Gd(III)-Gd(III) distance measurements with chirp pump pulses

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Abstract

The broad EPR spectrum of Gd(III) spin labels restricts the dipolar modulation depth in distance measurements between Gd(III) pairs to a few percent. To overcome this limitation, frequency-swept chirp pulses are utilized as pump pulses in the DEER experiment. Using a model system with 3.4 nm Gd-Gd distance, application of one single chirp pump pulse at Q-band frequencies leads to modulation depths beyond 10%. However, the larger modulation depth is counteracted by a reduction of the absolute echo intensity due to the pump pulse. As supported by spin dynamics simulations, this effect is primarily driven by signal loss to double-quantum coherence and specific to the Gd(III) high spin state of $S = 7/2$. In order to balance modulation depth and echo intensity for optimum sensitivity, a simple experimental procedure is proposed. An additional improvement by 25% in DEER sensitivity is achieved with two consecutive chirp pump pulses. These pulses pump the Gd(III) spectrum symmetrically around the observation position, therefore mutually compensating for dynamical Bloch-Siegert phase shifts at the observer spins. The improved sensitivity of the DEER data with modulation depths on the order of 20% is due to mitigation of the echo reduction effects by the consecutive pump pulses. In particular, the second pump pulse does not lead to additional signal loss if perfect inversion is assumed. Moreover, the compensation of the dynamical Bloch-Siegert phase prevents signal loss due to spatial dependence of the dynamical phase, which is caused by inhomogeneities in the driving field.

The new methodology is combined with pre-polarization techniques to measure long distances up to 8.6 nm, where signal intensity and modulation depth become attenuated by long dipolar evolution windows. In addition, the influence of the zero-field splitting parameters on the echo intensity is studied with simulations. Herein, larger sensitivity is anticipated for Gd(III) complexes with zero-field splitting that is smaller than for the employed Gd-PyMTA complex.

Keywords: chirp pulses, distance measurements, sensitivity enhancement

1. Introduction

EPR-based distance determination using Gd spin labels is becoming an important alternative to the common approach based on nitroxide spin labels [1]. The majority of experiments to date rely on the four-pulse DEER (double electron-electron resonance) sequence to access distance information between a pair of Gd(III) centers [2]. Modifications of this approach involve the use of non-identical spin labels, in particular a Gd(III) center and a nitroxide [3, 4], as well as different experimental schemes, such as relaxation-induced dipolar modulation (RIDME) [5] or continuous-wave EPR [6].

Distance measurements involving Gd spin labels have been successfully demonstrated on a number of systems, including model compounds, peptides, nanoparticles, proteins and DNAs [7–21]. Moreover, a promising biochemical property of Gd spin labels is the stability under reducing conditions, which recently enabled Gd-Gd distance measurements of peptides and proteins embedded in cellular environments [22, 23].

Because Gd(III) ions are in an $S = 7/2$ high spin state, high magnetic fields above 1 T are required to diminish unwanted contributions from the zero-field splitting (ZFS) to the obtained distance information [7]. Unlike with nitroxides at high magnetic fields, there is no pronounced orientation selection for Gd spin labels, which eases data analysis for the case of Gd-Gd distance measurements. However, there is one specific limitation in Gd-Gd DEER, namely that the dipolar modulation depth $\lambda$ is only on the order of a few percent due to the broad EPR spectrum. This disadvantage is partially compensated by the large echo signal $V_0$, which facilitates a large DEER sensitivity $\eta = V_0 \cdot \lambda$. In particular, the sensitivity of ordinary Gd-Gd DEER is optimized by observation of the central $1/2 \leftrightarrow -1/2$ transition (CT) at the central peak of the spectrum and pumping the satellite transitions [24].

In this work, we investigate how Gd-Gd DEER experiments can be improved with chirp pump pulses synthesized by an arbitrary waveform generator (AWG). A key advantage of arbitrarily shaped pulses is the improved excitation bandwidth, as demonstrated in a number of recent studies [25–32]. For chirp pulses, pulse performance can be quantified by the critical adiabaticity factor $Q_{\text{crit}}$ using the Landau-Zener equation [31]. Moreover, limitations in excitation bandwidth imposed by the resonator can be compensated by adaptation of the frequency sweep of the chirp pulse to the resonator profile, which establishes ultra-wideband (UWB) EPR [26, 28].
We have demonstrated recently that the sensitivity of Gd-Gd DEER can be enhanced by rearranging the equilibrium populations and by using a chirp pump pulse [30]. The focus of this previous study was on the long and intense chirp pulses required for efficient population transfer over frequency ranges beyond 1 GHz. In the present work, we elaborate further on the pump pulse. In principle, the DEER pump pulse should have a large adiabaticity factor for efficient inversion, which also requires long and intense pulses. However, experiments on $S = 1/2$ spins have shown that interference prohibits the use of long chirp pulses in four-pulse DEER [26, 27]. The key subject of this work is to identify and eventually alleviate additional limitations specific to the Gd(III) high-spin state with respect to chirp pump pulses.

The paper is organized as follows. In Section 2, the compounds used in this study, i.e. the Gd-rulers $I_n$ of the type Gd-spacer-Gd with the Gd-PyMTA complex as the spin label, and experimental details are described. Experimental results and discussions follow in Section 3. First, we show that optimization of the modulation depth is not necessarily sensitivity optimization, since the pump pulse also has a distinct influence on the echo intensity $V_0$ (Section 3.1). Then, a rather simple experimental procedure for sensitivity optimization is presented and potential complications related to multi-quantum excitation by the pump pulse are addressed (Section 3.2). In Section 3.3, we use spin dynamics simulations to study the intensity of the (unmodulated) DEER echo $V_0$ as a function of pulse and ZFS parameters. Further on, we show how sensitivity is improved even further with two consecutive chirp pump pulses (Section 3.4). Finally, the new methodology is tested with Gd-rulers having long Gd-Gd distances up to 8.6 nm in Section 3.5. At several instances, we refer to the supporting information (SI) for related in-depth information and further discussions.

