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Optimization of pulsed DEER measurements for Gd-based labels: choice of operational frequencies, pulse durations and positions, and temperature

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Abstract

In this work, the experimental conditions and parameters necessary to optimize the long-distance (~ 60 Å) Double Electron-Electron Resonance (DEER) measurements of biomacromolecules labeled with Gd(III) tags are analyzed. The specific parameters discussed are the temperature, microwave band, the separation between the pumping and observation frequencies, pulse train repetition rate, pulse durations and pulse positioning in the electron paramagnetic resonance spectrum. It was found that: (i) in optimized DEER measurements, the observation pulses have to be applied at the maximum of the EPR spectrum; (ii) the optimal temperature range for K_a-band measurements is 14–17 K, while in W-band the optimal temperatures are between 6–9 K; (iii) W-band is preferable to K_a-band for DEER measurements. Recent achievements and the conditions necessary for short-distance measurements (<15 Å) are also briefly discussed.

Keywords

Gd(III) spin labels; long distance pulsed DEER; K_a -and W-microwave bands

1. Introduction

The utility and potential of Gd(III)-based high-spin labels (GdL) for distance measurements by Double Electron Electron Resonance (DEER) have been demonstrated in a number of reports on model compounds, DNA and proteins [1–3]. This new family of spin labels is particularly useful at the microwave (mw) frequencies higher than the conventional X-band frequencies, at which the DEER measurements on nitroxide pairs are well established. The GdLs are especially attractive because of their lack of orientational selectivity, high effective B_1 due to the high spin ($S = 7/2$), and the possibility to access shorter distances due to the lack of exchange interaction and a broad EPR spectrum.

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From the first experiments using GdLs, it has become apparent that the realization of their full potential is not as straightforward as in case of the nitroxide labels [4–6] due to the considerably larger number of parameters that affect the absolute DEER effect, $\eta = \lambda V$ (where λ is the probability of flipping the spins by the pumping pulse, and V is the amplitude of the electron spin echo (ESE) signal generated by the observation pulses [1]). Among additional factors affecting η , as compared with nitroxides, are: redistribution of state populations with temperature; the dependence of the linewidth of the central $-1/2 \leftrightarrow 1/2$ transition on the choice of Gd(III) ligand and operational frequency; the possibility of using short pumping and observation pulses without spectral overlap; the variety of options for positioning of the pumping and observation pulses in the EPR spectrum.

In this work, therefore, using rather basic considerations, we will try to set a route for determining the optimal experimental conditions for maximizing either η , or the η -to-noise ratio. Such an optimization, while not particularly important for short and middle distance measurements, is critical for long distance measurements, when the labels are over 60 Å apart.

2. EPR spectrum of GdLs and the evaluation of signal amplitude and spin flip probability

The evaluation of η is based on the shape and structure of the EPR spectrum of the Gd(III) ions, which are mostly determined by the crystal field interaction (*cfi*). The key *cfi* parameter, D , varies for commonly used GdLs within rather narrow limits: $D/g\beta \in [20, 50]$ mT [7,8]. For the purpose of this work, it is sufficient to address only two cases, the upper and lower limits of D , and to interpolate the results for the intermediate D -values.

The Gd(III) complexes can be adequately characterized by the D and E parameters that account for the quadrupolar part of the *cfi* [9]. Higher-order terms, although present for Gd(III), are substantially smaller and can be safely neglected [10]. The fine-structure spin Hamiltonian in the laboratory coordinate frame **XYZ** is thus:

$$H_{fs} = g\beta B_0 S_z + D \left\{ S_{z_c}^2 - \frac{1}{3} S(S+1) \right\} + E(S_{x_c}^2 - S_{y_c}^2) \quad (1)$$

where **X_C**, **Y_C** and **Z_C** are the principal axes of the *cfi*. Here we only consider the high-field approximation, for which $D/g\beta B_0 \ll 1$. Taking into account that $D/g\beta \approx 50$ mT for all known Gd(III) complexes used in GdLs, this approximation is well satisfied for K_a-band ($B_0 \approx 1$ – 1.3 T) and higher-frequency mw bands. As it is evident from Eq (1), in an orientationally disordered situation with an axial *cfi* tensor ($E = 0$), the singularities of the $m_S \leftrightarrow m_S + 1$ transition line, to first order in *cfi*, are located at:

$$B_0^\perp(m_s) = \frac{h\nu_{mw}}{g\beta} + \frac{D}{2g\beta}(2m_s + 1) \quad \text{and} \quad B_0^\parallel(m_s) = \frac{h\nu_{mw}}{g\beta} - \frac{D}{g\beta}(2m_s + 1) \quad (2)$$

where h is Planck's constant, ν_{mw} is the mw frequency, and $B_0^\perp$ and $B_0^\parallel$ are the resonance magnetic fields corresponding to the perpendicular and parallel orientations of the principal *cfi* axis with respect to the magnetic field vector, **B₀**. The total width of the $m_S \leftrightarrow m_S + 1$ transition line is thus:

$$\Delta B_0(m_s) = |B_0^\perp - B_0^\parallel| = \frac{3}{2} \frac{D}{g\beta}(2m_s + 1) \quad (3)$$

and the total width of the EPR spectrum (the difference between $B_0^{\parallel}$ values of the outermost transitions) is $12D$. The total spread of the Gd(III) spectrum is therefore expected to be somewhere between 0.25 T and 0.6 T for different complexes. Derived in the first order of perturbation theory, Eq. (2) allows one to evaluate the width of all transitions except the $-1/2 \leftrightarrow 1/2$ transition. For the latter, the second order approximation in cfi (for K_a -band and higher) is sufficient to correctly reproduce the lineshape [8,11]. The characteristic width of this line was found to be approximately $D^2/(g^2\beta^2B_0)$.

For a general rhombic cfi tensor ($E/D \in [0, 1/3]$), the positions of singularities in the EPR spectrum depend on the E/D ratio [12,13]. In the experimental spectra, however, no singularities are generally observed [8]. The spectra are usually very smooth and virtually featureless, except for the prominent central line of the $-1/2 \leftrightarrow 1/2$ transition. The lack of cfi -related singularities in the experimental spectra was explained by broad statistical distribution of the cfi parameters [8]. The physical model introduced in [8] allowed us to reproduce the experimental spectra of the Gd(III) complexes in glassy samples. This model was similar to the superposition approximation of Newman and Urban [14], and was based on the assumptions that (i) the cfi tensor associated with a single ligand is axial and (ii) the cfi tensors of several ligands are additive. The distributions of D in this case are bimodal and can be approximated by two Gaussians with the maxima at $\pm D_c$, where D_c is the central D -value for each of the Gaussian components. The distribution of E/D , $P(E/D)$, in this model can be approximated as

$$P(E/D) \propto (E/D) - 2(E/D)^2 \quad (4)$$

Using this model, we performed numerical simulations of the EPR spectra varying D , temperature, and ν_{mw} . The representative examples of subspectra for various EPR transitions simulated for $D/g\beta = 50$ mT, $T = 20$ K, and the W-band ν_{mw} are shown in Figs. 1a and 1b. In order to demonstrate the line shape dependence on ν_{mw} (B_0), Fig. 1b also shows the subspectrum of the $-1/2 \leftrightarrow 1/2$ transition simulated for K_a -band. Amplitudes of the EPR lines are directly proportional to the differences in state populations.

