Spectroscopic selection of distance measurements in a protein dimer with mixed nitroxide and Gd$^{3+}$ spin labels

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The pulse DEER (Double Electron–Electron Resonance) experiment, also known as PELDOR (Pulsed Electron Double Resonance), has become very popular in recent years for measuring nanometer distances in biological macromolecules in structural biology applications. The most common application of DEER is to measure distances between two nitroxide spin labels (SLs) attached at specific points in the macromolecule of interest. Effective methods to detect the echo reduction effect. See DOI: 10.1039/c2cp40219j

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The high-spin Gd$^{3+}$ ($S = 7/2$) SLs were shown to behave similarly to $S = 1/2$ SLs in DEER measurements, allowing the use of well-established data analysis procedures developed for the $S = 1/2$ case. Distance measurements utilizing Gd$^{3+}$ based SLs reaching up to ~6 nm were recently reported for both a DNA duplex and a protein homodimer.

The best performing Gd$^{3+}$ chelates used so far are rather large and therefore limited to labeling surface sites on proteins. Consequently, it is of interest to consider a situation where a buried site in the protein is labeled with a small nitroxide and the surface site with a bulky Gd$^{3+}$ tag. Furthermore, to solve complex biochemical problems involving an assembly of proteins, it is sometimes beneficial to use more than a single type of spin label. Such complex labeling schemes enable the measurement of more than a single distance on the same sample with additional resolution based on spectroscopic selection of different pairs of labels. This approach was previously demonstrated on a mixture of $^{15}$N and $^{14}$N nitroxide based biradicals and between copper(n) and a nitroxide on a model compound. Such an approach also distinguishes between homo- and hetero-dimers.

The potential of DEER distance measurements between a nitroxide SL and a Gd$^{3+}$ ion was recently demonstrated on a model compound with a rigid spacer and a Gd$^{3+}$–nitroxide distance of ~2.5 nm, using X-band and Q-band (34 GHz) spectrometers. At W-band such an orthogonal spin labeled system should exhibit orientation selection only due to the nitroxide because of the isotropic g of Gd$^{3+}$, the isotropic character of its central transition (to second order), and the large distribution of its zero field splitting (ZFS). Because of this broad distribution setting the pump or observer pulse to the broad, featureless background of the Gd$^{3+}$ spectrum that makes contributions from all Gd$^{3+}$ transitions, except the

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central transition, with relative intensities determined by the Boltzmann distribution, there is no orientation selection for virtually any position in the Gd\(^{3+}\) spectrum.\(^{18,20,25}\) Accordingly, the DEER data analysis needs to include only two angles, which define the orientation of the inter-spin vector with respect to the g-principal axis system of the nitroxide. This is a significant simplification compared to the case of two nitroxides where five angles need to be considered.\(^{15}\) In proteins, the existence of orientation selection depends on the extent of mobility of the SL, which is a function of the protein local motion and the flexibility of the SL tether. This mobility translates into an orientation and distance distribution upon freezing. For highly flexible SLs, the separation between the maximum of the |S| and C0/2 transitions of the Gd 3+ spectrum and the maximum of the \(T_\text{exp}\) that the \(T_\text{exp}\) is orientation independent. For Gd\(^{3+}\), \(\lambda\) has to be scaled by the relative populations of the M\(S_\text{z}\) = ±1/2 levels in the case that the pump pulse is set to the central transition of the Gd\(^{3+}\) spectrum and g(\(\Delta\omega\)) corresponds to the lineshape of the central transition.\(^{26}\) The signal to noise ratio \((S/N)\) relevant for DEER is given by:\(^{6}\)

\[
S/N \propto V_0 \lambda \exp \left( -\frac{T_{\text{exp}}}{T_{\text{obs}}(\lambda)} \right) \frac{1}{\sqrt{T_{\text{obs}}(\lambda)}} \quad (3)
\]