2. Materials and methods

2.1. Sample preparation

2.1.1. Gd-rulers

The syntheses of Gd-rulers $I_n$ with $n = \{ 3, 5, 7, 9, 11 \}$ (see Fig. 1) will be published elsewhere. The stock solutions of Gd-ruler $I_3$, $I_5$ and $I_7$ had concentrations of 5 mM in $D_2O$ (pH 8.0), containing 28 mM, 38 mM and 30 mM NaCl, respectively. The stock solutions of Gd-ruler $I_9$ and $I_{11}$ had concentrations of 2 mM in $D_2O$ (pH 8.0), containing 20 mM NaCl. For the EPR experiments, the stock solutions were diluted with $D_2O$ and glycerol-d$_8$ (1:1 in volume). For Gd-ruler $I_3$, 20 µl of a solution with a concentration of 600 µM was used. For all the other Gd-rulers, the sample volume was 40 µl with a concentration of 50 µM. In the case of Gd-ruler $I_3$, also different concentrations were prepared. These concentrations are found in Fig. S12 in the SI, where the actual concentration study is presented.

2.1.2. Gd(III)-DOTA complex

According to the procedure reported in [15], a stock solution of Gd(III)-DOTA complex (DOTA = 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraaceticate) with a concentration of 40 mM was prepared. The stock solution was diluted with a 1:1 mixture in volume of water/glycerol to obtain the solution with a concentration of 200 µM that was used for the EPR experiments.

2.2. EPR experiments

All experiments were performed on a customized Bruker Elexys E580 Q-band spectrometer [35] with a previously described extension by a 12 GS/s AWG (Agilent M8190A) [30]. The temperature was 10 K and the repetition rate of the pulse sequences was 342 MHz in the case of Gd-ruler $I_3$ and 716 µs for all the other Gd-rulers. An exception is the acquisition of resonator profiles $v_1(f)$ based on transient nutation experiments, which were all acquired at a repetition time of 342 µs. The timing and sequence parameters for these transient nutation experiments were identical to the ones described previously in [30].

2.2.1. Four-pulse DEER

In order to record the DEER signal $V(t)$, the pulse sequence $\pm(\pi/2)_{\text{obs}}-\tau_1-(\pi)_{\text{obs}}-(\tau_1+t')-(\pi)_{\text{chirp}}-(\tau_2-t')-(\pi)_{\text{obs}}-\tau_2-\text{echo}$ was used, where all observation pulses had a length of 12 ns and the time $t'$ is incremented by Δt. The chirp pulses originated from the AWG and the relevant pulse parameters are described further below. Due to the chirp pulse, there was a time-shift between the time $t'$ of the pulse sequence and the actual dipolar evolution time $t$. This time shift varied with the pulse parameters and depended most critically on the total length of the...
pulse. In this work, typical offsets between \( t' \) and \( t \) were on the order of 0.8 times the pulse duration. Note that this offset is determined by the frequency sweep and flip angle of the chirp pulse, as well as by the lineshape of the pumped spins. For Gd-ruler 1, the timings were \( t_1 = 400 \text{ ns}, \Delta t = 8 \text{ ns}, \) and \( t_2 = 4.5 \mu s \) or \( 6 \mu s \). For Gd-ruler 1, we used \( t_1 = 2 \mu s, t_2 = 15 \mu s \) and \( \Delta t = 40 \text{ ns} \). For Gd-ruler 1, 9 and 11, the timings were \( t_1 = 1 \mu s, \Delta t = 100 \text{ ns} \) and \( t_2 = 18 \mu s, 20 \mu s \) and \( 22 \mu s \), respectively. For reference, an illustration of the pulse sequence with \( t_1 = 400 \text{ ns}, t_2 = 4.5 \mu s \) and \( t' = 40 \text{ ns} \) is shown in Fig. S22 in the SI.

For pre-polarized DEER experiments [30], two chirp pulses, with pulse duration of \( 2 \mu s \) each, preceded the above DEER sequence. The time gap between the two pre-polarization pulses was \( 300 \text{ ns} \) and the time gap between the last pre-polarization pulse and the DEER sequence was \( 3 \mu s \). The number of shots per point was \( 1000 \times 2 \) in these experiments.

DEER data were processed with the software DeerAnalysis [36]. Regularization parameters for the five Gd-rulers were 1, 1, 10, 100 and 100, respectively. Details on the separation of the background signals are provided in Section 6.3 of the SI.

### 2.2.2. Parameters of chirp pulses

For the chirp pulses used in this work, we refer to the start and end frequencies of the frequency sweep as \( f_1 \) and \( f_2 \), respectively. The (absolute) difference between \( f_1 \) and \( f_2 \) defines the sweep width \( \Delta f \). Accordingly, we specify the frequency range of a chirp pulse by \( \Delta f \) and \( f_2 \). Unless the sweep direction is explicitly indicated, \( f_1 = f_2 - \Delta f \). In all instances, the end frequency \( f_2 \) is specified relative to the observation frequency \( f_{obs} \). Hence, \( f_2 \) also denotes the closest nominal frequency spacing between the observation frequency and the pump frequency. In brief, \( f_2 \) is also referred to as frequency spacing between pump and observation frequencies. A typical value for this offset is \(-300 \text{ MHz} \). The deviations between the nominal frequency spacing and the actual frequency spacing are discussed in the SI (see Fig. S3).

