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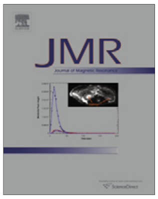

Simultaneous acquisition of pulse EPR orientation selective spectra

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ABSTRACT

High resolution pulse EPR methods are usually applied to resolve weak magnetic electron-nuclear or electron–electron interactions that are otherwise unresolved in the EPR spectrum. Complete information regarding different magnetic interactions, namely, principal components and orientation of principal axis system with respect to the molecular frame, can be derived from orientation selective pulsed EPR measurements that are performed at different magnetic field positions within the inhomogeneously broadened EPR spectrum. These experiments are usually carried out consecutively, namely a particular field position is chosen, data are accumulated until the signal to noise ratio is satisfactory, and then the next field position is chosen and data are accumulated. Here we present a new approach for data acquisition of pulsed EPR experiments referred to as parallel acquisition. It is applicable when the spectral width is much broader than the excitation bandwidth of the applied pulse sequence and it is particularly useful for orientation selective pulse EPR experiments. In this approach several pulse EPR measurements are performed within the waiting (repetition) time between consecutive pulse sequences during which spin lattice relaxation takes place. This is achieved by rapidly changing the main magnetic field, B_0, to different values within the EPR spectrum, performing the same experiment on the otherwise idle spins. This scheme represents an efficient utilization of the spectrometer and provides the same spectral information in a shorter time. This approach is demonstrated on W-band orientation selective electron-nuclear double resonance (ENDOR), electron spin echo envelope modulation (ESEEM), electron–electron double resonance (ELDOR) – detected NMR and double electron–electron resonance (DEER) measurements on frozen solutions of nitroxides. We show that a factors of 3–6 reduction in total acquisition time can be obtained, depending on the experiment applied.

1. Introduction

Pulse EPR spectroscopy consists of a variety of high resolution methods that provide structural information. These include hyperfine spectroscopic techniques that determine the local environment of paramagnetic centers, such as transition metal ions or radicals, via the hyperfine interaction of the unpaired electron(s) with nearby nuclear spins. This family of techniques includes one and two dimensional electron spin echo envelope modulation (ESEEM), electron-nuclear double resonance (ENDOR) and electron–electron double resonance (ELDOR)–detected NMR [1] Another family of techniques measures distances between electron spins and includes pulse double electron–electron resonance (referred to as DEER or PELDOR) and double quantum coherence spectroscopy [2–4]. These have recently become highly popular in the context of the structure of biomolecules and their complexes [4] but have been also applied to polymers, materials and micellar solutions [5–7].

High resolution pulse EPR techniques can, in principle, be carried out at any operational frequency. The EPR spectra of orientationally disordered systems, such as polycrystalline solids or frozen solutions, are inhomogeneously broadened due to the anisotropy of the magnetic interactions. At high magnetic fields the g-anisotropy becomes well resolved and it often determines the EPR lineshape, also for organic radicals. Other sources of considerable inhomogeneous broadening that are field independent are the zero field splitting for high spin systems and the anisotropy of the hyperfine interaction of paramagnetic metal ions. Due to microwave (mw) power limitations the bandwidth of the applied mw pulses is often much smaller than the width of the inhomogeneously broadened EPR spectrum and therefore the pulses affect only a small part of the electron spins. Consequently, the chosen magnetic field determines which subset of spins is excited and when the anisotropy that governs the spectrum is well resolved it also determines the subset of molecular orientations with respect to the external magnetic field that will participate in the

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experiment. It is therefore a common practice to carry out pulse EPR experiments at a number of magnetic field settings within the EPR spectrum. Such a series is referred to as orientation selective measurements and the analysis of the spectra obtained provides orientation information – namely relative tensors orientation. For example orientation selective pulse ENDOR measurements carried out on a sample with a well resolved *g*-anisotropy provide the orientation of the hyperfine interaction of the coupled nuclei with respect to the *g*- principal axis system [8–10]. This in turn yields important structural information, provided that the orientation of **g** in the molecular frame is known. Actually, orientation selective measurements can be referred to as two dimensional (2D) experiments, where the magnetic field represents the second dimension. In the case of 2D experiments, like Hyperfine Sublevel Correlation Spectroscopy (HYSCORE), such experiments will be three dimensional. The acquisition of orientation selective experiments is often time consuming because many measurements have to be carried out; the acquisition of a single ENDOR spectrum of a biological systems can easily take several hours and more.

The four-pulse DEER sequence [5] is one of the most popular techniques for measuring nanoscale inter-electron spin distances. It is most often applied to biomolecules (proteins and nucleic acids) labeled with nitroxide stable radicals. At X-band frequencies the *g*-anisotropy of nitroxides is barely resolved and the inhomogeneous linewidth is determined primarily by the ^{14}N hyperfine interaction. Accordingly, by setting the pump pulse to the $M_I = 0$ line, to a good approximation all possible orientations of the inter-spin vector relative to the external magnetic field contribute to the DEER experiment [3]. This simplifies considerably the data analysis because the DEER trace depends only on the inter-spin distance distribution [2,3] However, when such experiments are carried out at W-band (95 GHz) frequencies or higher, where the *g*-anisotropy becomes well resolved, the DEER traces depend also on the relative orientation of the *g*-tensors of the two nitroxide lables and on the orientation of the inter-spin vector with respect to the molecular frame (total of 5 angles). To extract this information, DEER traces should be collected along the EPR powder pattern and this, again, is time consuming [11–15].

