the PELDOR modulation depth increased by almost a factor of 3 with the adiabatic pump pulse (Figure 4a). However, it is clearly visible that the prolonged sech/tanh pump pulse ($t_{\mathrm{pump}} = 200$ ns) causes a distortion of the timetrace. This is also visible in the Fourier-transformed dipolar Pake pattern shown in Figure 4b (see Figure S4 in the Supporting Information). This originates from offset-dependent dipolar evolution during the sech/tanh inversion pulse, which is 10 times longer than the rectangular pulse.

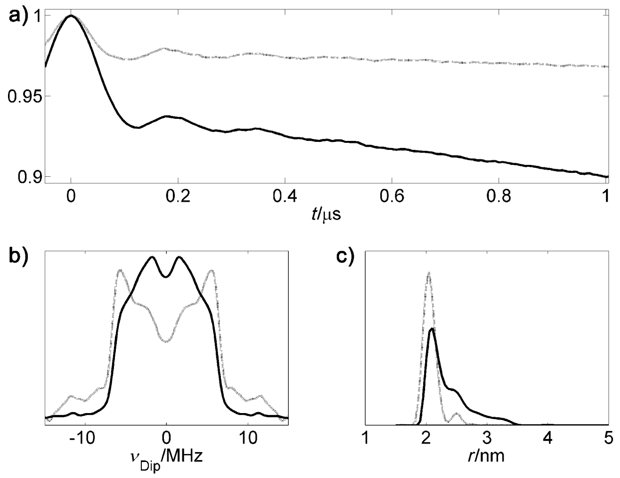

Figure 4. a) Normalized PELDOR timetraces of the Co^{2+}–nitroxide biradical recorded with a 20 ns rectangular pump pulse (dashed) or with the adiabatic pump pulse (solid). b,c) The Fourier transformations for both cases (b) as well as the distance distributions (c).[17] The less well-resolved dipolar Pake pattern for the adiabatic pulse PELDOR (dashed) is clearly visible.

The sech/tanh pulse does not invert all the spins at once but sequentially (Figure S2 in the Supporting Information). Thus, spins with different Larmor frequency have different dipolar evolution times. To overcome this limitation an alternative pulse scheme is proposed, where a second sech/tanh inversion pulse with opposite sweep direction is applied directly after the last π pulse of the probe pulse sequence (Figure 5). This accomplishes an equal net dipolar evolution time throughout all offsets. The introduction of a second pump pulse was already proposed by the group of Freed,[16] however, for the purpose of prolonging the dipolar evolution time.

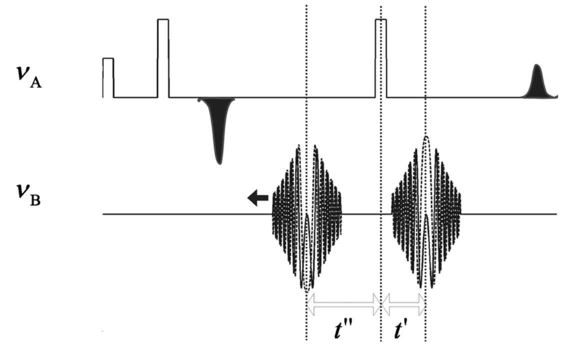

Figure 5. Modified pulse sequence to achieve an offset-independent dipolar evolution time $t = t'' - t'$. The two adiabatic pulses have opposite sweep directions with respect to each other. This is accomplished by inverting the sign of the Y component (dashed) of the adiabatic pump pulse. The X component (solid) remains unchanged.

In Figure 6 PELDOR experiments performed with one and two broadband inversion pulses are compared. The additional damping of the PELDOR oscillations by the long pump pulses could indeed be avoided by the two pump-pulse sequence.