Moreover, almost all chirp pulses used in this work take into consideration the resonator profile \( \nu_1(f) \) [26]. Here, we consider idealized resonator profiles parametrized by the quality factor \( Q_L \), the resonance frequency \( f_0 \), and the field strength \( \nu_1 \). Representative parameters are \( Q_L = 110, f_0 = 34.3 \text{ GHz} \) and \( \nu_1 = 40 \text{ MHz} \). The frequency range and resonator parameters define the actual form of the pulse’s frequency sweep function and also yield the adiabaticity factor \( Q_{\text{cen}} \). Due to the various transition moments related to Gd(III) ions, the adiabaticity is only indicated for the CT and referred to as \( Q_{\text{CT}} \). Note that due to the idealized resonator profiles, the given adiabaticity factors are not as quantitative as in our work at X-band frequencies [31]. Nevertheless, \( Q_{\text{CT}} \) scales linearly with the pulse length \( t_p \) and quadratically with \( \nu_1 \) for a given frequency range.

The edges of all chirp pulses are smoothened with a sine function parametrized by \( t_r \). For chirp pump pulses, we used \( t_r = 10 \text{ ns} \), whereas for pre-polarization pulses, we used \( t_r = 30 \text{ ns} \).
3. Results and discussion

3.1. DEER with a single chirp pump pulse

The performance of distance measurements with a single chirp pump pulse was evaluated with Gd-ruler $I_3$, whose echo-detected EPR spectrum cast in frequency scale is shown in red in Fig. 2a. The black dashed vertical line at the center denotes the position of the central line. Also shown in blue is the frequency response of a resonator with parameters $f_0 = 34.27$ GHz and $Q_L = 120$ fitted to experimental $v_1(f)$ data (brown).

In order to study the influence of the frequency positioning of the pump pulse relative to the observed $A$ spins, we monitored the amplitude $V_0$ of the DEER echo ($V(t)$ at $t = 0$) as a function of the frequency offset between observation and pump frequencies. The frequency offset was varied by adjusting the end frequency $f_2$ of the chirp pulse, while keeping the other parameters fixed ($\Delta f = 750$ MHz, $t_p = 128$ ns). The real and imaginary components of $V_0$ are shown in Fig. 2b, where the normalization $V_{ref}$ is the echo amplitude obtained in absence of the pump pulse. The data reveal a gradual reduction of $V_0$ as the chirp pulse approaches the $A$ spins. Furthermore, the phase of the echo is gradually shifted due to a dynamic Bloch-Siegert phase [37]. When considering the magnitudes of the echoes (data not shown), the reduction of the echo $V_0$ to 75% and 50% of $V_{ref}$ was at a frequency offset of -500 MHz and -260 MHz, respectively.

Given the significant reduction of $V_0$ by the pump pulse, the experimental DEER sensitivity $\eta = V_0 \cdot \lambda$ was examined for a variety of parameters of the pump pulse. In total, 42 pump pulses with a frequency offset $f_2$ of either -300 MHz or -400 MHz were tested. Four representative frequency ranges are indicated by the orange arrows in Fig. 2a. A collection of raw DEER data is found in Fig. S1a in the SI, together with further information on the actual parameters of these 42 pulses. Interestingly, the obtained echo amplitudes $V_0$ and modulation depths $\lambda$ show a correlation (see Fig. 2c).

The benefit of large modulation depth $\lambda$ is therefore offset by a smaller echo amplitude $V_0$, such that maximum modulation depth does not necessarily result in maximum sensitivity.

Further correlations were obtained when taking into consideration the adiabaticity factor of the pump pulse on the central transition, $Q_{CT}$. This factor quantifies the adiabaticity of the pump pulse and unifies all essential pulse parameters in one single parameter [31, 38, 39]. As is seen in Fig. 2d, the echo amplitude $V_0$ decreases as the adiabaticity of the pump pulse increases. Such a scaling can be explained by coherence transfer to unobserved multi-quantum coherence due to inversion of the $A$ spin’s neighbor transitions (see also Section 3.3 and [13, 31]). The two round circles in the figure with gray color correspond to data obtained with $\Delta f = 250$ MHz. In this case, the performance of the pulse cannot be quantified solely by its adiabaticity, because edge effects become significant if $\Delta f/2$ is comparable to the field strength $v_1$ of the passed transition [38]. In addition, also $\lambda$ and $Q_{CT}$ correlate in a sense that $\lambda$ increases with adiabaticity (see Fig. S1c in SI). Since adiabaticity relates to the inversion efficiency of the pumped $B$ spins, this result is not surprising and not specific to the high spin state of Gd(III).

One would expect a similar scaling behavior for distance measurements between $S = 1/2$ spins. For the DEER sensitivity $\eta = V_0 \cdot \lambda$, the competition between echo reduction and modulation depth results in optimum sensitivity for adiabaticities $Q_{CT}$ on the order of 1 - 2 (see Fig. 2e). In this regime, the pump pulse cannot be considered as an adiabatic pulse and therefore does not provide a perfect population inversion. Note that for distance measurements between a Gd(III) center and a nitroxide, a related result was obtained for the optimum flip angle of a monochromatic pump pulse [15]. The found correlations may be used to set up DEER experiments with close to optimum sensitivity. For instance, one could determine the sweep width $\Delta f$, which results in an op-
timum adiabaticity \( Q_{CT} \) for a fixed offset \( f_2 = -300 \) MHz and pulse length \( t_p \). This procedure does not require any calibration of the pump pulse, except the measurement of experimental \( v_1(f) \) data to quantify adiabaticity. The DEER experiment with Gd-ruler 13 in [30] was set up in such a way. In general, however, these correlations are only valid for the ZFS parameters of the Gd-PyMTA label of Gd-ruler 13 at Q-band frequencies and for the set of pulse parameters sampled by the 42 experiments presented above. Entirely different scaling between modulation depth and echo reduction may apply for compounds labeled with different Gd(III) complexes, or for pulses with different parameters, in particular for a different frequency offset \( f_2 \) with respect to the observation frequency.