All orientation selective pulse experiments are usually carried out consecutively, namely one field is chosen, data are collected until the signal to noise ratio is satisfactory, data are saved, the field changed and a new set of data is acquired. The repetition time of the EPR experiments, T_R, is usually determined by the electron spin lattice relaxation time, T_1, and it is generally accepted to use $T_R \sim 5\ T_1$, (Fig. 1a). The T_1 of nitroxides at low temperatures is on the order of a few ms and this limits significantly the rate of signal averaging. The length of the EPR pulse sequences is usually smaller than 100 μs and the spectrometer remains essentially idle for most of the time, just waiting for the spins to relax back to equilibrium. In addition, in most pulsed EPR experiments only a small part of the spins that span the full inhomogeneously broadened EPR spectrum contribute to the signal, while the majority of the spins are idle.

In this work we propose a parallel acquisition scheme which allows acquiring several orientation selective pulse EPR measurements in parallel during the waiting repetition time by rapidly changing the main magnetic field B_0, as illustrated schematically in Fig. 1b. This scheme represents an efficient utilization of spectrometer time and provides the same information in a shorter time, thus effectively increasing the *S*/*N* of pulsed EPR measurements per unit time. This approach is also useful in the complete absence of orientation selection. In this case all the spectra obtained along the EPR line will be similar and can be summed together to obtain a single spectrum with significantly better *S*/*N* ratio. Here however, one should pay attention to spectral changes occurring due to the small change in the field, as maybe the case for ENDOR but not for DEER. We demonstrate the utility of this approach on W-band orientation selective ENDOR, ESEEM, ELDOR–detected NMR, and DEER on frozen solutions of nitroxides.

2. Results

Fig. 1 illustrates the proposed concept of parallel acquisition. The traditional approach to obtain several pulsed EPR experiments, along the broad EPR line, with waiting time T_R between subsequent pulse sequences is shown in Fig. 1a. In Fig. 1b we illustrate the parallel acquisition concept where after acquiring the signal at one B_0, we change it to $B_0 + \Delta B_0$, acquire the signal there, and repeat this *n* times, as can be accommodated by T_R. We then reset B_0 to the initial position and start the experiment all over again to acquire the next sequence for signal averaging (number of shots). We first applied this approach to hyperfine spectroscopy type experiments and then to measurement of distance between electron spins.

A series of orientation selective ^{1}H ENDOR spectra of a frozen solution of 4-hydroxy-TEMPO in toluene, acquired with the Davies ENDOR pulse sequence (Fig. 2a) and parallel acquisition mode is shown in Fig. 3. The echo-detected (ED) EPR spectrum and the magnetic field covered in the g_{xx} region (10 field positions) are shown in Fig. 3a and the corresponding spectra are presented in Fig 3b. Several spectra were also acquired using the traditional approach and they are displayed in Fig. 3b as well for comparison. As expected, the ENDOR spectra exhibit a significant lineshape evolution as the field is varied; the outer edge shoulders at ±5 MHz disappear as the magnetic field is lowered. Other variations are noticed on the fine structure of peaks at ±2.5 MHz. The spectra acquired in parallel and those acquired using the traditional approach at the same fields with equal number of accumulations are practically identical and no significant source of noise is introduced with the addition of the rapid sweep magnetic field coils.

The three-pulse ESEEM sequence (Fig. 2b) belongs to another family of techniques that provide information on the hyperfine interaction with the neighboring nuclei. This pulse sequence produces nuclear coherences that evolve with time *T* and are manifested in the appearance of modulations in the echo decay. To observe these nuclear modulations the mw pulses should excite the allowed EPR transitions ($\Delta M_S = \pm 1$), ($\Delta M_I = 0$) and their branched forbidden transitions ($\Delta M_S = \pm 1$), ($\Delta M_I = \pm 1$) simultaneously. The frequency differences between these types of transitions are the associated nuclear frequencies. In Fig. 4a we present 10 three-pulse ESEEM time domain traces acquired in parallel along the whole 4-hydroxy TEMPO spectrum and the corresponding Fourier transform ESEEM spectra are shown on Fig. 4b. The magnetic fields chosen are indicated on the figure insert. ^{14}N modulations were observed only along the g_{xx} and g_{yy} positions since there the cancellation condition, $A/2 \approx \nu_I$, required for intense forbidden transitions [16,17] is fulfilled ($\nu_I \sim 10$ MHz, $A_{xx} \approx A_{yy} \approx 13$–17 MHz). The low field spectra show two peaks at 18.4 MHz and 22.7 MHz that correspond to the two single quantum ^{14}N transitions [18], ν^{β}_{sq1} and ν^{β}_{sq2} of the electron spin β manifold and low frequency peaks at ~1.5–3 MHz that are assigned to the single and double quantum transitions within the α manifold (ν^{α}_{sq1}, ν^{α}_{sq2} ν^{α}_{dq}). As the g_{zz} field position is approached the hyperfine couplings increases considerably, reaching $A_{zz} \approx 90$ MHz at the g_{zz} region. Here $A \gg \nu_I$ and the transition probability of the forbidden transition is considerably reduced. Moreover, the nuclear frequencies become too large to be covered by the mw pulses. Therefore no nuclear modulations were observed above 3377.7 mT. Comparison between the spectra acquired in parallel and those acquired using the traditional approach reveal no significant difference in signal to noise and no additional artifacts are introduced into the spectra when using the parallel acquisition approach.