While the cfi is the major factor responsible for the EPR spectrum shape, there are additional minor factors such as the broadening due to the unresolved hyperfine interaction (hfi) with ligand and matrix nuclei and with magnetic isotopes of Gd (^{155}Gd and ^{157}Gd , $\sim 30\%$, $I = 3/2$). To account for these factors, the simulated spectra were convoluted with a Gaussian line with a width between the maximum slope points of 1 mT. Obviously, such a convolution only affects the shape of the relatively narrow line of the $-1/2 \leftrightarrow 1/2$ transition, making it similar to that observed experimentally.

The simulated EPR spectra represent a starting point for estimating the optimal absolute DEER effect, $\eta = \lambda V$. The spin flip probability, λ , and the ESE signal amplitude, V , will be separately evaluated below.

(a) ESE signal amplitude

In the commonly used experimental setup of the four-pulse DEER [6], the refocused primary ESE signal is detected using a boxcar integrator. The shape of the refocused primary ESE, $V(t)$, is [15]:

$$V(t) \propto \int d(\Delta\omega) G(\Delta\omega) (\alpha_1 \beta_1) (\beta_2^*)^2 \beta_3^2 e^{-i\Delta\omega t} \quad (5)$$

$$\alpha_k = \cos\theta_k - i\cos\phi_k \sin\theta_k; \quad \beta_k = -i\sin\phi_k \sin\theta_k$$

$$\theta_k = \frac{\omega_{Nk} t_{pk}}{2}; \quad \tan\phi_k = \omega_{1k} / \Delta\omega; \quad \omega_{Nk} = \sqrt{\omega_{1k}^2 + \Delta\omega^2}$$

In this expression, ω_{1k} and t_{pk} are the amplitude and duration of the k -th observation pulse, $\Delta\omega$ is the frequency offset from the resonance, $G(\Delta\omega)$ is the EPR spectrum amplitude at the given offset, and t is the spin echo coordinate. The mw amplitude, ω_{1k} , is proportional to the square root of incident mw power and to the transition matrix element. The acquired signal, V_o , is the integral over t :

$$V_o = \int_{-\infty}^{\infty} V(t) dt \propto \int_{-\infty}^{\infty} V(t) dt \int d(\Delta\omega) G(\Delta\omega) (\alpha_1 \beta_1) (\beta_2^*)^2 \beta_3^2 e^{-i\Delta\omega t} = \int_{-\infty}^{\infty} dt e^{-i\Delta\omega t} \int d(\Delta\omega) G(\Delta\omega) \alpha_1 \beta_1 (\beta_2^*)^2 \beta_3^2 \propto G(0) \sin\theta_1^0 \sin^2\theta_2^0 / 2 \sin^2\theta_3^0 / 2 \quad (6)$$

where $G(0)$ is the EPR spectrum amplitude at $\Delta\omega = 0$, i.e., in resonance with $\omega_{mw} = 2\pi\nu_{mw}$, and $\theta_k^0 = \omega_{1k} t_{pk}$ are the nominal turning angles of the mw pulses. Eq. 6 implies that the acquired ESE signal, V_o , is proportional to $G(0)$ and to the product of the sines of the *nominal* turning angles. (In the experiment, the width of the boxcar gate is usually finite. The ‘truncated’ V_o remains, however, proportional to V_o , as can readily be seen from the echo simulations presented in the Supplementary Materials (SM)). As discussed above, the spectrum of Gd(III) ions is a sum of subspectra of various transitions. For these transitions, the θ_k^0 values at a given incident mw power are different, although their ratios are determined by transition matrix elements and fixed. It is evident from Eq. 6 that the first maximum of the ESE signal from any particular subspectrum is reached at the nominal turning angles ($\pi/2$, π , π). Due to different transition probabilities, it is impossible to simultaneously achieve the maximum for all of the subspectra. It is quite simple, however, to find the optimal signal (as is always done in an experiment, by adjusting the incident mw power), since the ratios of the nominal turning angles of different transitions are known and fixed, while the shape and the relative statistical weight of each subspectrum is already calculated in the simulation. Therefore, V_o is evaluated as a maximum of the sum of the individual signals, by means of tuning the respective sets of θ_k^0 . As an example, Fig. 1c shows the spectra obtained at the turning angles tuned to maximize the observable ESE signal at the center of the EPR spectrum and at a position shifted from the center by ~ 10 mT.

(b) Spin flip probability

A similar procedure can be applied to evaluate the maximal spin flip probability at the pumping position. It is well known [16] that the first maximum of the flip probability is reached when the nominal flip angle of the pumping pulse, θ_p^0 , is about π . As discussed above, identical nominal flip angles cannot be obtained simultaneously for all of the subspectra. Therefore, the maximum overall flip probability has to be evaluated using the procedure described below.

Before starting the evaluation, we should mention that even for the shortest pumping pulse (5 ns) used in our experiments, the B_1 amplitude (~ 3 mT) is substantially smaller than the characteristic width of any subspectrum, except that of the $-1/2 \leftrightarrow 1/2$ transition. While the frequencies of the $m_s \leftrightarrow m_s + 1$ and $m_s \leftrightarrow m_s - 1$ transitions of any given spin may become very close to each other or coincide within a certain (very narrow) range of orientation of the *cfi* frame relative to $\mathbf{B}_0$, for the most part, the difference between these transition frequencies exceeds the pumping pulse amplitude. Therefore, we can safely neglect the possibility of

multi-quantum transitions and assume that the $m_s \leftrightarrow m_s + 1$ and $m_s \leftrightarrow m_s - 1$ transitions are contributed to by different spin sub-ensembles. This allows one to evaluate the normalized (by population) flip probability, $(\lambda')^{ms}$, from the m_s state as the sum of partial probabilities, $(\lambda')^{ms \leftrightarrow ms+1} + (\lambda')^{ms \leftrightarrow ms-1}$. Such an approach should probably result in a slight overestimate of the flip probability when the pumping pulse is in resonance with the maximum of the EPR spectrum, since the probability of multi-quantum transitions around this spectral position is maximal. Such a possible overestimate, however, does not affect the conclusions of this paper, and we will continue to evaluate the flip probability in such a simple manner. The partial probability $(\lambda')^{ms \leftrightarrow ms+1}$ is calculated in the standard manner as:

$$(\lambda')^{ms \leftrightarrow ms+1} = \int d(\Delta\omega) G^{ms \leftrightarrow ms+1}(\Delta\omega) (\beta_p^* \beta_p) \quad (7)$$

where β is defined in Eq. 5 (the subscript “p” stands for “pumping”), and $G^{ms \leftrightarrow ms+1}(\Delta\omega)$ is the shape of the normalized subspectrum of the corresponding transition. The total flip λ^t , is therefore

$$\lambda^t = \sum n_s (\lambda')^{m_s} \quad (8)$$

where n_s is the population of the respective state. Then, as was done in the echo signal evaluation, the first maximum of λ^t is determined by varying (around π) the nominal flip angles in Eq. 7. Although the entire procedure seems to be complicated, it is actually not, since $(\lambda')^{ms \leftrightarrow ms \pm 1}$ varies only weakly within the interval of nominal flip angles from 150° to 210° .

Evaluation of the absolute DEER effect, η —To evaluate η , one needs to include the constraints imposed by the properties of the biomacromolecules and by the instrumentation. The contemporary home-made pulsed K_a^- and W-band instruments (see e.g. [17,18]) allow the minimal duration of a nominal π -pulse to be set somewhere between 5 and 10 ns. It was also reported [19] that commercial Bruker instruments allow the setting of similar minimal pulse durations, at least for one of the $Q(K_a)$ band instrument options. The parameters of commercial W-band instrument are not yet as good as [18], but nevertheless, are under constant improvement [20]. It seems reasonable, therefore, to define the lower and upper limits of the pulse durations in our analysis as 5 and 10 ns. Since V_o does not depend on the pulse duration (see Eq. 6), the duration of the observation pulses, t_p^o , is only limited by the characteristic phase relaxation time, T_m . In protonated matrices, T_m of Gd(III) ions is about 1–2 μ s, and the sensible upper limit of t_p^o can be set at 100–200 ns to avoid unnecessary loss of the signal due to the phase relaxation. The finite duration of the observation pulses, however, effectively introduces a distortion in the time domain pattern, as described in refs [6,21]. To avoid the distortion, the observation pulse durations should not exceed ~10% of the period, t_m , of the dipolar modulation. The latter is related to the interpair distance as:

$$t_m \cong 2 \frac{r^3}{100} (ns) \quad (9)$$

where the distance, r , is in Å. Currently, we usually work with compounds in which the minimal average separation between the labels is ~13–15 Å. To be able to detect such a distance in DEER, the observation pulse duration should be ~5 ns. Therefore, depending on the distance between the labels, the duration of the observation pulses can be set somewhere between 5 ns and 100–200 ns.

The durations of the pumping and observation pulses stipulate the necessary frequency separation, $\Delta\nu$, between the pumping (ν_p) and observation (ν_o) mw frequencies, and accordingly, the Q -value of the resonator, and the pumping and observation EPR positions. The required $\Delta\nu$ can be evaluated from the frequency profiles (spectra) of the pumping pulse and the refocused echo. Such spectra are presented in Fig. 2. Acceptable lack of interference between the pumping and observation pulses can be achieved when the edge of the echo spectrum is set at first “zero” of pumping pulse spectrum, as is shown in Fig. 2. The trivial calculations show that for such a situation $\Delta\nu \cong 0.87/t_p^p + 1/(2t_p^o)$. (Note that the profiles presented in Fig. 2 were calculated for ideal rectangular pulses. Real pulses, which have a finite ‘rise/fall’ time or specially shaped ‘smooth’ pulses generate less prominent sidebands. The $\Delta\nu$ evaluated above may be considered, therefore, as an upper limit for necessary frequencies separation). Therefore, depending on the durations of the pulses, $\Delta\nu$ should be maintained between ~ 300 MHz ($t_p^p = t_p^o = 5$ ns) and ~ 100 MHz ($t_p^p = 10$ ns and the observation pulse is long). One more constraint that is imposed by the DEER measurement is temperature, which, for the reasons discussed below, should be kept between 5 – 20 K. The calculations of η were performed for two mw bands (K_a and W) and for various positions of pumping and observation pulses, various pulse durations, and temperatures, within the limits defined by these constraints. We discuss below the data obtained for each mw band.

3. Optimal conditions for DEER measurements

a. K_a -band

Figure 3 presents some of the results of $\eta(T)$ calculations performed for $D/g\beta = 50$ mT and for positioning of the pumping and observation frequencies in resonance with parts of the EPR spectrum marked as “a”, “b” and “c” in the inset. (At this stage, η does not incorporate limitations imposed by phase and spin lattice relaxation. The effect of these factors on η is discussed below.) For dependences 1 and 2, the pumping frequency (ν_p) was in resonance with the maximum of the EPR spectrum (position “a” in the inset), and the pumping pulse had durations of 5 and 10 ns, respectively. The observation frequency (ν_o) was set at $\nu_p + 100$ MHz or $\nu_p + 300$ MHz, in resonance with positions “b” and “c”, respectively. For lines 3 and 4, ν_o was set in resonance with the EPR position “a”, while ν_p was set in resonance with positions “b” and “c” for pumping pulses of 10 ns and 5 ns, respectively. (In accordance with the $\Delta\nu$ evaluation discussed above, the 100 MHz separation between the pumping and observation frequencies corresponds to long observation pulses and a 10 ns pumping pulse, while $\Delta\nu = 300$ MHz corresponds to a situation when $t_p^p = t_p^o = 5$ ns). As follows from Fig. 3, so far the maximal η is apparently reached when ν_o is set at the *maximum* of the EPR signal, and the *shortest* (5 ns) pumping pulse is applied.

Obviously, the refocused primary echo signal used for the observation is subject to phase relaxation. The phase relaxation of GdLs at the temperatures (and GdL concentrations) used for the DEER experiment is caused by random modulation of electron-nuclear interaction by means of nuclear spin diffusion, and does not depend on temperature. It depends, however, on the magnitude of the electron-nuclear interactions, which, in turn, depend on the observation position due to variation in the composition of the EPR spectrum. In W-band, the composition of the observed signal can substantially vary with temperature, which can result in implicit temperature dependence of the phase relaxation. This effect should be taken into account in fine optimization of W-band measurements. Nevertheless, it is generally true that the echo signal collected at the center of the spectrum decays slower than that collected at other field positions, at least up to a pulse separation of about 10 μ s.

