Selective Distance Measurements Using Triple Spin Labeling with Gd^{3+}, Mn^{2+}, and a Nitroxide

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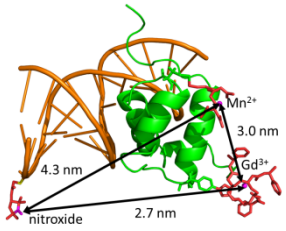
ABSTRACT

Distance measurements by pulse EPR techniques, such as double electron-electron resonance (DEER, also called PELDOR), have become an established tool to explore structural properties of bio-macromolecules and their assemblies. In such measurements a pair of spin labels provides a single distance constraint. Here we show that by employing three different types of spin labels that differ in their spectroscopic and spin dynamics properties it is possible to extract three independent distances from a single sample. We demonstrate this using the *Antennapedia* homeodomain orthogonally labeled with Gd^{3+} and Mn^{2+} tags in complex with its cognate DNA binding site labeled with a nitroxide.

TOC GRAPHICS



Double electron-electron resonance (DEER, also called PELDOR) experiments provide valuable long-range distance restraints to probe the structure of biological macromolecules and their assemblies by measuring the dipolar interaction between unpaired electron spins at different sites.[1] For diamagnetic systems, DEER experiments are commonly performed following the covalent attachment of two, usually identical, spin labels at well defined, strategically chosen locations within the biological macromolecule. While the majority of DEER applications have been carried out using the well-established nitroxide spin label, other options for spin labelling have emerged in the last decade, which are based on Gd^{3+} [2-3], Mn^{2+} [4,5,6] and Cu^{2+} ions[7-8] as well as trityl radicals.[9-11] Cu^{2+} has the advantage of being a simple S=1/2 and is attractive at common X-band frequencies where its spectra width is limited. Trityl radicals are unique as they allow for room temperature distance measurements.[11] Among the metal ions Gd^{3+} and Mn^{2+} labels are particularly attractive for measurements at a high field, such as W-band (95 GHz, ~3.5 T), though Q-band (~34 GHz, ~1.2 T) is also possible.[12] The use of Gd^{3+} complexes as spin labels at W-band has been shown to be advantageous over the commonly used nitroxide label due to the absence of orientation selection[13-14] and their stability in living cells.[15-16]

The most popular method for introducing Gd^{3+} and Mn^{2+} spin labels is via the thiol group of a native or genetically engineered cysteine residue as is commonly done with nitroxide spin labels. An alternative method is to employ an unnatural amino acid that has been genetically encoded to be incorporated in response to an amber stop codon.[17-18] While DEER applications using Gd^{3+} and Mn^{2+} have so far involved mostly Gd^{3+}–Gd^{3+} and Mn^{2+}–Mn^{2+} distance measurements, the possibility to use different chemistries to attach different types of spin labels triggered the exploration of distance measurements between different types of spin labels. So far, Gd^{3+}–nitroxide[19-22] and Mn^{2+}–nitroxide[23-25] distance measurements have been reported both in model compounds and in bio-

macromolecules. The advantage of this approach is the increase in sensitivity as compared to the "homogenous" metal-metal spin labeling scheme, although orientation selection is reintroduced to a limited extent.[21] Trityl-nitroxide distance measurements,[26-27] as well as distance measurements between a paramagnetic metal ion that is an integral part of a metalloprotein, such as Cu^{2+} and Fe^{3+}, and a nitroxide[28] or a trityl spin label,[29] have also been reported.

When using two spin labels (similar or different), the EPR-based distance measurements produce a single distance (except for oligomeric proteins); this implies that *n* different samples need to be prepared, if structural information is to be obtained from *n* distances. This is labor intensive. The possibility of performing spectroscopically selective DEER measurements using two types of paramagnetic centers in a three spin system and obtaining information beyond a single distance has been demonstrated on model systems, nanoparticles and trans-membrane model peptides.[23,30-33] In addition, measurements were carried out on a homodimeric protein, produced by mixing monomers that were each labeled with a different spin label (Gd^{3+} and nitroxide), and spectroscopic selection of Gd^{3+}–nitroxide, nitroxide–nitroxide and Gd^{3+}–Gd^{3+} distances was demonstrated.[22] However, as the two labels were attached to the same site of the protein, the different distances extracted reflected the different chemical structures of the spin labels rather than giving three independent structural constraints on the protein. An alternative approach for introducing two different spin labels takes advantage of two orthogonal labeling chemistries by using a cysteine to label one site and an unnatural amino acid to label the other site.[19]