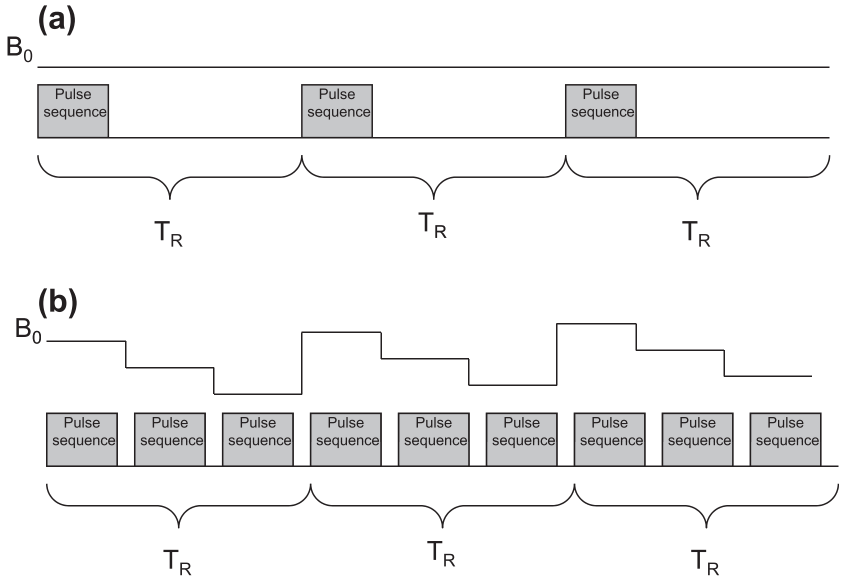

Fig. 1. Comparison of (a) conventional, sequential and (b) parallel pulse EPR signal acquisition schemes.

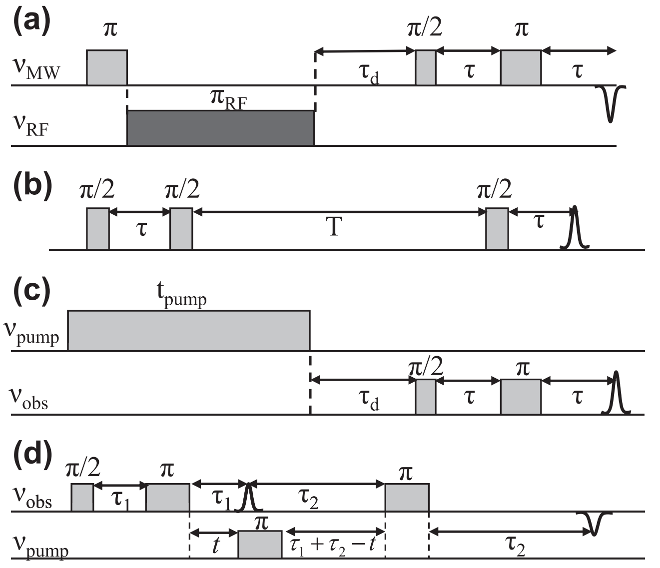

Fig. 2. Pulse sequences used for parallel acquisition in this work, (a) Davies ENDOR, (b) three-pulse ESEEM, (c) ELDOR–detected NMR, (d) four-pulse DEER.

The last example of hyperfine spectroscopic techniques is the ELDOR–detected NMR experiment, acquired with the pulse sequence shown in Fig. 2c. Like ENDOR it gives the NMR frequencies of coupled nuclei, though with reduced resolution [19,20]. The long high turning angle mw pulse at ν_{pump} drives a forbidden transition and is followed by an echo detection sequence on the allowed EPR transition ($\Delta M_S = \pm 1$), ($\Delta M_I = 0$) at ν_{obs}. The difference between the allowed and forbidden transitions corresponds to $\Delta\nu = \nu_{\mathrm{pump}} - \nu_{\mathrm{obs}}$ and yields the frequencies of the NMR (or ENDOR) transitions. The ELDOR–detected NMR spectrum of 4-hydroxy-TEMPO in toluene recorded with the magnetic field set to the $M_I = -1$ g_{zz} component is presented in Fig. 5a. It shows ^{1}H and ^{14}N signals, the assignment of which is given on the spectrum. In Fig. 5b we shows the 2D representation of the ELDOR–detected NMR spectra [21] of this sample, acquired as function of magnetic field in the region of g_{zz} with the emphasis on the two $\nu^{\beta}_{sq1,2}$ lines. Here the aim was to determine the field dependence of these lines at this specific region to determine the ^{14}N A_{zz} and Q_{zz}. The field positions for each trace are indicated in Fig. 5c. Such 2D plots are useful for obtaining the ^{14}N A_{zz} and its quadrupole coupling constant. The details will be available in a forthcoming publication. The 2D plot in Fig. 5b was acquired in two series of measurements with five ELDOR–detected NMR spectra acquired in each. This limit of five spectra per acquisition was imposed by the excitation bandwidth of both types of pulses, since we wanted to avoid overlap between the pump and detection pulses in subsequent experiments. This may occur when the excitation bandwidth of the pulses is larger than the B_0 field difference between subsequent experiments.

Finally we present DEER data acquired using the parallel acquisition approach. W-band four-pulse DEER experiments were carried out on a rigid bi-radical shown in Fig. 6a. Fig. 6b depicts the echo-detected EPR spectrum, the positions and the bandwidth of the pump and observer pulses used in the parallel acquisition mode. Fig. 6d shows the DEER traces acquired using the parallel mode compared with the traces accumulated using the traditional four-pulse sequence, namely obtained in three individual experiments. These are very similar, except for the different S/N that arises from different number of accumulations and small differences in the background decay of the DEER traces. The latter is attributed to the phase differences between different experiments that were tuned independently by observing the echo intensity on the scope. We were not able to correct the phase after the data acquisition because our spectrometer does not have quadrature detection.