### 3.2. An experimental shortcut to optimize sensitivity

As discussed in the previous section, sensitivity optimization critically depends on the relative scaling between the modulation depth \( \lambda \) and the echo amplitude \( V_0 \). In this section, a fairly simple experimental procedure to maximize DEER sensitivity in situ is presented.

While the echo amplitude \( V_0 \) can be obtained directly from a single data point at \( t = 0 \), an accurate extraction of \( \lambda \) requires several data points. However, dipolar modulation leads to a reduction of the echo amplitude \( V(t) \) for \( t > 0 \) due to both inter- and intra-molecular contributions. One can therefore obtain qualitative information on the actual \( \lambda \) by comparing \( V(t) \) at \( t = 0 \) and at a later time \( t_\eta \). This procedure requires only two data points and results in the two-point parameters \( \lambda_{2\eta} = 1 - V(t_\eta)/V_0 \) and \( \eta_{2\eta} = V_0 - V(t_\eta) \). For the 42 datasets of the previous section, we found rather good correlation between these two-point parameters and the actual \( \lambda \) and \( \eta \), independent on whether \( t_\eta = 1 \) \( \mu s \) or \( t_\eta = 4 \) \( \mu s \) was chosen (see Fig. S1e in SI).

Sensitivity can thus be optimized in situ by searching the maximum of \( \eta_{2\eta} \). First, a pulse length \( t_\eta \) is chosen which is short enough for the expected distance. Second, \( \eta_{2\eta} \) is recorded while changing both the sweep width \( \Delta f \) and the end frequency \( f_2 \), thus optimizing the frequency positioning of the chirp pulse (see first section of the SI). In this way, we obtained modulation depths beyond 10% with 128 ns long chirp pulses.

The best dataset of Gd-ruler 13 from the previous section is shown in black in Fig. 3. The form factor in panel (a) shows a modulation depth of 10%. Optimization of \( \eta_{2\eta} \) resulted in the gray curve with modulation depth of 13% and a 24% improvement in \( \eta \). The pulse parameters at this sensitivity optimum were \( \Delta f = 900 \) MHz and \( f_2 = -75 \) MHz. In agreement with the results of the previous section, the pump pulse had a reduced inversion efficiency \( Q_{CT} = 2 \).

While the distance distributions in panel (b) are in good agreement, the form factor of the optimized pulse shows two spikes around \( t = 4 \) \( \mu s \) (see arrows). These artifacts are due to residual coherence excitation of the A spins by the pump pulse and typical for small frequency offsets \( |f_2| \) between pump and observer pulses \( f_2 = -75 \) MHz. Here, these artifacts could in principle be suppressed by digital filtering of the DEER data. However, in situations where such a small offset \( |f_2| \) is not admissible, the pulse parameters which maximize \( \eta_{2\eta} \) up to a given minimum offset may be used instead. Moreover, the consecutive chirp pulses presented below in Section 3.4 do typically not require such small offsets.

### 3.3. Choice of fringe frequencies

Besides possible shortcomings due to residual excitation of \( A \) spins by the pump pulse, optimization of \( \eta_{2\eta} \) in high-spin systems may select pulse parameters which lead to higher harmonics of dipolar frequencies by inducing \( |\Delta f| > 1 \) flips of the \( B \) spins. An example dataset is shown in gray in Fig. 4, together with the reference data in black from Fig. 3 above. The form factor in panel (a) shows a larger modulation depth for the higher harmonic data. In the distance distributions in panel (b), an additional peak due to the second harmonic is observed \( (|\Delta f| = 2) \). The vertical dashed black line indicates the position of the peak distance at 3.41 nm as well as the position of the second harmonic distance peak at 2.71 nm. In the distance domain, these two positions are related by a multiplication factor of \( 2^{-1/3} \).

Under our current experimental conditions, we could only observe such distinct harmonics for pulses with extraordinary high adiabaticity factors \( Q_{CT} > 5 \). These were achieved by application of the pump pulse at the resonator maximum, which enforced observation of the central line outside the resonator.
bandwidth, thus severely reducing the sensitivity $\eta$. However, for cases where (i) multi-mode resonators are used and (ii) echo reduction effects at large adiabaticity factors are tolerable, $|\Delta m_3| > 1$ excitation with chirp pulses may become more prominent. Further data is found in Section 2 of the SI, including a significant narrowing of distance distributions which resembles RIDME data recorded with Gd-ruler $I_3$ [5].

3.3. Dependence of echo intensity on ZFS splitting

In this section, the echo reduction effect is studied using spin dynamics simulations of the $A$ spins. In particular, these simulations give insight into the behavior with respect to the ZFS parameter $D$ to predict the sensitivity of Gd(III) complexes with different $D$ values. Detailed information on the simulation method is found in the Section 8.1 of the SI.

At first, the validity of our simulation approach was tested by comparison to experimental data. Experimentally, the normalized DEER echo amplitude $V_0/V_{ref}$ was recorded as a function of the frequency offset between observation and pump frequencies, as described above in Fig. 2b. Here, the pulse length $t_p$ was 192 ns and the sweep width $\Delta f$ was 850 MHz. The experimental data for real (black) and imaginary (gray) components are shown by the solid lines in Figs. 5a and b for Gd-ruler $I_{H1}$ that contains Gd-PyMTA and for a solution of Gd-DOTA complex, respectively.