In the present work we show that by using three different spin labels based on Gd^{3+}, Mn^{2+}, and nitroxide tags it is possible to extract three independent distances, Gd^{3+}-Mn^{2+}, Gd^{3+}-nitroxide and Mn^{2+} -nitroxide, from a single sample.[34] We demonstrate this using the *Antennapedia* homeodomain labeled with Gd^{3+} and Mn^{2+} tags, introduced via orthogonal labeling, in complex

with its cognate DNA binding site[35], which was labeled with a nitroxide spin label (see Fig. 1a). More specifically, the unnatural amino-acid *p*-azido-L-phenylalanine (AzF) was introduced at position 22 and labeled with a Gd^{3+} chelate, C3,[17] and the Mn^{2+} ion was introduced via a MTS-EDTA tag attached to Cys39. Finally, the nitroxide was introduced through a reaction of the oligonucleotide d(CTCTAATGGCTT*TC), containing a phosphorothioate (PT) group between the second and third last nucleotides (indicated by a star), with IAM-PROXYL. PT groups in DNA are known to be amenable to selective alkylation.[36-37] The complementary strand was not labeled (see Fig. 1b-d for the reaction schemes). Experimental details are given in the Supplementary Information, SI.

The selection and assignment of the three different distances was based on: (i) The different spectra of the three spins, particularly at W-band, which provide increased spectral resolution. (ii) The different nutation frequencies of nitroxide (S=1/2), Mn^{2+} (S=5/2) and Gd^{3+} (S=7/2) which, for the central transitions, follow the ratio 1:3:4. (iii) The longer electron spin-lattice relaxation time, T_1, of nitroxides relative to Gd^{3+} and Mn^{2+}. Initially we performed a series of W-band echo-detected EPR (ED-EPR) measurements at different conditions to optimize the EPR spectra of the different paramagnetic centers, which have very different spectroscopic properties. The conditions found for maximizing the ED-EPR intensity of each of the spin labels are summarized in the SI and the spectra are shown in Figure 2. The nitroxide spectrum (Fig. 2a) is best observed while optimizing the pulse lengths for a S=1/2 system, which will lead to too long pulses for the Mn^{2+} and Gd^{3+} central transition. For example, for the same power setting a $\pi/2$ pulse of 30 ns for a nitroxide corresponds to $3\pi/2$ and 2π pulses for Mn^{2+} and Gd^{3+}, respectively. Similarly, for optimized observation of Mn^{2+} and Gd^{3+} (Fig. 2b,c), in addition to the too short pulses for the nitroxide, a fast repetition rate, suitable for the short T_1 values of Mn^{2+} (~121 μs)

and Gd^{3+} (115 μs) will saturate the nitroxide (T_1 =45 ms) signals (see Fig. S1). These concepts were used in the past to select between Gd^{3+} and nitroxides.[20, 22, 31] The Mn^{2+} spectrum reveals the presence of a small contribution from Mn^{2+} ions that are not bound to the EDTA tag and display much narrower peaks. We attribute this to migration of a small amount of Mn^{2+} ions from the protein to the DNA (see Fig. S2).