3. Discussion

In this work we have presented a new approach for acquiring several pulse EPR spectra along an inhomogenously broadened EPR spectrum that saves considerable measurement time. Several pulse experiments are carried out at different magnetic fields within one repetition time using a rapid field jump which allows us to change B_0 within μs. This is particularly important for measurements at liquid Helium temperatures which are costly. DEER measurements on biological systems have become extremely popular in the last decade. So far the number of high field DEER measurements has been rather limited [11,12,14,22,23] but they can be attractive when the relative orientations of the radicals are of

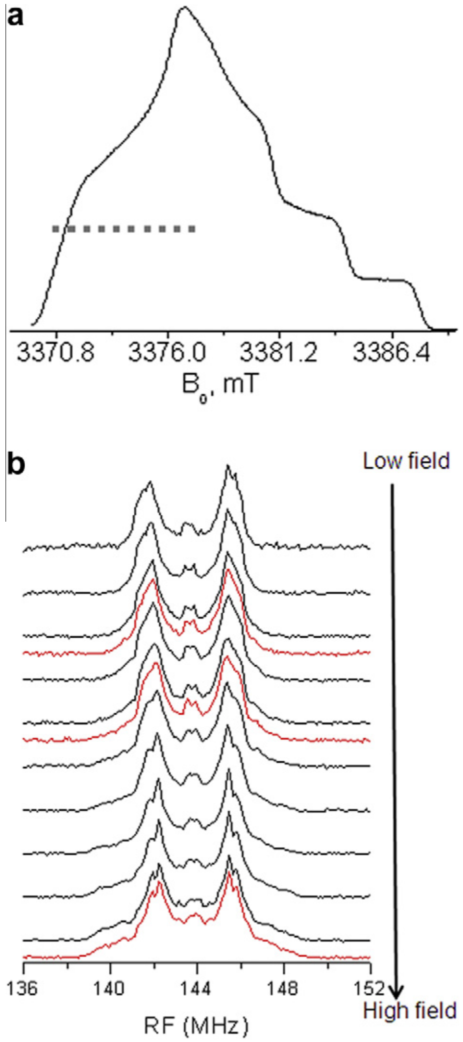

Fig. 3. (a) ED-EPR spectrum of a frozen solution of 4-hydroxy-TEMPO in toluene and the field positions where spectra were acquired using parallel acquisition. Ten ^{1}H Davies ENDOR spectra acquired in parallel (black), and using the conventional approach (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

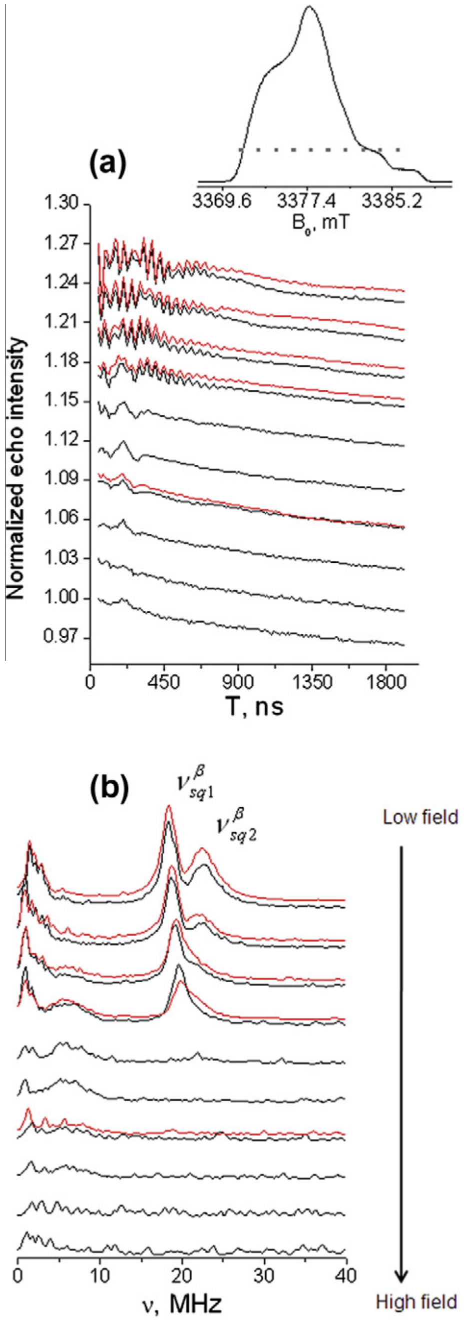

Fig. 4. (a) Time domain three-pulse ESEEM traces of a frozen solution of 4-hydroxy-TEMPO in toluene acquired in parallel (black), and with the traditional approach (red) along the whole nitroxide EPR spectrum. Field positions are indicated in figure insert showing the ED-EPR spectrum. The bottom time domain trace is shown to scale and subsequent traces are shifted by 0.03 to ease the comparison. (b) FT ESEEM spectra of the traces in (a). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

interest or/and when the sample amount is limited. For example, the sample size at W-band is usually an order of magnitude smaller than at X-band, while the concentrations are comparable. In this case a series of orientation selection spectra should be recorded and acquiring them simultaneously saves considerable time, particularly in biological samples where the low concentrations require acquisition times as long as 12 h [24]. Even for samples with high conformational distribution where no orientation selection is observed, this methodology is useful because the absence of orientation selection cannot be determined a priory and one has to establish the lack of orientation selection by acquiring spectra at several fields. In this case data can be added up, improving the *S*/*N* ratio.