The simulated data points are illustrated by the circles in Figs. 5a and b. As is readily seen, the simulations agree to the overall trend in experimental data. In particular, the excursion of the imaginary component due to a dynamical Bloch-Siegert phase shift [37] is comparable. When comparing the real component, simulated data show a stronger reduction effect. The stronger reduction effect is observed even better when comparing the corresponding magnitudes in Fig. 5c. Here, data related to Gd-PyMTA and Gd-DOTA spin labels are shown in black and gray, respectively, and simulated data are vertically offset below experimental data. Distinct features at experimentally relevant offsets $f_2 < 0$, such as the point where the magnitudes related to Gd-PyMTA and Gd-DOTA cross or the points where the magnitudes are 50%, appear horizontally displaced by roughly 100 MHz. For the phase of $V_0/V_{ref}$ (see Fig S27a in SI), a displacement of roughly 50 MHz to the same direction can be observed, which could be explained by an overestimation of the pulse amplitudes in the simulations. In general, the precision of such a simulation depends on how well the orientation-averaged spin dynamics of the Gd(III) centers and the field amplitudes inside the microwave resonator can be modeled. For our present purposes, the overall reproduction of experimental data by our simulations is considered to be sufficient.

As seen in Fig. 5c, the reduction of the echo intensity $V_0/V_{ref}$ is more pronounced for Gd-DOTA ($D < 1$ GHz) than for Gd-PyMTA (mostly $D > 1$ GHz). In the context of distance measurements between Gd(III) centers and nitroxides with monochromatic pulses, comparison of $V_0/V_{ref}$ between Gd-DOTA and Gd-DTPA ($D > 1$ GHz) also revealed a stronger relative reduction effect for Gd-DOTA than for Gd-DTPA [15]. Since the echo $V_{ref}$ in absence of the pump pulse itself depends strongly on $D$, the absolute echo intensity $V_0$ in the presence of the pump pulse is required to calculate the DEER sensitivity $\eta = V_0 \cdot \lambda$. For this purpose, $V_0$ and $V_{ref}$ were simulated for a broad range of $D$ values from $D = 0.15$ GHz to $D = 2.5$ GHz. Here, each $D$ value corresponds to an average over orientations and a polynomial distribution of $E$ values (see [40]), as indicated in Fig. 5d for $D = 1$ GHz. The frequency offset $f_2$ between pump and observer frequencies was kept fixed at -300 MHz. Note that a Gaussian weighting over ten different $D$ values was used above in the simulations related to Gd-PyMTA and Gd-DOTA spin labels.

Fig. 6 shows the results for the simulation of $V_0$ and $V_{ref}$. Due to the second order broadening of the CT, the DEER echo $V_{ref}$ in absence of the pump pulse is larger for smaller $D$ values. The monotonic increase in $V_{ref}$ towards small $D$ stops for $D < 0.4$ GHz, which results in a turnover of the $V_{ref}$ curve at the smallest considered $D$ values. Further information on the origin of this effect is found in the SI (see Fig. S25).

As a consequence of the dependence of $V_{ref}$ on $D$, the DEER echo $V_0$ in presence of the pump pulse is also larger for smaller $D$ values. The echo reduction from $V_{ref}$ to $V_0$ is strongest at $D = 0.6$ GHz and almost vanishes at the extremities for very small or very large $D$ values (see Fig. S24). The strong reduction effect at $D = 0.6$ GHz is due to spectral coverage of the $A$ spin’s neighbor transitions by the pump pulse. Hence, in-
version of the neighbor transitions results in coherence transfer to double-quantum coherence, which does not contribute to the observed echo in the DEER experiment [13]. At the smallest $D$ of $0.15$ GHz, the neighbor transitions occupy (by first order) a frequency range of ±0.3 GHz (compare Fig. 5d), such that spectral coverage by the pump pulse goes to zero and $V_0 \approx V_{\text{ref}}$. For large $D > 2$ GHz, the neighbor transitions are broadened to more than 8 GHz, which also results in negligible echo reduction. Based on our simulations, we could not identify any reduction mechanism other than spectral overlap with neighbor transitions, unless the pump pulse has direct spectral overlap with the observed A spins or spatial inhomogeneity of the driving field $V_1$ is assumed (see Section 8.2 of the SI).

Notably, the scaling of the relative effect $V_0/V_{\text{ref}}$ and the absolute intensity $V_{\text{ref}}$ can readily be understood qualitatively. When observing the central line of the Gd(III) spectrum, the echo signal $V_{\text{ref}}$ arises mainly due to CT contributions, which exhibit second order broadening. In this case, the echo reduction is primarily driven by spectral overlap between the pump pulse and the neighbor transitions of the CT, which exhibit first order broadening. Loosely speaking, a bisection of $D$ therefore enhances the relative echo reduction effect by a factor of two and the absolute intensity by a factor of four, given that pump and observation parameters are unchanged. Using this simplified viewpoint, one realizes that absolute sensitivity improves towards smaller $D$. Clearly, the actual scaling depends on the pump and observation parameters. An experimentally relevant example is the bisection of $D = 1.2$ GHz to $D = 0.6$ GHz, as these are close to the mean values for Gd-PyMTA and Gd-DOTA, respectively. Here, our simulation showed an enhancement of the relative echo reduction effect by 1.7 and an enhancement of the absolute intensity by 3.8 when going from $D = 1.2$ GHz to $D = 0.6$ GHz.

Based on the simulations, a larger relative DEER sensitivity $\eta$ for Gd(III) complexes with ZFS parameters that are smaller than for Gd-PyMTA, such as for instance Gd-DOTA, is anticipated. Because the modulation depth $\lambda$ itself is not included in the simulations, an experimental evaluation of $\eta$ for smaller ZFS is required. In any case, one would expect that for a given inversion profile of the pump pulse, the narrowing of the satellite transitions at smaller $D$ values results in a larger modulation depth $\lambda$. Nevertheless, there are other critical factors that influence the experimental DEER sensitivity. In particular, the environment of the spin label determines the phase memory time $T_2^*$ and may also influence the echo reduction effect. When comparing experimental DEER data of different Gd(III) complexes in different environments, advantages of smaller ZFS parameters therefore may be offset by the changes induced by the environment.