Due to, commonly, non-exponential phase relaxation, we chose the 10 μ s time base as a benchmark because the pulse separation in known DEER experiments rarely exceeds this value [22] (some examples of primary echo kinetics collected at different observation positions are shown in the Supplementary Materials, SM). The introduction of phase relaxation into DEER optimization does not change, therefore, the conclusion about the optimal pulse settings made in the first paragraph of this Section. There are, however, some additional factors that should be considered.

In commonly used single mode volume resonators (SMVR), if the measurements are thoroughly optimized, then the Q value of the resonators has to be set to accommodate the required frequency separation between the pumping and observation frequencies, $\Delta\nu$, and $\Delta\nu \propto 1/Q$. The detected signal amplitude, on the other hand, is proportional to Q . Therefore, for fair comparison of $\eta(T)$ presented by curves 3 and 4 in Fig. 3, the former has to be multiplied by $\sqrt{3}$, that is, the square root of the ratio of the respective frequency separations or Q -values. Such a correction transforms line 3 into line 3' that lies above line 4 over the entire temperature range, indicating that *longer* pumping (10ns) and long observation pulses are preferable in optimal DEER measurements. Calculations performed for $D/g\beta = 20$ mT resulted in a similar conclusion, although the absolute values of η were found to be about three times higher due to the more narrow spectrum.

The next factor that is critical for η optimization is related to the spin-lattice relaxation. The standard signal acquisition procedure in pulsed EPR is to repetitively accumulate the ESE signal. If noise is independent of the signal, then the signal-to-noise ratio (S/N) increases as $\sqrt{N}$, where N is a number of accumulations. The optimal pulse repetition rate (RR), RR_{opt} , depends on the spin-lattice relaxation rate. It is empirically (and commonly) chosen as $RR_{opt} = (t')^{-1}$, where t' is the time interval between the pulse trains that allows the magnetization to recover to ~95% of its equilibrium value. The optimal repetition time, t' , is temperature-dependent, and the next step in optimization of DEER measurements is to obtain the temperature dependence of t' .

The theory of spin-lattice relaxation is complicated and lacks quantitative predictive power [9]. The only way to judiciously obtain the temperature dependence of t' is to measure it. Since t' may depend not only on temperature, but on D -values as well, measurements were performed for commonly used GdLs, such as Gd538, Gd595, MS-325 and GdDOTA [1,7], whose D -values cover the range from $D/g\beta \sim 20$ mT (GdDOTA) to ~ 50 mT (MS-325). The structures of these compounds are presented in SM. For these measurements, we employed the inversion recovery technique, in which a short inversion pulse (8 ns) and long observation pulses (~ 40 ns) were used. The samples were water-glycerol (1:1, v:v) solutions of the respective GdLs.

The concentration of GdLs did not exceed 50 μ M, in order to make the effects of spectral diffusion negligible, while keeping the ESE signal strong enough to enable robust measurements. The measurements were performed within the temperature interval from 6 K to 20 K because below 6 K it is difficult to maintain a stable temperature using available cryogenic equipment [23]. Representative results from these measurements are shown in Fig. 4 and in the SM. It was found that the dependence of the magnetization recovery on t , $\ln f(t)$, is not linear (see SM, Section 2). This indicates that the spin-lattice relaxation rates are distributed rather than characterized by a single $(T_1)^{-1}$ [24]. To good accuracy, these kinetics can be approximately linearized in the coordinates $(\ln f(t), t^{0.7})$. The collected data on the magnetization recovery allow one to find the temperature dependence of the optimal repetition time, $t'(T)$. The typical dependences of $RR_{opt} \equiv (t')^{-1}$ on temperature are presented in Fig. 4.

As follows from simple analysis of the data presented in Fig. 4, the temperature dependence of RR_{opt} of Gd595 and Gd538, can be empirically expressed (in Hz) over the studied temperature range as $RR_{\text{opt}} = 2.31 \cdot T^{2.46}$ and as $RR_{\text{opt}} = 3.15 \cdot T^{2.33}$, respectively. Similar dependences were found for GdDOTA and MS-325. In general, the RR_{opt} dependence on temperature can be expressed as $RR_{\text{opt}} = \alpha \cdot T^{\rho}$; $2.5 \leq \rho \leq 2.3$. Comparison of the data obtained for high and low D (MS-325 and GdDOTA) show that α does depend on D , but this dependence is not significant and can be approximated as $\sim \sqrt[3]{D}$. Therefore, the optimal repetition rate, RR_{opt} , in DEER measurements depends on the temperature, on average, as $T^{2.4}$, and the S/N ratio due to this factor will increase with temperature in proportion with $T^{1.2}$.

The optimal repetition rate can now be incorporated into the *adjusted* absolute DEER effect, $\eta^a(T)$ so $\eta^a(T) = \eta(T) \sqrt{RR_{\text{opt}}} \propto \eta(T) \cdot T^{1.2}$. Assuming that the experimental temperature dependence of RR_{opt} remains valid down to 5 K, $\eta^a(T)$ can be readily expressed as $\eta^a(T) = \eta(T) \cdot (T/5)^{1.2}$, reflecting the relative correction of $\eta^a(T)$ for the optimal repetition rate in the temperature range between 5 K and 20 K. Fig. 5. shows $\eta^a(T)$ derived from $\eta(T)$ presented in curve 3' in Fig. 3. The $\eta^a(T)$ dependence presented in Fig. 5 shows a broad maximum between 13–17 K, which could suggest the optimal temperature range for DEER measurements in K_a-band. Such a suggestion would, however, be acceptable only if spectral diffusion effects induced by the spin-lattice relaxation are ruled out in this temperature interval.

It is well-known that a *single* arbitrary spin flip during the DEER sequence completely eliminates the DEER effect [24]. The spin flip probability for the longitudinal relaxation process described by a single T_1 is $1 - \exp(-t/T_1)$, and it was shown in [24] that $T_1 \sim 10t_b$ (where t_b is the time base of the DEER measurements) leaves the DEER effect practically unperturbed. Therefore, the temperature of the measurements has to be chosen in such a way, as to ensure that $RR_{\text{opt}} \sim 1/(3T_1) < 1/(30t_b)$. As a result, if in the long-distance measurements the time interval between the observation pulses is $\sim 10 \mu\text{s}$, the temperature should be kept to maintain $T_1 \sim 100 \mu\text{s}$ or longer, which limits RR_{opt} to $\sim 3.3 \text{ kHz}$ and the temperature to 18–20 K, in accordance with the data presented in Fig. 4. However, if the spin-lattice relaxation times are distributed, then RR_{opt} has to be decreased as compared with the case of a single T_1 [24]. Such a situation has been analyzed in [24] for the case of spin-lattice relaxation rates ($W \propto 1/T_1$) complying with the Margenau distribution:

$$\phi_M(W) = \sqrt{\frac{3W^*}{2\pi}} \frac{1}{W^{3/2}} \exp\left(-\frac{3W^*}{2W}\right) \quad (10)$$