The number of experiments that can be acquired simultaneously generally depends on the system of interest (EPR spectral width and spin lattice relaxation time), the particular experiment and the available hardware. In the DEER experiment only the spin excited by the observer pulses are required to return to thermal equilibrium. This allows pumping the spin packets that were the observer spins at the previous acquisition step. Accordingly for the DEER experiment a minimum B_0 field jump is on the order of the excitation bandwidth of the observer pulses. Similar considerations for the size of the B_0 field jumps apply for other experiments that use hard pulses at a single frequency, such as Mims ENDOR, ESEEM and HYSCORE. The experiments that are the most suited for the parallel acquisition scheme are those that require highly selective MW pulses such as Davies ENDOR. These experiments usually utilize only a small fraction of the spins and span only up to 10 MHz of the total EPR spectral width. Hence such experiments can be densely accommodated within the EPR spectrum.

For the ELDOR–detected NMR experiment few additional considerations should be taken into account. Because in this

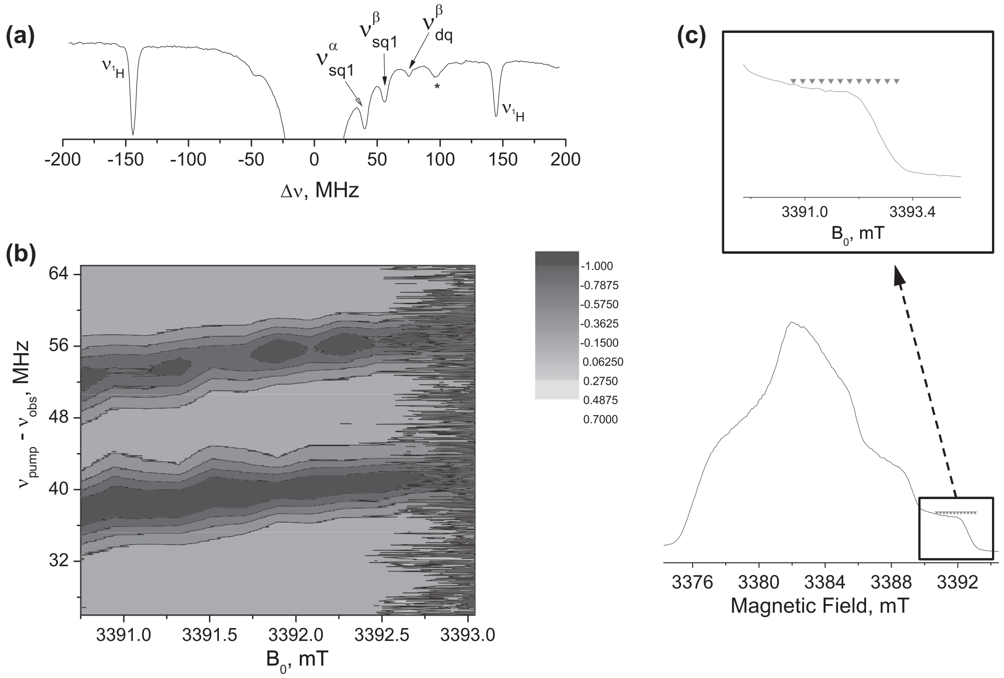

Fig. 5. (a) An ELDOR–detected NMR spectrum acquired at 3392.5 mT. (b) The 2D ELDOR–detected NMR spectra acquired with parallel acquisition scheme. Field positions for each 1D trace are indicated in the enlarged square.