where \(T_{\text{obs}}(\lambda)\) and \(T_{\text{obs}}(\lambda)\) are the phase memory time and the spin-lattice relaxation time of the observer spins, and \(T_{\text{exp}}\) is the time between the first pulse and the focused echo in the four pulse DEER experiment (see Fig. S2, ESI†). According to eqn (3), the nitroxide-Gd\(^{3+}\) distance is measured best by setting the observer frequency to the central transition of the Gd\(^{3+}\) spectrum to obtain a large \(V_0\) that can be accumulated very fast due to the short \(T_\text{1}\). The pump pulse should be set to the nitroxide spectrum to achieve a large \(\lambda\) because the nitroxide spectrum is narrower.\(^{23}\) We note that the \(T_\text{1}\) of the pumped spins is irrelevant as long as it is not too short.

The separation between the maximum of the \((-1/2) \to |1/2\rangle\) transition of the Gd\(^{3+}\) spectrum and the maximum of the

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**Optimizing experimental conditions for W-band Gd\(^{3+}\)-nitroxide DEER measurements.** When performing pulse EPR measurements on a pair comprising a nitroxide with \(S = 1/2\) and a Gd\(^{3+}\) with \(S = 7/2\), their considerably different spin physics characteristics have to be considered. The spin-lattice relaxation time of Gd\(^{3+}\) is several orders of magnitude shorter than that of the nitroxide (\(\sim 100 \mu s\) and \(\sim 100\) ms, respectively, at 10 K) and the transition probabilities of Gd\(^{3+}\) are larger. Accordingly, at a given MW power, the \(\pi\) and \(\pi/2\) MW pulses are much shorter for Gd\(^{3+}\) than for nitroxides. This allows selective distance measurements.

**Fig. 1b** shows the 10 K ED (Echo Detected) EPR spectrum of the mixed labeled ERp29 sample recorded under conditions optimized for Gd\(^{3+}\). The spectrum consists of a sharp, \(\sim 1.1\) mT wide line due to the central \((-1/2) \to |1/2\rangle\) transition, superimposed on a broad background arising from all other transitions. The contribution from the nitroxide spectrum (marked by an arrow in Fig. 1b) is barely observable under these conditions due to insufficient MW power. **Fig. 1c** shows the spectrum of the same sample acquired under MW power optimized for the nitroxide. Under these conditions, the signal of the nitroxide spectrum is strong and the Gd\(^{3+}\) signal is attenuated.

The pairwise time evolution of the echo intensity in a DEER experiment is given by:\(^{1-3}\)

\[
V(t) = V_0(1 - \lambda (1 - \cos \omega_d (1 - 3 \cos^2 \theta) t)) \quad (1)
\]

where \(V_0\) is the echo intensity at \(t = 0\), \(g_1\) and \(g_2\) are the g values, \(r\) is the inter-electron distance, and \(\theta\) is the angle between the inter-electron vector and the magnetic field.

The modulation depth, \(\lambda\), for a pumped nitroxide is given by:

\[
\lambda = \int_{-\infty}^{\infty} \frac{e_{\text{pump}}}{\epsilon_{\text{pump}}/2} \sin^2 \left( \frac{t_p \sqrt{(\omega^2 + \Delta \omega^2)}}{2} \right) g(\Delta \omega) d(\Delta \omega) \quad (2)
\]

where \(e_{\text{pump}}\) and \(t_p\) are the amplitude and duration of the pump pulse, respectively, \(\Delta \omega\) is the off-resonance frequency, and \(g(\Delta \omega)\) is the lineshape of the nitroxide EPR spectrum. Eqn (2) are valid when there is no orientation selection, namely \(\lambda\) is orientation independent. For Gd\(^{3+}\), \(\lambda\) has to be scaled by the relative populations of the M\(S_\text{z}\) = ±1/2 levels in the case that the pump pulse is set to the central transition of the Gd\(^{3+}\) spectrum and g(\(\Delta \omega\)) corresponds to the lineshape of the central transition.\(^{26}\) The signal to noise ratio \((S/N)\) relevant for DEER is given by:\(^{6}\)