resulting in the magnetization recovery kinetics $f(t)$:

$$f(t) = \int_{-\infty}^{\infty} \phi(W) \exp(-Wt) dW = \exp(-\sqrt{6W^*t}) = \exp(-\sqrt{3} \sqrt{t/T_1^*}) \quad (11)$$

that is $\ln(f(t)) \propto t^{0.5}$. In this case, the “most probable T_1 ” should be maintained at $\sim 40t_b$, in order to avoid unwanted distortions of the DEER effect. The experimental dependence of $\ln(f(t)) \propto t^{0.7}$ suggests that the distribution of spin-lattice relaxation rates for Gd(III) is more narrow than $\phi_M(W)$ and the “effective T_1 ” should likely be maintained somewhere between $10t_b$ and $40t_b$. One can conclude that for the long-distance measurements, the temperature has to be maintained to allow $RR_{\text{opt}} \sim 1\text{--}1.5 \text{ kHz}$, which limits the temperature range to 11–16 K. Coincidentally, this range is very close to the temperature interval where $\eta^a(T)$ reaches its maximum, as evident from Fig. 5.

Thus, the above analysis allowed us to determine the optimal conditions for *long-distance* DEER measurements in K_a -band: pulse durations and positioning, separation between the pumping and observation frequencies, and the temperature. One of the findings, i.e. the setting of the observation frequency (rather than the pumping frequency) in resonance with most intense part of GdL EPR spectrum, is opposite to the usual frequency setting employed in DEER investigations of nitroxides [6].

b. W-band

The analysis performed above for K_a -band to find optimal η^f was repeated for W-band. Fig. 6 presents some results of $\eta(T)$ calculations for W-band performed for the same parameters as for K_a -band. Although the behavior of $\eta(T)$ in W-band is different from that observed in K_a -band, due to different state populations and the narrowing line for the $-1/2 \leftrightarrow 1/2$ transition (cf. the data presented in Figs. 3 and 6), the optimal DEER effect, η , is reached under the same conditions, that is, when ν_o is in resonance with the *maximum* of the EPR signal and the *shortest* (5 ns) pumping pulse is applied. Similarly, after the Q -value adjustment, the long observation and longer pumping (10ns) pulses become preferable. The signal loss due to the phase relaxation in W-band for the central part of EPR spectrum is smaller than for the off-center positions (at least, for the 10 μ s time base), and is similar to that observed in K_a -band.

The temperature dependence of RR_{opt} in W-band was, as in K_a -band, determined experimentally, and it was found that the magnetization recovery is not exponential. To good accuracy, the recovery kinetics can be linearized in coordinates $(\ln f(t), t^{0.8})$, (see SM, Section 3) close to those observed in K_a -band $(\ln f(t), t^{0.7})$. The dependence of RR_{opt} on the temperature (found from analyzing the recovery kinetics) is presented in Fig. 7. As follows from comparison of Figs. 4 and 7, the temperature dependence of RR_{opt} in W-band is significantly weaker than in K_a -band, while the absolute RR_{opt} at low temperatures is substantially higher. For example, in W-band at 6 K, $RR_{opt} \approx 1.6$ kHz (cf. ~ 200 Hz in K_a band), while at 20 K $RR_{opt} \approx 3.5$ kHz for both mw bands. The reason for such a difference is not clear yet and, although intriguing, is certainly beyond the scope of this paper.

The adjusted DEER effect, $\eta^a(T) = \eta(T) \sqrt{RR_{opt}(T)/RR_{opt}(5K)}$, is presented in Fig. 8. As follows from Fig. 8, in W-band, apparently, a higher measurement temperature results in a better S/N in DEER measurements. However, the higher temperature could be prohibitive due to the adverse effects of spectral diffusion (*vide supra*). The non-exponential magnetization recovery indicates that in W-band, as in K_a -band, T_1 is statistically distributed, although this distribution is probably more narrow. Therefore, for *long-distance DEER measurements* that require the time base of $t_b \sim 10 \mu$ s, the measurement temperature should be maintained to provide for $RR_{opt} \sim 1.5 - 2$ KHz (assuming that in W-band RR_{opt} could be slightly higher than in K_a -band due to the more narrow T_1 distribution). Such RR_{opt} values are realized at the temperatures of 6 – 9 K (see Fig. 7).

Thus, the optimal conditions for long-distance DEER measurements in W-band are similar to those found for K_a -band and require similar inverted (as compared with the DEER of nitroxides) pulse settings. The main difference between two bands is in the different temperature ranges for optimal measurements. With this, the analysis of optimal condition for “in-band” DEER measurements is completed. The obvious next step is to examine, which mw band is preferable for DEER measurements.

c. Comparison of the η^a values between the bands

To compare the η^f values from different bands, one needs to account for additional ν_{mw} – dependent parameters which were omitted in the “in-band” calculations. To understand what

is missing, we will consider a general expression for the power, P_e , generated by the precessing magnetic momentum [25]:

$$P_e \propto \nu_{mw} C^2 f(T, \nu_{mw})^2 \alpha Q_L V_s \quad (12)$$

In this expression, ν_{mw} is the operational mw frequency, C is spin concentration, $f(T, \nu_{mw})$ is the population difference between states, and α , Q_L and V_s are the filling factor, loaded Q -value, and sample volume, respectively. For similar types of resonators, the filling factor, α , is independent of ν_{mw} and can be omitted in following discussion. The sample volume, $V_s \propto \nu_{mw}^{-3}$, and $Q_L \propto \nu_{mw}$. The amplitude of the transient signal, $V_0 \propto P_e$ and therefore:

$$V_0 \propto C f(T, \nu_{mw}) / \sqrt{\nu_{mw}} \quad (13)$$

In our simulations of the EPR spectra, the population difference, $f(T, \nu_{mw})$, has already been explicitly included. Assuming that the concentration of GdLs in the sample is the same in K_a - and W-band measurements, the 'inter-band' adjustment factor becomes simply

$\sqrt{\nu_{mw}^{K_a} / \nu_{mw}^W} \approx 1 / \sqrt{3}$. An example of optimal η^f values obtained using this adjustment is presented in Fig. 9. As is evident from Fig. 9, the gain in η^f for the measurements performed in W-band as compared to K_a -band is about a factor of 3 for $D/g\beta = 50$ mT, and about half that for $D/g\beta = 20$ mT. The dependence on D is understandable because the line of the $-1/2 \leftrightarrow 1/2$ transition for $D/g\beta = 20$ mT is already rather narrow in K_a -band. The further narrowing of this line in W-band is insignificant, as well as the corresponding increase of its amplitude.