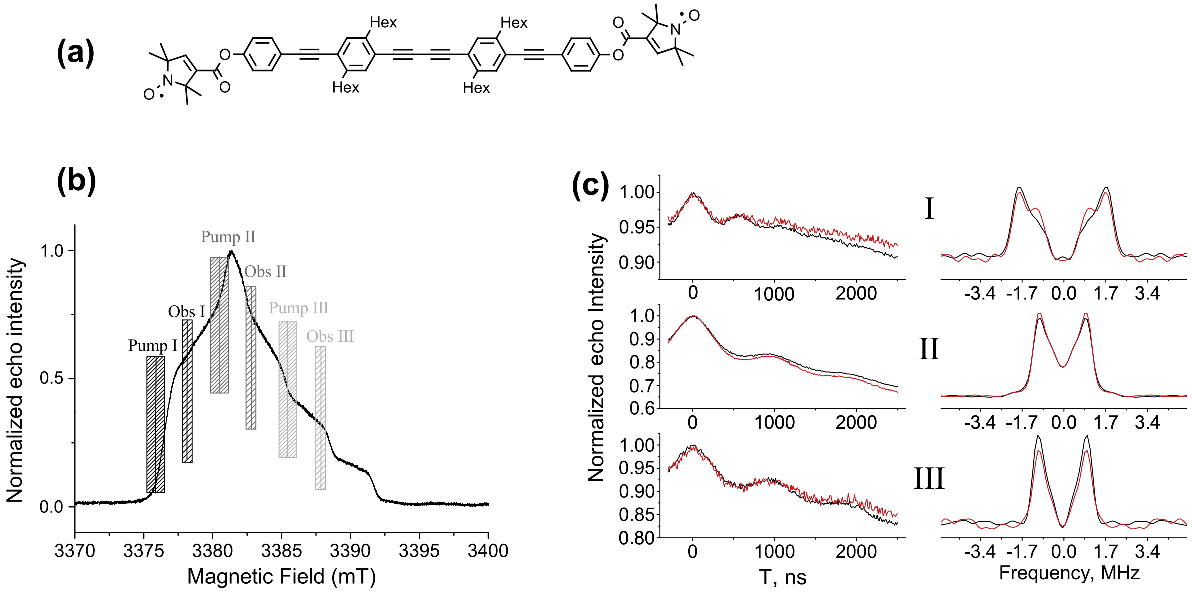

Fig. 6. (a) The biradical used for the DEER measurements and its ED-EPR spectrum (b). Rectangles represent the positions for the pump and observer pulses for three parallel DEER experiments (I, II, III) and their width represents the pulse bandwidth. (c) Comparison of conventional (red) and parallel acquisition (black) of DEER data (left) normalized time domain DEER traces and (right) FT DEER spectra after baseline subtraction recorded with settings I, II and III. The field positions for each DEER measurement are indicated in Fig. 2b. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

experiment two different MW frequencies are utilized, we can encounter a situation where more than one type of observer spins are affected during the same scan due to non relaxed pumped spins. Consider for example two spin packets A and B that resonate at different magnetic field positions and will therefore be detected at different B_0 steps. In addition, consider two forbidden transitions A′ and B′ that are associated with the spin packets A and B, respectively (See Fig. 7). In conventional ELDOR–detected NMR experiment when the pump frequency affects A′ transition and spins A are detected a spectral line is observed in the ELDOR–detected NMR spectrum (Fig. 7a). If at the B_0 step when B spins are detected the pump frequency is on resonance with the A′ forbidden transition it will not affect the spectrum of the B spins, but will affect the spectrum of the A spins at the next B_0 step, since by definition the spins do not relax to equilibrium between subsequent steps (Fig. 7b). This artifact line will appear shifted by ΔB_0 from the real spectral line and will have weaker intensity due to relaxation between subsequent steps.

Another possible source of artifacts in ELDOR–detected NMR spectra acquired in parallel happens when the pump frequency applied at the B_0 step where we detect B spins, will affect the A spins directly (Fig. 7c). This will result in a hole in the spectrum similar to the one that appears around the $\Delta\nu = 0$ frequency in the conventional ELDOR–detected NMR spectrum. This problem can be avoided by either acquiring only part of the ELDOR–detected NMR spectrum such that the pump and observer frequencies never overlap for any B_0 step. Alternatively, by acquiring only half of the ELDOR–detected NMR spectrum such that no spins are affected by the pump frequency before they are detected. The latter means to acquire the positive part of the ELDOR–detected NMR spectrum $\nu_{\text{pump}} > \nu_{\text{obs}}$ while incrementing the B_0 field position and to acquire the negative part $\nu_{\text{pump}} < \nu_{\text{obs}}$ while decrementing it. In this case we still may encounter the situation when the detection pulses affect the forbidden transition for another spin packet during the preceding B_0 step, but this should have minor affect since low power mw pulses of ~200 ns duration should not affect significantly the forbidden transitions which require μs long high power pulses to be affected.

Both types of problems, artifact lines at ΔB_0 and pseudo holes discussed in the previous section, can be avoided by extending the idea of the random acquisition protocol [25]. We propose here to change randomly the pump frequency at each B_0 step; in this way all artifacts discussed above will appear randomly during subsequent scans and will only contribute to the noise, while real lines will sum up constructively. In this approach the noise level for each experiment in the particular B_0 step will be higher compared to the conventional ELDOR–detected NMR, but the possibility to acquire several spectra in parallel will compensate for it. In terms of required hardware this will require either a mw source with setting time within the time of the B_0 step, hundreds of μs in our case, or a an additional mixing of the mw with some rapidly changing RF source. Despite these potential shortcomings we showed here that under our experimental conditions the resulting ELDOR–detected NMR spectra are essentially artifact free as is evident from Fig. 6b.

It is the spectral properties of the system under study that dictates the requirements for the parallel acquisition hardware. The broader the EPR spectrum, the larger should be the magnetic field range, the sweep coils provide. For a particular current driver, increasing the range will come at the expense of the settling time, because coils with a larger number of turns will have a higher