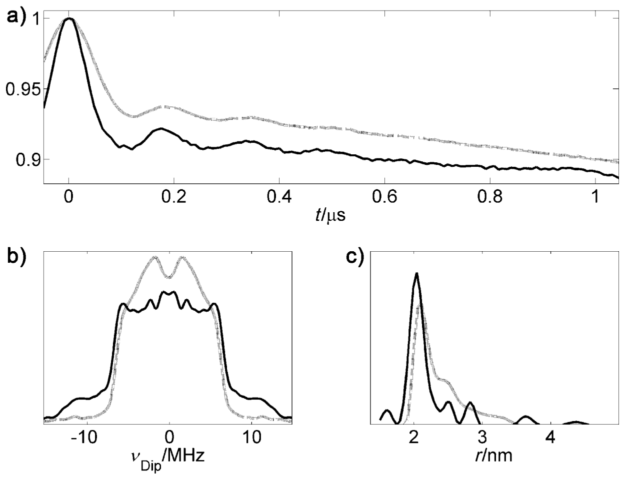

Figure 6. a) PELDOR timetrace of the Co^{2+}–nitroxide biradical. The result of a four-pulse sequence with one adiabatic inversion pulse is shown in gray. The modified four-pulse sequence with an offset independent dipolar evolution time t is achieved by incorporating a second adiabatic inversion pulse with opposite sweep direction and gives rise to the timetrace in black. The second inversion pulse has a 3/2 times higher bandwidth (see text for details). b, c) Fourier transform dipolar frequency spectra (b) and distance distributions obtained with Tikhonov regularization (c).[16]

The finite steepness in the transition region between the passband and the stopband causes a fraction of spins at these offset frequencies to be unaffected by the second broadband pulse. Because the dipolar evolution time t for such spins is differently defined (starting 1 µs after the evolution time of the two pump-pulse sequence and moving in the opposite direction), they lead to a residual single pump-pulse PELDOR signal evolving in the opposite direction. Such artifacts introduced by the incomplete second inversion of spins with a Larmor frequency close to the edges of the passband of the first adiabatic inversion pulse can be strongly reduced if the second adiabatic inversion pulse is designed with a broader passband width (see Figures S3–S5 in the Supporting Information).

In the case of two nitroxide radicals, the increase in the modulation depth of the PELDOR time trace caused a gain in the signal-to-noise ratio (S/N) by a factor of 2.2 (see Table S1 in the Supporting Information). The modulation depth of 0.73 is almost the maximum that can be achieved for a PELDOR experiment with a nitroxide–nitroxide biradical, where the same paramagnetic species is probed and pumped. In the case of the cobalt–nitroxide biradical system, an even larger enhancement in pump efficiency was achieved for pumping on the cobalt spin system. The gain in S/N is 2.5 if the rectangular pump pulse is replaced by a 200 ns shaped pump pulse. However, the prolonged pump pulse acts as a filter for higher dipolar frequencies. This could be almost completely avoided by using two pump pulses. The experiments with two pump pulses then showed a further increase in S/N by a factor of 3.1 compared to that of the rectangular pump pulse. These values were achieved for a strongly overcoupled microwave resonator to achieve very large bandwidth. Whereas these are not the optimal conditions for a nitroxide–nitroxide PELDOR experiment with classical rectangular pump pulses, such a bandwidth is necessary for PELDOR experiments on paramagnetic centers with broad EPR spectral width. Therefore this result is very promising for applications of PELDOR on natural metal cofactors in enzymes. Such pulses will also be of great importance at higher magnetic fields and therefore higher microwave frequencies, where usually the excitation power is much more limited and the EPR linewidth is strongly increased.

For the nitroxide–nitroxide compound studied, with a large distance of $r = 3.4$ nm[15] between the two N-O groups, a single broadband inversion pulse was sufficient, because the oscillation period is considerably longer than the the pump-pulse duration t_{pump}. In the case of the cobalt–nitroxide compound, with an interspin distance $r = 2.07$ nm,[6c] the four-pulse PELDOR sequence with one adiabatic inversion pulse led to a strong damping of the higher frequencies in the dipolar spectrum. This could be almost circumvented by introducing a second broadband time-reversed inversion pulse. Additionally the five-pulse broadband inversion PELDOR sequence does not have any loss in dipolar evolution time window, as long as the length of the first inversion pulse t_{pump} does not exceed the first time period τ_1.

The application of shaped pump pulses makes it possible to not only increase the inversion bandwidth for the electron spin system, but also to tailor inversion pulses to a specific demand. For example, the λ parameter can be varied while maintaining a fixed bandwidth, the λ parameter can be optimized for a given orientation selection at high fields, and a small stop-band can be created in the inversion pulse in order to avoid overlap with the probe-pulse sequence. More sophisticated optimization methods can be readily employed to develop the necessary pulses. Therefore such pulses might also be very useful in the future to investigate higher correlations in systems with more than two spins and to optimize high-field orientation-selective PELDOR experiments for maximum sensitivity.

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Keywords: adiabatic pulses · broadband inversion pulses · EPR spectroscopy · PELDOR · transition-metal ions

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