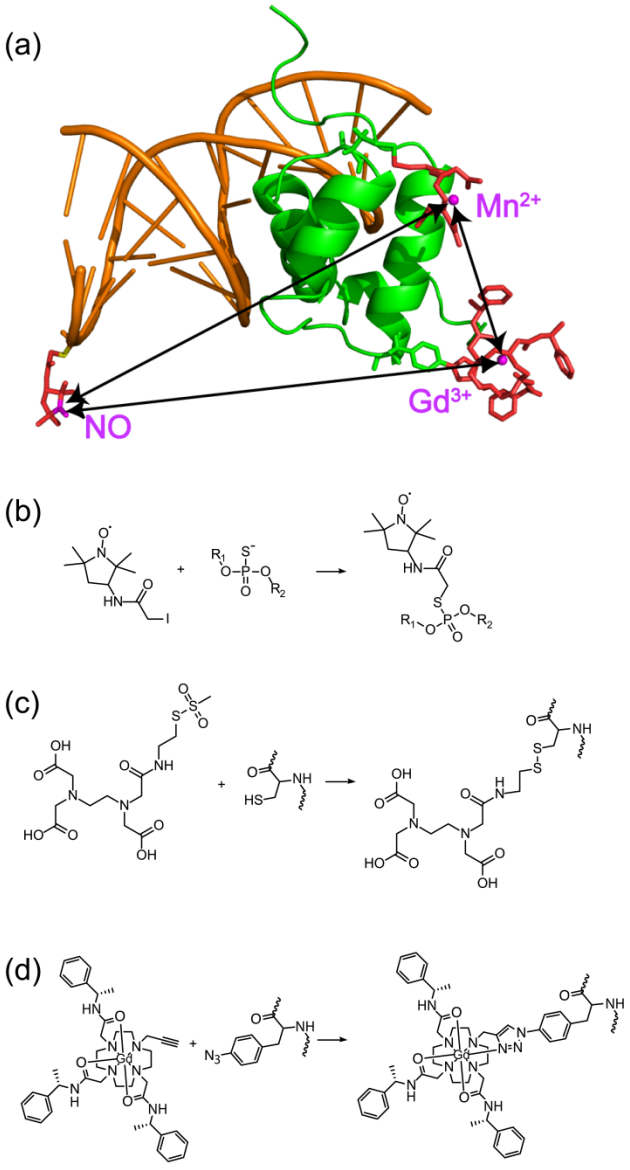


Figure 1. Labeling schemes used. (a) Cartoon representation of the complex between the *Antennapedia* homeodomain and its cognate DNA. The three different tags used in the present work were modeled on the NMR structure of the complex (PDB ID: 1AHD)[38] showing the tags in red and the paramagnetic centers in magenta. (b) Reaction scheme of a PT group in DNA with IAM-PROXYL. (c) Reaction scheme of a cysteine residue with EDTA-MTS. (d) Cu(I)-catalyzed click reaction of an AzF residue with the C3-Gd tag.

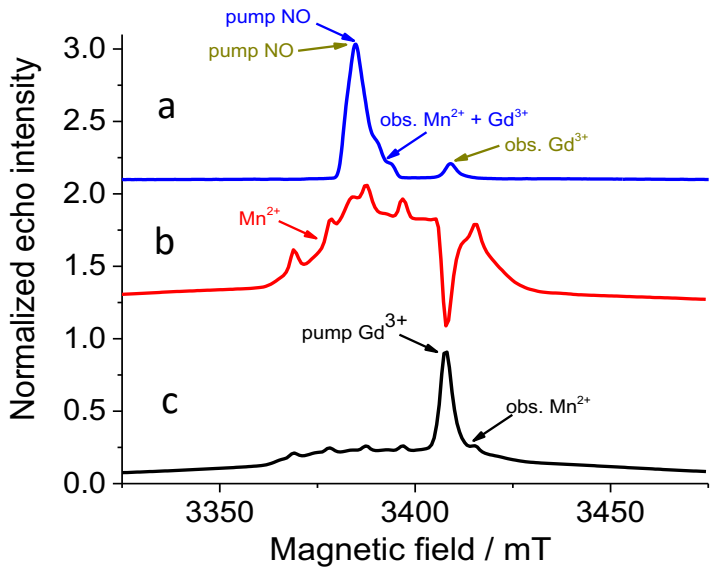


Figure 2. W-band ED-EPR spectra of the *Antennapedia* homeodomain-DNA complex recorded under different conditions (see SI) suited to highlight each one of the labels separately. Measurements were optimized for the observation of (a) nitroxide (blue), (b) Mn^{2+}(red), and (c) Gd^{3+}(black). The positions of the pump and observe pulses for the selection of different distances are also indicated. The top trace shows in blue the set up for Gd^{3+}-NO and Mn^{2+}-NO distances and in green the set-up for Gd^{3+}-NO distance selection using a dual-mode cavity. The bottom trace shows the pulse position for Mn^{2+}-Gd^{3+} selection.

To deterime the distances between the three labels we performed a series of DEER measurements under different conditions optimized for selective detection of the different distances (Fig. 3). The full experimental details of the different measurements are given in the SI. The pulse frequencies for selecting individual distance distributions are also summarized in Fig. 2. All DEER traces were background-corrected and analyzed using the DeerAnalysis software[38] and for each trace the regularization parameter recommended by DeerAnalysis was used for obtaining the distance distributions. To measure the Gd^{3+}–Mn^{2+} distance we positioned the pump pulse on the Gd^{3+} peak and the observe frequency 100 MHz up-field, close to the last peak of the