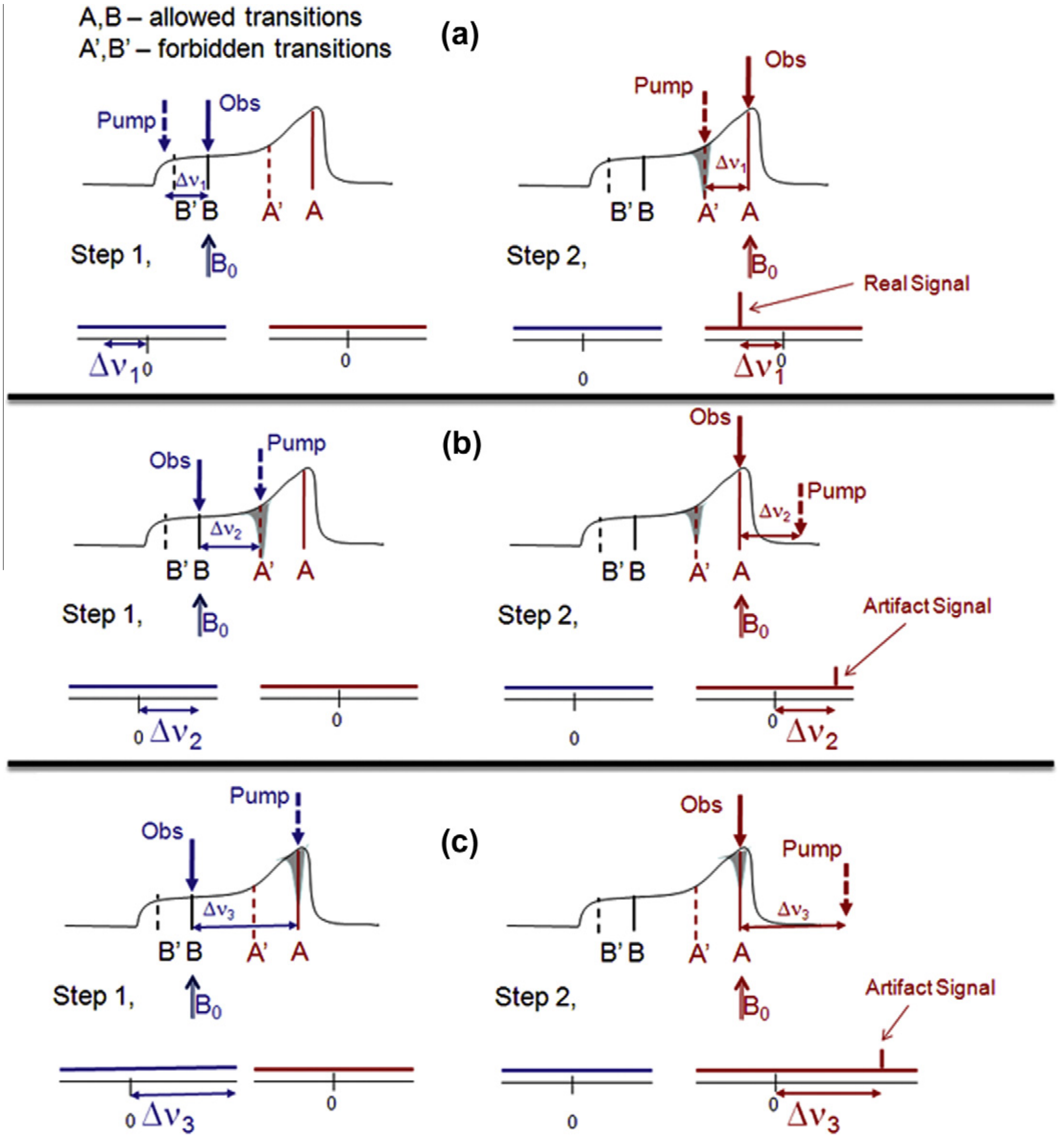

Fig. 7. A scheme explaining the source of (a) "normal" spectral lines of ELDOR–detected NMR spectra recorded with parallel acquisition, (b) one type of artifact and (c) another type of artifacts that may appear such spectra. Solid line – allowed transitions, dashed line forbidden transitions, solid arrow – position of observer pulse, dashed arrow – position of pump pulse. See text for details.

inductance. In our set-up the sweep range of the rapid sweep coils is 40 mT, which is twice the width of the nitroxide spectrum at W-band. The settling time in our system for a field jump of 10 mT is <500 μs, measured from the time of the trigger to the current driver to the time the field is stable enough to allow the pulsed EPR measurement.

Another hardware bottleneck one has to be aware of is the rate of the signal acquisition. The echo intensity is normally measured by either a boxcar integrator and an Analog to Digital Converter (ADC) or a digitizer. The digitizer seems to be better suited for the parallel acquisition scheme since it acquires data continuously with high time resolution. In our system the time that it takes to zero boxcar integrator is ~1.5–2 ms and therefore no more than six parallel experiments are plausible if the repetition time of a single experiment is 10 ms.

The two above considerations that limit the number of experiments acquired in parallel become even more significant for paramagnetic centers with shorter relaxation times such as transition metal ions. For Cu^{2+} and VO^{2+} measurements at 6 K parallel acquisition will be useful and can be implemented already at X-band because of the large *g* and hyperfine anisotropies, respectively. It should be mentioned that with the modern developments of high power current drivers, which give long, tens of amperes current pulses it should be possible also to implement parallel acquisition scheme for the paramagnetic centers with shorter relaxation time such as high spin systems. In the particular case of Mn^{2+} ($S = 5/2$, $I = 5/2$) it should be possible to acquire in parallel experiments with the fields set to each of the six hyperfine components of the central $|-1/2\rangle \rightarrow |1/2\rangle$ transitions, which at high fields are often intense and narrow. These can then be added improving the S/N by a factor of $(6)^{1/2}$, which is equivalent to a reduction of measurement time by a factor of 6. The total field range required for this is ~45 mT.

The last experimental issue we want to discuss here is the heating introduced by the rapid sweep coils. With our helium cooled system with a flow cryostat we were able to operate the rapid sweep coils down to the 6 K. If one wants to perform these experiments at even lower temperatures the construction of the superconducting rapid sweep coils should be considered. On our setup it takes about 2 min from turning on the rapid sweep coils, until the temperature is stable enough for measurements and no further phase drift is observed.

