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Refocused Out-Of-Phase (ROOPh) DEER: A pulse scheme for suppressing an unmodulated background in double electron-electron resonance experiments

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ABSTRACT

EPR pulsed dipolar spectroscopy (PDS) is indispensable for measurements of nm-scale distances between electronic spins in biological and other systems. While several useful modifications and pulse sequences for PDS have been developed in recent years, DEER experiments utilizing pump and observer pulses at two different frequencies remain the most popular for practical applications. One of the major drawbacks of all the available DEER approaches is the presence of a significant unmodulated fraction in the detected signal that arises from an incomplete inversion of the coupled spins by the pump pulse. The latter fraction is perceived as one of the major sources of error for the reconstructed distance distributions. We describe an alternative detection scheme – a **Refocused** **Out**-**Of**-**Phase** DEER (ROOPh-DEER) – to acquire only the modulated fraction of the dipolar DEER signal. When Zeeman splitting is small compared to the temperature, the out-of-phase magnetization components cancel each other and are not observed in 4-pulse DEER experiment. In ROOPh-DEER these components are refocused by an additional pump pulse while the in-phase component containing an unmodulated background is filtered out by a pulse at the observed frequency applied right at the position of the refocused echo. Experimental implementation of the ROOPh-DEER detection scheme requires at least three additional pulses as was demonstrated on an example of a 7-pulse sequence. The application of 7-pulse ROOPh-DEER sequence to a model biradical yielded the interspin distance of 1.94 ± 0.07 nm identical to the one obtained with the conventional 4-pulse DEER, however, without the unmodulated background present as a dominant fraction in the latter signal.

1. Introduction

From advent of the first proof-of-principle experiments more than three decades ago [1,2] pulsed dipolar EPR spectroscopy (PDS) transformed into a versatile and broadly applicable method for nanometer scale distance measurements between paramagnetic centers in a variety of biological and chemical systems [3]. Specifically, researchers are finding the method to be particularly useful for obtaining long range (*ca.*, 2–8 nm) distance constraints in biomolecules and molecular complexes [3–6] as well as in polymers and hybrid nanomaterials [7–9]. Recently, DEER has been applied to measure interspin distances up to 16 nm in a fully deuterated tetradecameric protein complex [10]. Nowadays, applications of PDS continue to grow at a rather rapid pace.

While a number of different pulse schemes have been developed for PDS over the years [11–15], pulsed electron-electron double resonance (PELDOR), also known as double electron-electron resonance (DEER) [1,16–18] became the most frequently utilized variant of the method. DEER relies on the observer and pump pulses at two resonant frequencies for manipulating coupled electronic spins in order to measure the dipolar interaction without many unwanted contributions, primarily the electron-nuclear hyperfine interactions that typically appear in the echo-detected EPR signals.

The past decade documented a remarkable progress in increasing concentration sensitivity and the distance range of DEER experiments. This progress was enabled by advances in both EPR instrumentation and pulse sequence development. In terms of the instrumentation, an increasing availability of commercial power amplifiers at Q-band (34 GHz) and higher mm-wave frequencies made possible to carry out DEER experiments at magnetic fields/resonant frequencies higher than X-band (9 GHz). High field

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(HF) EPR spectroscopy is advantageous because of smaller sample volumes, increased sensitivity [19–21], and higher orientation selection for randomly oriented samples [22–31]. Another significant improvement of DEER sensitivity has been achieved by employing arbitrary waveform generators (AWG) to produce frequency-chirped pulses with a dramatically improved excitation bandwidth resulting in a much larger fraction of the spins inverted by the pulses [32]. The broadband excitation using chirped as well as composite pulses for PDS has been the subject of a rapid development over the past few years [33–36,15,37–43].

Pulse sequences also improved significantly. For example, it has been shown that effect of nuclear spin diffusion on the electron-spin phase relaxation could be reduced significantly by utilizing the Carr-Purcell decoupling scheme [44,45] in a form of a 5-pulse DEER sequence. Such a sequence provides for longer dipolar evolution times required for measurements of longer interspin distances [46]. Extension of the single echo detection scheme to an echo train was shown to increase the signal-to-noise ratio up to a factor of 2.6 when the number of the detected refocused spin echoes was increased to 21 [47]. Further optimization of a pulse EPR experiment could be achieved by carefully choosing conditions for dynamical decoupling as was demonstrated by a systematic study of transverse relaxation for protonated and deuterated nitroxides in both protonated and deuterated *o*-terphenyl glasses [48]. Another notable development was an introduction of a 7-pulse DEER sequence [49] incorporating *sech*/*tanh* -shaped pulses to reduce artifacts caused by multiple inversion pulses.