Thus, these estimates indicate that the use of W-band for DEER measurements has a definite advantage over K_a -band under optimal experimental conditions. This advantage and the ~30-fold difference in sample volume, or the number of labeled molecules used in the measurements (for equal concentrations, and also if conventional resonators are employed), could be a decisive factor for choosing this mw band. We have already noticed, however, that so far in the W-band, it is possible to either perform experiments with short pulses [17] or small sample volumes [26,27], but not with both. Nevertheless, it requires only ~10 W of mw power to generate an ~6 ns π pulse in the W-band SMVR, having $Q \sim 300$ if Gd(III) is used as the label. The 2 W commercial solid state W-band amplifiers and protection switches which survive up to 10 W are readily available [28], so it is reasonable to expect that higher power amplifier will appear on the market quite soon, allowing us to perform the experiment *with short pulses and small sample volumes*.

4. Some related experimental data and conclusion

It is understandable that this research remains only partially complete because of the lack of direct quantitative measurements of η^f that would support the conclusions made in Sections 2 and 3. While highly desirable, such measurements are metrological, tedious and quite different from DEER experiments. Even to start, they would require an implementation of a long list of instrumentation modifications, different procedures for sample preparation and improvements in synthetic procedures, which are necessary to account for numerous factors affecting signal amplitude, noise, and spin flip probability. Modified instruments should include a set of resonators with various Q -values (or a variable Q -value resonator), a properly matched mw tract, a means of monitoring the coupling of resonator and VSWR, and a refined pulse preparation unit in which low-power mw leakage is eliminated, etc.

There are, however, reported data in the literature [1, 26], which, although collected in incompletely controlled and not optimal instrumental environments, might qualitatively

support or at least illustrate the conclusions made in Sections 2 and 3. These data can also suggest some ideas about the mw band preferences and a degree of possible further improvements in DEER experiments.

The first dataset presented in Fig. 10 is related to the issue of positioning the pumping and observation pulses. During a DEER investigation of double-labeled DNA in K_a-band [1], a number of measurements at various pumping/observation positions were made, but only the data for the conventional pumping/observation positioning were reported. In Fig. 10, the time-domain pattern recorded for the conventional pulse setting is presented along with the time domain pattern collected for the observation frequency being in resonance with the maximum of the EPR spectrum, and the pumping frequency corresponding to position 'b', as shown in the inset. The data are presented in a format convenient for evaluating the ratio of η^t values for the two sets of pumping/observation pulses, *i.e.*, as a difference between the initial and current signal amplitudes. The purpose of such a presentation is as follows. As is well known, the asymptotic DEER signal is:

$$V(t_b \rightarrow \infty) \equiv V_a = V_0(1 - \lambda^t)e^{-C\lambda^t t_b} \quad (14)$$

when the time base of the measurements is large enough to apparently complete the intra-pair dipolar evolution. For $C\lambda^t t_b \ll 1$

$$V_a \approx V_0(1 - \lambda^t(1 + Ct_b)) \quad (15)$$

and

$$(V_0 - V_a)/(1 + Ct_b) = V_0\lambda^t \equiv \eta \quad (16)$$

In the measurements, the results of which are presented in Fig. 10, all experimental conditions were kept the same, and the decays due to the phase relaxation at a given time base are also about the same. Therefore,

$$\eta_1/\eta_2 = (V_0 - V_a)^{(1)} / (V_0 - V_a)^{(2)} \quad (17)$$

where the indices (1) and (2) indicate a particular set of measurements.

Thus, the ratio of the η -values for two pumping/observation positions shown in the inset equals to the ratio of $(V_0 - V_a)$ at these positions. As is evident from Fig. 10, this ratio equals to 1.44 ± 0.04 . We have also calculated the η -values for these experimental settings using Eqs. 5–9 and found the calculated ratio η_1/η_2 to be about 1.3. The reasonable agreement between the experiment and calculations confirms that DEER of GdLs with the inverted positioning of the pumping and observation pulses allows one to increase the absolute DEER effect.

A more complicated issue is the comparison of the data obtained in different mw bands. The DEER kinetics shown in Fig. 11 were collected for double-labeled DNA with the intent to estimate the long-distance limit in the W-band DEER measurements of GdLs [26]. The W-band instrument used in those measurements is described in detail elsewhere [27]. The K_a-band DEER kinetics are also presented in the same Figure. In both bands, the experiments were performed using the samples of the same concentration of double-labeled DNA (~40 μ M). The temperature of the measurements (~10 K), the separation between the pumping and observation frequencies (~120 MHz), the repetition rate (2 kHz), and the resonator types

were the same in both bands. The “3dB” bandwidths of the resonators were very similar, (~60 MHz), and adequate to accommodate the pumping pulse without substantial distortions. The best pulse parameters achieved at the given experimental conditions were 30 ns ($\pi/2$) for observation pulses in both bands, while the pumping pulses were 12 ns and 15 ns, in K_a - and W-band, respectively. The time bases for the DEER measurements and the data collection times were 7 μ s and 9 μ s, and 120 and 80 min, for K_a - and W-band, respectively. In both cases, the data were acquired using a boxcar integrator, with the echo signal being integrated between the half-height points. The pumping pulse was applied to the maximum of the EPR spectrum. Although the experimental conditions were about the same in both bands, they were quite far from optimal. Nevertheless, the pumping flip probabilities, λ^t , can be compared quantitatively. As is evident from the data presented in Fig. 11, in W-band λ^t is smaller than in K_a band, and the ratio of λ^t values can be estimated as 1:1.24. The calculation using Eqs. 7–9 results in this ratio being equal to 1:1.2, quite close to that observed experimentally.

As mentioned above, a direct quantitative comparison of the signal amplitudes is not possible from these data without having additional information on the performance of the mw-components in K_a - and W-bands. Alternatively, one can compare another experimentally important characteristic, namely, the ratio of the normalized DEER effect to noise, (ρ_n/N (ρ_n is defined in Fig. 11 and Fig. 11 caption). Taking the available factors such as the differences in flip probabilities, accumulation time and time base into account, the ρ_n/N was found to be 3.4 :1 in favor of W-band. (The factor 3.4 was comprised from the factor of ~2, due to phase relaxation decay caused by different time bases, the factor ~1.2, which was due to the difference in accumulation times and the factor 1.4, which was due to the difference in noise levels at equalized ρ_n 's). Based on this data as initial information and on the general knowledge of the performance of mw components in different bands, and on the analysis performed in Sections 2 and 3, we believe that the advantage of the W-band will most likely remain also under the optimal experimental conditions. Similar results were obtained for GdDNA conjugates labeled by a different tag, Gd538, which has a larger value of D . These data are presented in the SM, Section 3.

The analysis and results presented above make it quite obvious what should be done for improvement of the DEER measurements and (as a result) for extension of the range of accessible distances. We discuss the feasible options below.