4. Conclusions

We proposed and demonstrated a novel approach for the acquisition of several pulse EPR spectra at different fields within the inhomogeneously broadened EPR spectrum. It provides an efficient utilization of the pulse EPR spectrometer time, reducing the experiment time by a factors of 3–6 depending on the experiment. Because most pulse EPR experiments are carried out at low temperatures and require cooling with liquid He, this improvement is manifested also in cost effectiveness. The applicability of this approach was demonstrated on three types of orientation selective hyperfine spectroscopy experiments, ENDOR, ESEEM and ELDOR–detected NMR as well as for electron–electron distance measurements with the four-pulse DEER sequence. The results show that the same spectral information compared to the traditional approach is obtained at a much shorter time.

5. Experimental

The field jump set-up: For the parallel acquisition experiments we used rapid sweep coils and a current generator described earlier [26] with slight modifications. In the present set-up we used two high current operational amplifiers (Burr–Brown (Texas-Instruments), OPA549T) connected in parallel to the two 30 turns Helmholtz coils. The characteristic of this set-up are: maximum sweep rate 0.17 mT/μs, maximum sweep range 40 mT, stabilization time <500 μs. The SpecMan4EPR software [27] controlling the spectrometer had been modified to accommodate parallel acquisition.

Samples: For the DEER experiments we have used a rigid biradical with an inter-spin distance of 3.6 nm [28] dissolved in o-terphenyl (0.5 mg/0.2 g, which corresponds to ~2.5 mM). For the ELDOR–detected NMR, ENDOR and ESEEM measurements a 2.5 mM solution of 4-hydroxy-TEMPO in toluene was used.

Spectroscopic measurements: All EPR experiments were performed on the home build W-band (94.9 GHz) EPR spectrometer [23]. Echo-detected EPR spectra were recorded with the two pulse echo sequence, $\pi/2$-τ-π-τ-echo, where the magnetic field is swept. The mw pulses were $t_{\pi/2} = 60$ ns and $t_\pi = 120$ ns, and $\tau = 400$ ns or $\tau = 550$ ns.

Davies ENDOR [29] spectra were acquired with a pulse sequence shown on Fig. 2a where the frequency of the RF pulse was varied between 136 and 152 MHz and the random acquisition mode [25] was applied. The length of the RF pulse was 30 μs, $\tau_d = 5$ μs, $t_{\pi/2} = 100$ ns, $t_\pi = 200$ ns and $\tau = 400$ ns. The repetition time was 10 ms for independently acquired spectra. For spectra acquired in parallel the repetition time was increased to 16 ms to accommodate ten 1.5 ms intervals, the delay from the beginning of each step to the acquisition was 1.2 ms, the ΔB_0 step was 0.7 mT and 10 spectra were acquired per one RF frequency setting. All ENDOR spectra were normalized from 0 to 1 to facilitate the comparison. The temperature was 40 K.

Three-pulse ESEEM [17] were acquired with the pulse sequence shown in Fig. 2b where the time interval *T* was incremented from 50 ns in steps of 12.5 ns. Other parameters were: $\tau = 400$ ns, $t_{\pi/2} = 20$ ns, repetition time 10 ms. For data acquired in parallel the repetition time was increased to 16 ms to accommodate ten 1.5 ms intervals, the delay from the beginning of each step to the acquisition was 1 ms and the ΔB_0 step was 1.6 mT, 10 spectra were acquired per one T setting. To obtain the FT ESEEM spectra the maximum intensity was normalized to unity, background decay was removed by subtracting a 3rd order polynomial, the resulting data set was zero filled to 512 points, apodized by a Hamming function and fast Fourier transformed to obtain final spectra. The spectra are displayed in the magnitude mode. The temperature was 40 K.

ELDOR–detected NMR spectra [30] were acquired with the pulse sequence shown in Fig. 2c where the length of the pump pulse was 500 μs, $t_{\pi/2} = 200$ ns, $t_\pi = 400$ ns, $\tau_d = 10$ μs and $\tau = 500$ ns. The ΔB_0 step was 0.208 mT with a length of 2 ms and a settle time of 1.3 ms. The ELDOR–detected NMR spectra were acquired in a series of five acquisitions per one pump pulse frequency setting. The temperature was 40 K.

The four-pulse DEER sequence [31] (Fig. 2d) was employed with a frequency separation of 65 MHz between the observe and pump pulses and the length of the pulses were $t_{\pi/2} = 40$ ns and $t_\pi = 80$ ns and the pump pulse duration was 25 ns. DEER traces were sums of eight τ_1 values starting with 350 ns and a step of 12.5 ns and a 8 step phase cycle was implemented: ($\pi/2_{\mathrm{obs}}$ +*x*, −*x*, +*x*, −*x*, +*x*, −*x*, +*x*, −*x*; π_{obs} +*x*, +*x*, +*x*, +*x*, −*x*, −*x*, −*x*, −*x*; π_{pump} +*y*, +*y*, −*y*, −*y*, +*y*, +*y*, −*y*, −*y*; π_{obs} +*x*, +*x*, +*x*, +*x*, +*x*, +*x*, +*x*, +*x*; receiver +, −, +, −, +, −, +, −). The extensive phase cycle was mainly implemented to suppress hardware artifacts and baseline offsets. The observe and pump pulses are incoherent and there is no relation between the *x* and *y* phases. The repetition time was 25 ms. For DEER traces acquired with the parallel acquisition scheme the ΔB_0 field step was 5.1 mT and the length of each step was 2 ms and allowing for 1.7 ms settling time. The overall repetition time, T_R, was kept at 25 ms and the temperature was 19 K.

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