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**Fig. 1** (a) Structure of the ERp29 dimer (PDB ID 2QC7) showing the labeling sites and the spin labels C1-Gd\(^{3+}\) (1) and MTSL (2) used in this study. Sample composition: 25% \(A = B = 1\); 50% \(A = 1\); \(B = 2\); 25% \(A = B = 2\). (b) and (c) EPR spectra of the ERp29 dimer acquired with MW power optimized for Gd\(^{3+}\) (b) and for the nitroxide SL (c). Other experimental parameters were the same for both spectra: \(\pi/2\) and \(\pi\) pulse durations were 60 ns and 120 ns, respectively; \(\tau = 550\) ns; \(T = 10\) K; repetition time: 50 ns.
The nitroxide spectrum at W-band is $\Delta \nu = 685$ MHz (Fig. 1c). Unfortunately, this is larger than the bandwidth of our current cavity ($\sim 100$ MHz). We therefore chose a configuration with $\Delta \nu < 100$ MHz, setting the observer pulse to the broad background of the Gd$^{3+}$ spectrum as shown in Fig. 1c, thus considerably compromising $T_0$ (by a factor of about 20) and reducing the signal-to-noise (S/N) ratio according to eqn (3). In this configuration, pulses given at the observer frequency affect, in principle, both the Gd$^{3+}$ and nitroxide spins. The latter, however, were suppressed by using a high repetition rate ($5$ kHz) that saturates the observer nitroxide spins due to their much longer $T_1$ at $10$ K.

Our chosen experimental configuration with $\Delta \nu = 65$ MHz created a very strong direct off-resonance effect of the pump pulse on the observer spins, resulting in a significant reduction of $T_0$. This effect is similar to the phase shift effect described earlier$^{27}$ but is much more pronounced due to the high transition probability of the high-spin Gd$^{3+}$ ion. A pump pulse with a flip angle of $\pi$ for the nitroxide spins amounts to a $\sim 4\pi$ pulse for the Gd$^{3+}$ spins. This difference produced a much stronger echo reduction compared to the situation of DEER applied to two nitroxide or two Gd$^{3+}$ labels, where the echo reduction effect is practically negligible. This effect is described and analyzed in detail in the ESL. To obtain DEER data with an acceptable S/N ratio, we reduced the duration of the pump pulse until $T_0$ was within $\sim 70\%$ of its initial intensity without the pump pulse.

**DEER distance measurements.** The Gd$^{3+}$–nitroxide four-pulse DEER data after the background subtraction are shown in Fig. 2a. The experiment was performed with a pump frequency, $\nu_{\text{pump}}$, set to the maximum of the nitroxide spectrum and the observer frequency set to $\nu_{\text{obs}} = \nu_{\text{pump}} + 65$ MHz. At this spectral position, many orientations are excited simultaneously. Moreover, the X-band room temperature EPR spectrum (Fig. S1, ESL) indicates a relatively unconstrained motion of the nitroxide spin SL at room temperature. This suggests little orientation selection at low temperatures. Accordingly, we proceeded to analyse the data neglecting orientation selection, using the common approach used for X-band DEER data (eqn (1) and (2)). The resulting distance distribution is shown in Fig. 2a. Details of the background subtraction procedure and data analysis are available in the ESL. The distance distribution shows a maximum at $5.86$ nm.

Next we measured the distances in the ERp29 dimers labeled with two C1-Gd$^{3+}$ SLs ($25\%$). To exclude the nitroxide spins from the DEER experiment, we set both the observer and the pump frequencies outside of the nitroxide EPR spectrum. The observer frequency was set to the maximum of the $| -1/2 \rangle \rightarrow | 1/2 \rangle$ Gd$^{3+}$ transition to maximize $T_0$ and the pump frequency was set to $\nu_{\text{pump}} = \nu_{\text{obs}} + 65$ MHz. The distance distribution shown in Fig. 2b (right) has a maximum at $6.04$ nm. This is identical to the distance measured in the previous work on the pure C1-Gd$^{3+}$ labeled sample.²⁰