- i. Our calculations have shown that shorter pumping pulses would result in a better absolute DEER effect, but this gain disappears if the Q -value is adjusted to accommodate a shorter pulse (see Fig. 3). The adjustment by the Q -value, however, is only necessary if the measurements are performed in a SMVR, so the obvious route to enhance the absolute DEER effect is to use different types of resonators. One option is to employ bimodal resonators. In a bimodal resonator, the separation between the pumping and observation frequencies does not directly depend on the Q -values of the modes. Therefore, the necessary frequency separation can be maintained without sacrificing the Q -value of the observation mode. Observation can be performed at a rather high Q -value, substantially boosting the signal amplitude. The implementation of a bimodal resonator in W-band measurements has recently been reported [29]. As we already mentioned that commercial W-band power amplifiers which are suitable for generating short mw pulses are gradually becoming more available, and the problem of receiver protection is, in principle, solved [28,30].
- ii. Another way to enhance the absolute DEER effect is to employ sapphire-based dielectric resonators. For example, in the K_a -band DEER measurements of Gd-labeled DNA, the use of a dielectric resonator resulted in a 3-fold increase of the

absolute DEER effect as compared to a SMVR, while the sample volume was four times smaller than that used with the SMVR [18]. Evidently, use of dielectric resonators makes DEER performance in K_a -band comparable with that achieved in conventional W-band measurements.

- iii. The third route that we recently explored and obtained promising results is to use the high power W-band instrument at Environmental Molecular Sciences Laboratory (EMSL) of PNNL equipped with a *non-resonant* cavity [31]. The design of this instrument is similar to that published in [17]. The details of the experiment are presented in the SM. The bandwidth of the instrument is about 500 MHz, which allows π -pulses of 6–8 ns duration to be readily accommodated. The increase of spin flip probability in comparison with that of obtained in the existing W-band instrument was quite impressive, ~4-fold, as shown by the data presented in SM, Section 3. The instrument, however, still suffers from low general sensitivity, and work to improve it is in progress.

In summary, (i) we were able to define optimal conditions in DEER measurements, *important for long distance measurements* such as pulse positioning, temperature of measurements and mw band; (ii) we presented some experimental support for these findings; (iii) based on these findings, we were able to recommend further feasible measures for additional improvements of DEER measurements. According to our estimates, when optimal conditions and improvements are implemented, the time base in DEER measurements can be extended up to ~20 μ s, which corresponds to the distances of ~100 Å accessible in DEER measurements of Gd– labeled biomacromolecules.

So far, our analysis and discussion have been mostly related to *long-distance DEER measurements*. To conclude this paper, we should briefly mention some peculiarities of *short distance measurements*. The short-distance measurements do not require a long time base, and therefore the concentration requirements in these measurements are relaxed. The main problems resulting from finite durations of the pumping *and* observation pulses in such kinds of measurements are not related to sensitivity, but to effective dead time and to excitation of the dipolar spectrum corresponding to $\Delta m_S=1$ [6,21]. To measure short distances, short pulses are essential, and as consequence, the separation between the pumping and observation frequencies should be large. In K_a -band, the problem of generating short pulses with adequate frequency separation was recently solved using a dielectric resonator [18] in pseudo-bimodal fashion. In W-band, the problem was solved using the W-band spectrometer at EMSL with a non-resonant cavity [31]. To test the performance of this spectrometer, we used a Gd(III) trimer, in which three GdLs are attached by flexible arms to a benzene ring. The distances between Gd(III) ions in this compound are short and may vary, in principle, between ~8–25 Å, assuming that free rotation is allowed around single bonds [21]. The *cfi* *D*-value of the GdL in this compound was close to 50 mT, and the EPR spectrum exhibited additional broadening due to static dipolar interactions. The latter can reach up to 100 mT, depending on the complex conformation and the spin state of the interacting GdLs. Nevertheless, the superior pulse parameters of the EMSL instrument allowed us to collect the DEER kinetics, for which the entire evolution is mostly completed during a time of ~50 ns, as evident from Fig. 12. The Gd(III) concentration in this experiment was $\approx 100 \mu$ M (33 μ M of trimers). At this concentration, the decay due to ‘volume’ dipolar interaction at time base of 300 ns is negligible (0.3%), as compared with decay due to dipolar interactions between Gd(III) ions in the trimer. Collection time domain patterns, therefore, can be treated as DEER kinetics of intra-pair interactions. Such kinetics level off somewhere around half of the period of dipolar evolution, and the ‘distance-time’ relationship is determined by Eq. 9. Since kinetics have apparently leveled off after ~50 ns, one can readily evaluate that for most configurations, distance separations between Gd(III) is ~17 Å. As follows from direct simulations, the observed kinetics can be easily reproduced

(see Fig. 12), assuming that the pair distance distribution function, $G(r)$, is the sum of two Gaussians, $G(r) = g_1(r) + 0.2 g_2(r)$; $g_1(r) \propto \exp -((x - 14)/14)^2$, $8.5 \text{ \AA} \leq x \leq 20 \text{ \AA}$, and $g_2(r) \propto \exp -((x - 18)/18)^2$, $11.5 \text{ \AA} \leq x \leq 23 \text{ \AA}$. Apparently, the $G(r)$ is defined between $8.5 \text{ \AA}$ and $23 \text{ \AA}$, which is in distance limits, allowed by molecule configurations. The first momentum of this distribution function is $\approx 15 \text{ \AA}$, while 70% of the pairs are separated by a distance less than $17 \text{ \AA}$, in good agreement with qualitative evaluation. The measurement of short distances in the Ka and W-bands, at present, do not, therefore, encounter any problems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

a. Subspectra of Gd(III) ions simulated for W-band ($\nu_{\text{mw}} = 95$ GHz) and $D/g\beta = 50$ mT, $T = 20$ K. Lines 1, 2 and 3 are the subspectra of $(-3/2 \leftrightarrow -1/2) + (1/2 \leftrightarrow 3/2)$; $(-5/2 \leftrightarrow -3/2) + (3/2 \leftrightarrow 5/2)$ and $(-7/2 \leftrightarrow -5/2) + (5/2 \leftrightarrow 7/2)$ transitions, respectively. The subspectrum of $-1/2 \leftrightarrow 1/2$ transition is presented separately in Fig. 1b. All subspectra are convoluted with a Gaussian line, which has 1 mT width between the maximal slope points. The transition probabilities for all subspectra are taken equal in this simulation.

b. Lines 1 and 2, simulated W-band and K_a -band ($\nu_{\text{mw}} \approx 30$ GHz) subspectra of $-1/2 \leftrightarrow 1/2$ transition, respectively; $D/g\beta = 50$ mT, $T = 20$ K. The arrows indicate the magnetic field axes corresponding to K_a - and W-bands.

c. Central part of simulated W-band spectra ($\nu_{\text{mw}} \approx 95$ GHz, $D/g\beta = 50$ mT, $T = 5$ K) optimized for observation at the maximum of the spectrum (solid line 1, observation position "a") and for observation shifted by 10 mT downfield (dotted line 2, observation position "b"). The nominal turning angles for echo generation in these two cases were $(0.528\pi; 1.056\pi; 1.056\pi)$ for line 1 and $(0.62\pi; 1.24\pi; 1.24\pi)$ for line 2. The increase in the amplitude for position b after the optimization is about 20%.