### 3.4. Consecutive chirp pulses

Instead of one single chirp pump pulse, the pump pulse may be split into two consecutive chirp pulses of half the total pump pulse duration $t_p$ that pump the Gd(III) spectrum on both sides. In order to investigate the effects associated to these consecutive chirps, data obtained with one single chirp pulse is compared to data obtained with the same single chirp pulse followed by a second one of equal duration, but frequency positioning mirrored at the observation frequency: An up-chirp that sweeps from $f_1$ to $f_2$ is therefore followed by a down-chirp that sweeps from $-f_1$ to $-f_2$. At first, we consider the echo reduction $V_0/V_{\text{ref}}$. Then, we turn to the modulation depth $\lambda$.

The echo reduction $V_0/V_{\text{ref}}$ is shown in Fig. 7a. The black curve represents single pulse data and is identical with the experimental data of Gd-ruler $I_{111}$ in Fig. 5c. The gray curve was obtained with the two consecutive pump pulses. Interestingly, a comparable echo intensity is observed at frequency offsets $|f_2|$ on the order of 100 to 200 MHz. The added pulse therefore does not necessarily result in a smaller echo. This can be explained by double-quantum coherence generation by the first pulse: If the first pulse perfectly inverts the A spin’s neighbor transition with the lower resonance frequency, single-quantum coherence is fully converted to unobserved double-quantum coherence. The second pulse inverts the neighbor transition with the higher resonance frequency in the same way and converts the double-quantum coherence further to triple-quantum coherence (see Fig. S26 for illustration). Consequently, the second pulse does not lead to any further coherence loss in this idealized case. In practice the inversion by the pump pulses is not perfect, such that one would expect some additional loss by the second pulse. In fact, large offset frequencies $|f_2|$ show a rather

![Figure 6: Simulation of echo integral as a function of $D$ in the presence ($V_0$, gray) and absence ($V_{\text{ref}}$, black) of a 192 ns long $\Delta f = 850$ MHz chirp pulse with offset $f_2 = -300$ MHz. See Section 8.1 of the SI for further details on simulations.](image)

![Figure 7: Echo intensity with consecutive pump pulses as a function of the pump offset frequency $f_2$ with $\Delta f = 850$ MHz and $t_p = 192$ ns. (a,b) Magnitude and phase of $V_0/V_{\text{ref}}$ of Gd-ruler $I_{111}$ obtained with one single pump pulse (black) and consecutive pump pulses (gray). The black curve is also shown in black in Fig. 5c.](image)
pronounced loss due to the second pulse, because the adiabaticity at such large offsets is not sufficient for population inversion (see also Fig. S7).

Interestingly, the second pulse results in a larger echo at very small offset frequencies $|f_2| < 120$ MHz, which indicates the compensation of an echo reduction mechanism at small offset frequencies. For pulses with smaller sweep widths $\Delta f$, compensation is even more apparent (see Fig. S6b). The generation of triple-quantum coherence described above cannot explain such a compensation. One possibility is the mutual compensation of the Bloch-Siegert phase due to symmetry of the pump frequency windows: The phase of the echo for a single pump pulse is shown in black in Fig. 7b. Addition of the second pulse almost fully compensates the Bloch-Siegert phase.

As supported by simulations in the SI (see Fig. S27 and Section 8.2 of the SI), the aforementioned compensation mechanism can be explained by spatial inhomogeneity of the driving field $\nu_1$. In this case, the Bloch-Siegert phase induced by one single pulse becomes spatially distributed and partially dephases the spatially averaged echo. Application of the second pulse largely cancels the spatial dependence of the Bloch-Siegert phase, which increases the echo intensity. Note that such a compensation of Bloch-Siegert phase in presence of spatial inhomogeneity is highly reminiscent of the ABSTRUSE echo refocusing scheme with chirp pulses [31, 41]. Since the inhomogeneity in our microwave resonator is not known precisely, it is difficult to quantify the significance of the proposed compensation mechanism. However, we could not identify any other compensation mechanism that is supported by our spin dynamics simulations. Alternative compensation mechanisms would therefore most likely be related to spin couplings which are not considered in our simulations.

For a fair comparison of sensitivity with one single chirp and consecutive chirps, the overall pulse duration needs to be the same. Fig. 9 shows form factors (a) and distance distributions (b) of data obtained with one single chirp (gray) and consecutive chirps (black). The single chirp data are the same as the data shown above in Fig. 3. For the consecutive chirps, optimization of $\eta_{\text{DP}}$ (see Fig. S2 in SI) resulted in the pulse parameters $\Delta f = 550$ MHz and $|f_2| = 150$ MHz and $Q_{\text{CT}} = 3.0$, which results in $V_0/V_{\text{ref}} = 0.62$ and $\lambda = 13.3\%$. Identical data as in Fig. 3.

The influence of consecutive chirps on DEER data, and in particular on the modulation depth $\lambda$, is shown in Fig. 8. The form factors $V(t)/V_0$ are shown in panel (a) for the up-chirp (orange), the down-chirp (blue), and the consecutive chirps (green). The parameters of the chirp pulses used here were $t_0 = 128$ ns, $\Delta f = 625$ MHz and $|f_2| = 300$ MHz. With the consecutive chirps, the achieved modulation depth of 17% is almost the sum of the modulation depth of the individual pulses, 10% and 9%. The loss of 2% may be due to the longer overall pulse duration with the consecutive chirps, therefore causing interference of the dipolar evolution (see also Section 3 of the SI). Notably, the same experiment performed with shorter pulses shows a much smaller loss of 0.6% in the modulation depth (see Fig. S11c).