It is important to note here that all of the abovementioned pulsed schemes achieved an increased accessible distance range by adding more pulses to the standard 4-pulse DEER sequence. For example, the DEER sequence based on the Carr-Purcell scheme requires at least one additional inversion pulse in order to utilize the full time interval instead of only half of it [46]. Each refocusing pulse of the Carr-Purcell sequence also requires an additional inversion pulse [46,49]. For a broadband inversion DEER, an additional pulse is required in order to compensate for the long duration of the inversion pulse, which causes a distribution of the zero time points in the trace [32,49]. While the additional pulses are beneficial for improving DEER distance range and resolution, the longer pulse train would generally result in some signal loss. Thus, in practice, depending on the sample and the relaxation times, such signal losses could even overweigh the gains. Another important problem associated with an increased number of pulses is appearance of signal artifacts caused by pulse imperfections. Such artifacts require either an additional signal post processing [46] and/or can be suppressed by optimizing pulse excitation profiles [50].

One of the major remaining problems in DEER experiments and a significant complication for the data analysis is the presence of an unmodulated background signal arising from an incomplete inversion of the electronic spins by the pump microwave pulses. One reason for the latter is a broad width of EPR spectra with respect to the narrow pulse excitation bandwidth achieved by commercial EPR spectrometers. The second reason is the requirement for avoiding the frequency overlap between the pump and observer DEER pulses for the individual spins in a dipolar pair that typically exhibits overlapping EPR spectra. In order to overcome this limitation the bandwidth of the refocusing pulses should be made sufficiently broad for non-selective excitation of the entire EPR spectrum of the spin pair [50]. For these reasons an unmodulated background is typically present in experimental DEER traces.

We note that experimental DEER signals also have another minor unwanted contribution originating from weak magnetic interactions of the observer spins with multiple electronic spins randomly distributed across the sample. If not taken into account, this contribution causes an artificial broadening of the measured distance distributions due to an enhanced damping of the dipolar oscillations. In practice, one could readily suppress such a signal damping by diluting the sample with solvent and/or diamagnetically labeled macromolecules albeit at the cost of lower signal amplitude [6]. However, the dominant part of the unwanted background due to the incomplete spin inversion discussed above would still remain unchanged for the diluted spins.

Typically, for biradical nitroxides at X- and Q-band EPR frequencies the fractions of the modulated and unmodulated DEER signals are usually comparable unless the advantage of a very broad non-selective excitation can be utilized [50]. However, the ratio of the modulated and unmodulated DEER signals may change dramatically in disfavor of the modulated signal with an increase in the magnetic field due to the dominance of *g*-factor anisotropy in the EPR spectra and/or lower achievable B_1. The insufficient excitation bandwidth becomes a particular problem for DEER employing transition metal ions as spin labels. For example, for DEER experiments carried out at W-band (95 GHz) using "spectroscopically easy" Mn^{2+} molecular tags, the reported modulation depth was only 0.4% [51]. Finally but not lastly, the modulated fraction of the DEER signals could be further reduced in samples with incomplete labeling. The latter could be a particular problem for protein sites with a poor solvent accessibility and/or containing irreversibly oxidized cysteines.

The presence of a large unmodulated background imposes a serious problem for DEER experiments and, particularly, for the data analysis. Currently, in order to efficiently separate the unmodulated fraction from the DEER signal, the length of the DEER signal acquisition has to be extended and/or the shape of the background signal has to be known *a priori*, preferably, from a control experiment containing only randomly dispersed spins. Generally, the background shape can only be fitted properly if the total length of the measured DEER trace is significantly longer than the time at which the dipolar modulation decays. In practice, for many spin systems a rather approximate separation of the unmodulated fraction allows for an estimate of the inter-spin distance if the length of the collected DEER trace is at least 1.25 of the dipolar modulation period [46]. However, a more accurate determination of distances and distance distribution requires an extension of the trace length to about 1.75 of the modulation period [52].

For more distant spins in a pair the period of the corresponding dipolar oscillation could be much longer than the phase memory time T_m. This would result in a substantial – up to several orders of magnitude – decrease in the spin echo amplitude due to a large inter-pulse separation and a loss in the signal-to-noise ratio for the DEER trace. Additional ambiguities in DEER data analysis arise for samples with a heterogeneous distribution of the interspin distances [53–56] such as, for example, observed for spin-labeled membrane proteins reconstituted into micelles or liposomes. For the latter systems the exact shape of the DEER component arising from the randomly distributed spins is usually unknown and this makes the separation of the modulated signal from the experimental DEER trace even more challenging [57–63].

Here we propose an alternative detection scheme for the DEER experiment – ***R***efocused ***O***ut-***O***f-***Ph***ase DEER (ROOPh-DEER) - to acquire only the modulated fraction of the DEER trace that is free from the unmodulated background caused by the incomplete inversion of the pump spins. While such a detection scheme could be realized in a pulse sequence in a number of different ways, at least three additional pulses are required for filtering out the unmodulated fraction of the DEER signal. Although the proposed pulse scheme requires a further optimization with respect to the signal losses caused by addition of the microwave pulses, its ability