Figure 2.

Spectra of the π -pumping pulse (1) and refocused primary echo, (2), generated by nominal rectangular ($\pi/2$; π ; π) pulses in dimensionless coordinates $\delta\nu t_p$; $\delta\nu$ is the frequency offset from the carrier frequency (in Hz), t_p is pulse duration (in s). The spectra are shifted relative to each other to show the minimal frequency separation (~ 1.4 – 1.5 unit), at which the spectral overlap can be neglected. The echo spectrum profile was calculated for the case of identical durations of all three pulses.

Figure 3.

Absolute DEER effect, $\eta(T)$, calculated for $D/g\beta = 50$ mT, K_a -band, in the temperature range between 5–20 K and for various positions of the pumping and observation pulses marked as “a”, “b” and “c” in the inset. For lines 1 and 2, the pumping frequency, ν_p , was in resonance with the maximum of the EPR spectrum, (position a in the inset) and the durations of pumping pulse were 5 ns and 10 ns, respectively. The ESE signal was observed at the spectrum positions b and c, which are shifted downfield from position c by ~ 3.57 mT (100 MHz) and 10.7 mT (300 MHz), respectively. For lines 3 and 4, the observation frequency, ν_o , was in resonance with the EPR maximum, while the 10 ns or 5 ns pumping pulse was in resonance with positions b and c, respectively. Dashed line 3' is obtained from line 3 by multiplying the latter by 3 to account for the difference in Q -values necessary to accommodate the different frequency separation. Inset: central part of spectrum of Gd(III). Note that the 100 MHz separation between the pumping and observation frequencies corresponds to ‘long observation pulses’ and a 10 ns pumping pulse while $\Delta\nu = 300$ MHz corresponds to situation when $t_p^p = t_p^o = 5$ ns.

Figure 4. K_a -band temperature dependence of the optimal repetition rate for Gd595 (open circles) and Gd538 (squares) presented in double-logarithm coordinates. Dashed and solid straight lines are obtained using the least squares method.

Figure 5.

Optimal ($\eta(T)$, line 1) and adjusted for the optimal repetition rate absolute DEER effect ($\eta^d(T)$, line 2) in K_a -band. Line 1 is the same as line 3' in Fig. 3. Line 2 was obtained from line 1 by multiplying it by $(T/5)^{1.2}$, that is the square root of the relative optimal repetition rate, $\sqrt{RR_{opt}(T)/RR_{opt}(5K)}$. To make the adjustment, the experimental dependence $RR_{opt}(T)$, presented in Fig. 4 was extrapolated to 5 K.

Figure 6. Absolute DEER effect, $\eta(T)$, calculated for W-band. The calculation parameters and line designations are the same as in Fig. 3.

Figure 7.

The relative temperature dependence of the optimal repetition rate, $RR_{opt}(T)/RR_{opt}(5\text{ K})$, for GdDOTA in the W-band. The experimental data were obtained in the temperature interval of 6–25 K. The line presented in Fig. 7 is drawn through the experimental data points using the least squares method, and is extrapolated to 5 K. At 6 K, $RR_{opt} = 1.63\text{ KHz}$.

Figure 8.

Lines 1 and 2, optimal absolute ($\eta(T)$) and adjusted optimal absolute DEER effects ($\eta^d(T)$), respectively, in W-band. Line 1 is the same as line 3 in Fig. 6. Line 2 is the product of line 1 and $\sqrt{RR_{opt}(T)/RR_{opt}(5K)}$ shown in Fig. 7.

Figure 9.

Inter-band (W-band vs. K_a -band) comparison of the η^a values for GdLs having $D/g\beta$ of 50 mT (a) and 20 mT (b). Lines 1 and 2 are the η^a values evaluated for W-band and K_a -bands, respectively. The shadowed rectangles show the temperature range, in which the DEER measurements can be performed without adverse effects of spectral diffusion. The arrows indicate the η^a values to be compared.

Figure 10.

(1) and (2) K_a -band DEER time domain patterns of double-labeled 2Gd595-DNA collected at the observation positions a and b (shown in the inset), which corresponds to interchangeable mw frequencies of 29.736 GHz and 29.856 GHz in the observation and pumping channels; temperature, 12 K; pulse parameters: pumping (π) pulse, 15 ns; observation pulses, 30, 45, and 45 ns; concentration, 20 μ M. Vertical bars indicate the time interval, where the intra-pair dipolar evolution is mainly completed.

Figure 11.

The normalized logarithms of W-(1) and K_a -band (2) DEER kinetics of double-labeled 2Gd595 DNA. Concentration of biomacromolecules in both experiments were $\sim 40 \mu\text{M}$. ν_p was in resonance with the EPR maximum. The offset of ν_o from ν_p was 120 MHz. Observation pulses, 30 ns ($\pi/2$); pumping pulses, 12 and 15 ns, in K_a - and W-band, respectively. The time bases in DEER measurements and data collection times were 7 μs and 9 μs , and 120 and 80 min, for K_a - and W-band, respectively. The inset shows the definition of the normalized DEER effect, ρ_n , which is the asymptote of the normalized DEER kinetics after the removal of the decay due to uniformly distributed spins. Shaded box indicates the noise level, N . ρ_n is equal to flip probability, λ^f when 100% of the biomacromolecules are double labeled.

Figure 12.

Solid line: normalized W-band DEER time domain patterns for Gd538 trimer (see the structure in SM) collected using the ESML instrument [31]. Microwave frequencies, $\nu_p = 95.104$ GHz, $\nu_o = 94.704$ GHz; π -pumping and observation pulses, 8 ns; temperature, 7 K. Pumping pulse was applied to the maximum of the EPR spectrum. Dashed line: simulated time domain pattern. It was calculated in the standard way as

$V(t) = 1 - \lambda^t (1 - \int_{-\infty}^{\infty} G(r) \cos D t dr)$, (D is common designation of dipolar interaction). $G(r)$, the distribution function used in the simulation, is shown in the insert. It is very likely that in this $G(r)$, the weight of the realizations of less than 12–13 Å are inflated, due to those factors already discussed in [21].