The distance distributions computed from the data in panel (a) are shown in panel (b) using the same color coding. All the distributions are in reasonable agreement. Notably, pumping both sides of the spectrum does not induce $\Delta n_2 > 1$ flips of the pumped spins (compare Fig. 4). Excitation of dipolar harmonics with chirp pulses is either related to the level connectivity of the inverted transitions or to the direct excitation of forbidden multi-quantum transitions passed by the chirp pulse [42, 43], thus requiring large adiabaticity factors and a large field strength $\nu_1$ (see also Section 2 of the SI).

![Figure 8](image.png)

**Figure 8:** Modulation depth enhancement with consecutive pump pulses with 128 ns length each, $\Delta f = 600$ MHz and $|f_2| = 300$ MHz. Orange curves obtained with a single up-chirp, blue curves with a single down-chirp, and green curves with consecutive up- and down-chirp. (a) Form factor with modulation depths of 10% (orange), 9% (blue), and 17% (green). (b) Regularized distance distributions. The normalized echo intensities $V_0/V_{\text{ref}}$ are 0.54 (orange), 0.72 (blue), and 0.54 (green). The primary DEER signal is shown in Fig. S11a.

The frequency spacing $f_2$ between pump and observation at optimum $\eta_{\text{DP}}$ is larger than for a single pump pulse. In this way, proximity problems such as residual excitation of the A spins by the pump pulse or potential deviations from the weak coupling limit of the dipole interaction [7, 20] are alleviated. Another point worth mentioning is that there is no significant overhead in setting up the experiment with consecutive chirps. The parameters of the two pulses are related by symmetry and are optimized concurrently when maximizing the two-
point sensitivity $\eta_{2P}$. In summary, the consecutive chirp pulses constitute the most sensitive realization of DEER measurements between pairs of Gd(III) ions that we could achieve so far. As with any four-pulse DEER experiment with chirp pulses, the approach has a shortcoming at short distances due to the extended pulse duration of the pump pulse. Previously, we could faithfully obtain a distance distribution with a mean distance of 2.5 nm with a 64 ns pulse on a different system [26]. Five-pulse DEER techniques are considered to be free of this limitation [27]. Due to the high-spin state of Gd(III) ions and the adiabatic pulses required for five-pulse DEER, however, we would expect significant complication in implementing such a scheme. In any case, the shorter dipolar evolution windows to measure distances below 2.5 nm alleviate sensitivity issues. The situation is opposite when determining long distances: Durations of chirp pulses become short compared to dipolar frequencies and longer dipolar evolution windows require high sensitivity. Besides possible distortions due to the duration of chirp pulses, the ultra-wideband frequency windows of the pump pulses may influence the obtained distance information. While orientation selection effects in Gd-Gd distance measurements are not significant [1], contributions of the ZFS may be altered [7, 20]. For further reference, an overview over the subtle differences in distance distributions obtained with Gd-ruler $I_1$ is provided in Fig. S14 in the SI. In cases where chirp pump pulses are not admissible and monochromatic pump pulses are used, it is worth mentioning that the concepts elaborated in this study can be translated to monochromatic pulses. In particular, we demonstrate in Section 7 of the SI how consecutive monochromatic pump pulses can be optimized in situ to achieve a sensitivity that is surprisingly competitive to chirp pulses. As a result, consecutive monochromatic pump pulses optimized with sub-nanosecond timing reach a modulation depth of 12% at $V_0/V_{\text{ref}} = 0.7$, which corresponds to a sensitivity that is 83% of the best result with consecutive chirp pulses. Instead of two consecutive monochromatic pulses, a single multi-frequency pulse as demonstrated in [44] might also be able to reach a similar performance.

3.5. Application to long distances

Small dipolar couplings related to spins at a long distance require long coherent observation windows, and therefore a high sensitivity. For that purpose, consecutive chirp pump pulses in combination with pre-polarization pulses [30] are applied for distances beyond 6 nm. At first, the modulation depth achieved with Gd-ruler $I_1$ is analyzed (6 nm). Fig. 10a shows form factors obtained with a dipolar evolution window determined by $\tau_2 = 10 \mu s$ (gray) and $\tau_2 = 18 \mu s$ (black). The phase memory time $T_2^*$ on the order of 9 $\mu$s explains the larger noise contribution of the data with the prolonged observation window (see Fig. S13a). In fact, the data with $\tau_2 = 18 \mu s$ was acquired 18 times longer than the data with $\tau_2 = 10 \mu s$. A peculiar feature of the data in Fig. 10a is the difference in the modulation depth. For the shorter time window, the modulation depth is beyond 20%, whereas for the longer time window, the modulation depth is reduced to 15%. Further data is provided in Fig. 10b, where the dependence of the two-point parameter $\lambda_{2P}$ on the delay $\tau_2$ is shown. Accordingly, prolongation of the observation window results in a reduction of the modulation depth. In a previous study, it has been shown that this reduction mechanism does not only depend on $\tau_2$, but also on temperature [10].

In order to gain further insight into the origin of this effect, we studied the dependence on $\tau_2$ of both $\lambda_{2P}$ and the echo signal $V_0/V_{\text{ref}}$ at various concentrations. The data and descriptions are found in Section 4 of the SI and are summarized as follows: First, no pronounced concentration dependence was observed, such that inter-molecular interactions can be excluded. Second, $V_0/V_{\text{ref}}$ changed only marginally with $\tau_2$ as compared to the changes in $\lambda_{2P}$ with $\tau_2$. The efficiency of refocusing the dipolar coupling, which evolves twice during the time interval $\tau_2$ if $t = 0$, is therefore not significantly influenced by $\tau_2$. Accordingly, $\tau_2$-dependent dipolar dephasing mechanisms related to unwanted spin flips can be excluded. One such mechanism is related to residual spin flips of unobserved spins by the observation pulses, which introduces additional dipolar evolution pathways [45]. Given the large frequency separation between pumped and observed spins, one would expect a majority of these additional evolution pathways to be negligible. Another mechanism is related to stochastic flips of the pumped spins by longitudinal relaxation (see also [10]). With the short longitudinal relaxation times encountered in Gd(III) complexes, one would actually expect such a $\tau_2$-dependent dephasing mechanism to be relevant. Our investigations made so far could not fully elucidate the reason for the reduction of the modulation depth at prolonged $\tau_2$ delays and further experiments are required. A relaxation driven mechanism appears most plausible, as also suggested by the temperature dependence of this effect [10]. Moreover, we expect this effect to be specific to high-spin Gd(III) ions, as we are not aware of such a reduction effect in distance measurements between nitroxides. The consequences of the reduction of $\lambda$ for long time windows are twofold: First, it causes an additional reduction in sensitivity $\eta$. Nonetheless, the most stringent reduction in $\eta$ at long $\tau_2$ remains the small echo signal $V_0$. Second, the altered apparent $\lambda$ at long $\tau_2$ may complicate quan-
A quantitative analysis of the inter-molecular background decay. 