Finally we measured the nitroxide–nitroxide distance in the ERp29 dimers with two nitroxide spin labels ($25\%$). There is no range in the nitroxide EPR spectrum without overlap with the Gd$^{3+}$ signal. Therefore we took advantage of the different relaxation properties of the two paramagnetic centers and carried out the measurements at $50$ K, where the Gd$^{3+}$ contribution to the EPR spectrum becomes negligible. The measurements were carried out at two observer frequencies and the two separate traces are shown in Fig. S4 (ESI†). Assuming that the two acquired DEER measurements sample most of the available distances (see the ESL for details) we summed the two DEER datasets. Fig. 2c (left) shows the combined DEER trace after the background subtraction. This gave a nitroxide–nitroxide distance distribution with a maximum at $5.5$ nm (Fig. 2c, right). The modulation depth in all three types of distance measurements is lower than expected from eqn (2) because of the statistical nature of the spin labeling.

We carried out model calculations based on the crystal structure of ERp29 (PDB ID 2QC7) to account for the experimentally determined Gd$^{3+}$–nitroxide and the nitroxide–nitroxide distance distributions. Our recent Gd$^{3+}$–Gd$^{3+}$ distance measurements showed that this structure prevails also in solution.²⁰ The distance distributions were modeled by crafting the spin labels onto the cysteine residues, randomly varying the dihedral angles, and eliminating those rotamers that had steric clashes with the protein. A comparison of the experimental and
calculated distance distributions of the different pairs of spin labels is shown on the right side of Fig. 2. The substitution of one of the C1-Gd\(^{3+}\) tags by a nitroxide spin label shifts the maximum of the calculated distance distribution from 6.05 nm\(^2\) to 5.81 nm. Substitution of the second Gd\(^{3+}\) tag by the nitroxide SL shortens the distance further to 5.6 nm. These shifts in the maxima of the calculated distance distributions agree well with the experimental results. The largest discrepancy (no more than 0.1 nm) is observed for the nitroxide–nitroxide distance distribution.

The modelled distance distribution is the narrowest for two C1-Gd\(^{3+}\) tags, whereas it becomes broader when one C1 tag is changed to a nitroxide SL and broadens even further upon the second substitution. This reflects the larger conformational space sampled by the nitroxide SL compared to the bulky C1-Gd\(^{3+}\) tag, which positions the Gd\(^{3+}\) ion in a more well defined location relative to the protein. This trend is not reproduced experimentally, which may be attributed to S/N limitations and the insufficiently long evolution time in the nitroxide–Gd\(^{3+}\) DEER measurements. Alternatively, the conformational sampling of the nitroxide SL may be non-uniform due to its hydrophobicity.

The present work shows that we can spectroscopically select Gd\(^{3+}\)--Gd\(^{3+}\), nitroxide–Gd\(^{3+}\), and nitroxide–nitroxide distance distributions, in the range of 6 nm, from a mixed labeled protein dimer using a very small quantity of protein (about 0.3 nmol in total). We note that the effective concentration of the homodimer using a very small quantity of protein (about 0.3 nmol in total). We note that the effective concentration of the homodimer is usually not feasible for the narrow band cavities used in most W-band EPR spectrometers. This limitation can be overcome by utilizing a dual mode resonator as reported recently. We expect that the Gd–nitroxide DEER sensitivity will increase by a factor of 20–40 with such a cavity.

When there is no interest to measure several distances from a single sample, realization of the full S/N advantage of the Gd\(^{3+}\)--nitroxide DEER measurement requires preparation of a sample in which 100% of the molecules of interest are labeled with both types of paramagnetic centers. This is readily achieved with heterodimers, where each monomer can be labeled separately with a different type of spin label. If an intramolecular distance is of interest, it is possible to utilize labeling schemes where two different labels are attached to the same molecule as is common for FRET ( Förster Resonance Energy Transfer) experiments. Random labeling will lead to Gd\(^{3+}\)--nitroxide pairs in only 50% of the sample as in the case of ERp29 shown here.

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Notes and references