![Graph](image)

Figure 11: DEER results for long distances using consecutive chirp pulses with 384 ns total duration and pre-polarization for Gd-ruler $I_7$ (green), $I_8$ (orange) and $I_{11}$ (blue). (a) Primary DEER signals $V(t)/V_0$ acquired with 258, 195 and 158 scans, respectively. (b) Form factors with modulation depths of the order of 15% (green), 6% (orange) and 8% (blue). (c) Regularized distance distributions with mean distances of 6.0 nm, 7.3 nm and 8.6 nm, respectively. The blue vertical lines indicate mean distances of 6.0 nm, 7.2 nm and 8.5 nm calculated by taking the bending of the spacer into account (See Section 6.4 of the SI). All data are illustrated with vertical offset and parameters of the chirp pulses are given in Table S2 in the SI.

A series of long-distance measurements on three different Gd-rulers is shown in Fig. 11. Data obtained with Gd-ruler $I_7$ (6 nm), Gd-ruler $I_8$ (7.2 nm), and Gd-ruler $I_{11}$ (8.5 nm), are shown in green, orange, and blue, respectively. As is already seen in the primary data in panel (a), dipolar oscillations are observed up to the longest distance, where $\tau_2$ was set to 22 $\mu$s. At the beginning of each curve, a short decay is observed which is an artifact related to the pre-polarization pulses used in these experiments [30]. Here, pre-polarization doubled the echo signal $V'_0$ (see Section 5 of the SI). The background decay in the primary data has been extracted according to the description in Section 6.3 of the SI. In the form factors in panel (b), modulation depths for the distances beyond 6 nm (orange and blue) are reduced to values below 10%. The significant differences in apparent modulation depths may also be related to the choice of pulse parameters (see Section 1.2.2 of the SI). The regularized distance distributions in panel (c) constitute well-defined distance peaks.

The dipolar evolutions windows used here allow to extract mean distances as well as the width of the distance distribution [46]. Mean distances predicted by taking the bending of the spacer into account [20] are shown by the blue vertical lines underneath the distance peaks (see Section 6.4 of the SI). Mean distances extracted from the experimental distributions (see caption and Section 6.3 of the SI) are slightly longer than the predicted distances. The deviation is up to 1.6% and presumably due to approximations in distance prediction and experimental uncertainty. In comparison to recent experiments at W-band [20], we notice apparent differences for the long Gd-ruler $I_{11}$. These are discussed in Section 6.3 of the SI.

Interestingly, all the distance distributions presented in Fig. 11c appear symmetric. As previous experiments on structurally closely related nitroxyl-spacer-nitroxyl dissolved in ortho-terphenyl have shown, the bending modes of these compounds result in an asymmetric distribution [33, 34]. Since the shape of the distance distributions in Fig. 11c may contain artifacts and broadening due to insufficient length of the dipolar evolution window, we used Gd-ruler $I_5$ with a 4.7 nm distance for a fully resolved distance distribution. For reference, the data is shown in Fig. S15 in the SI and also does not reveal the asymmetric fingerprint found for nitroxyl-spacer-nitroxyl in ortho-terphenyl. We tentatively attribute the absence of a pronounced asymmetry in our data to the longer persistence length at the glass transition temperature of the D$_2$O/glycerol solvent.

4. Conclusions

In summary, chirp pump pulses significantly improve Gd-Gd DEER sensitivity. To the best of our knowledge, modulation depths on the order of 20% combined with sensitive observation at the maximum of the Gd(III) spectrum were previously not feasible with conventional DEER techniques. This sensitivity improvement is expected to be of importance for biophysical studies, where low concentration, reduced labeling efficiency and proton-containing environments are commonplace. Moreover, the complication of finding suitable chirp pulse parameters to reach the sensitivity optimum is eliminated by means of a simple and fast setup protocol. According to this protocol, the only critical pulse parameter that remains to be adjusted is the length of the pump pulse, which needs to be adapted to the expected distance range. Importantly, the applicability of this protocol is not limited to Gd-Gd DEER. The main limitation related to the high-spin state of Gd(III) is the inverse relation between the modulation depth $\lambda$ and the echo intensity $V_0$. As demonstrated in our work, consecutive pump pulses partially alleviate this limitation. Another strategy is to employ a Gd(III) complex with ZFS parameters optimized to the experiment. In this respect, our spin dynamics simulations predict better DEER sensitivity for ZFS parameters smaller than the ones of the employed Gd-PyMTA label. Experimental variation of the ZFS by means of different ligands for the Gd(III) ion may also resolve the currently not fully understood aspects, such as the loss of the modulation depth at long evolution windows or other potential limitations of Gd-Gd DEER.

Important further considerations on Gd-Gd DEER with chirp pulses include the experimental performance at higher fields, where microwave power is reduced, and a comparison to alternative techniques, such as the RIDME technique.
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References