Mn^{2+} multiplet to maximize the Mn^{2+} signal (Fig. 3, black trace). These conditions ensured that there was no overlap with the nitroxide spectrum, thus avoiding any contributions from distances involving the nitroxide (Gd^{3+}-nitroxide, Mn^{2+}-nitroxide). Furthermore, the repetition rate used was such that the nitroxide is saturated. The distance distribution consists of a major peak at 3 nm and two a shoulder at 3.6 and a smaller peak at 4.5 nm. To examine the impact of DNA binding on this distance we also measured the Mn^{2+}–Gd^{3+} distance in the homeodomain in the absence of DNA under the same conditions (black trace in Fig. 3). Here the distance distribution gave a strong peak at 2.8 nm along with a weak peak at 4.5 nm, indicating that the binding caused a slight shift of the maximum of the distance distribution and a broadening of the distribution. The broadening can also include a small contribution from Mn^{2+} ions that migrated to the DNA.

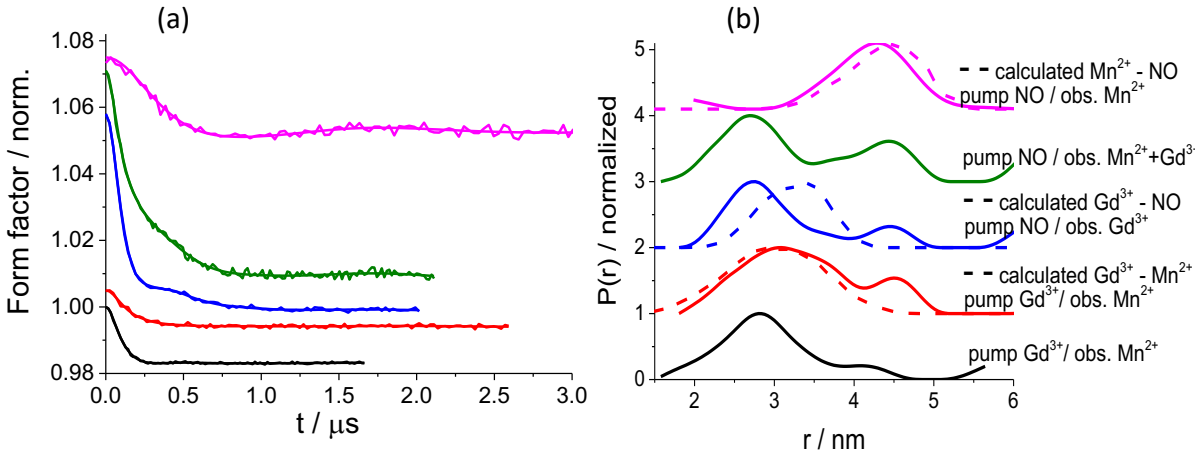


Figure 3. (a) Series of DEER traces (after background removal) and fits obtained for the homeodomain/DNA complex designed to select the three different distances in the sample along with DEER traces of control samples (see text). The traces were shifted vertically relative to each other for improved visibility. The pulse set up is indicated next to the traces in (b) and as indicated in Fig. 2 and the Table S2. The blue trace was obtained with a dual-mode cavity. The black trace corresponds to the homeodomain alone and the magenta trace corresponds to the homeodomain/DNA complex where the protein was labeled only with Mn^{2+} only. (b) Distance distributions obtained from these traces with the DeerAnalysis[38] software. The primary DEER data are shown in Fig. S4. Dashed lines indicate the distance distributions calculated for the three labels modeled on the NMR structure of the *Antennapedia* homeodomain-DNA complex (PDB ID: 1AHD).[39]

The distance between the nitroxide label on the DNA and the Gd^{3+} label on the homeodomain was measured with a dual-mode cavity (blue trace in Fig. 3).[21, 40] This allowed positioning the

pump pulse on the maximum of the nitroxide spectrum and the detection frequency on the peak of the Gd^{3+} spectrum ($\Delta\nu$ = 695 MHz). At this observe position the contribution of the signal from Mn^{2+} is negligible compared to that from Gd^{3+} (see Fig. 2c) and therefore any contributions from the Mn^{2+}-nitroxide distance are equally negligible. The ED-EPR spectrum was measured under these conditions also for a τ value of 4.5 μs (see Fig. S3), relevant for the DEER measurements, showing that the relative contribution of the Mn^{2+} remained low, namely its phase memory is similar to that of the Gd^{3+} . Under this set-up the nitroxide and Mn^{2+} contribute to the spectrum at the pump pulse position, but because the pump π pulse is optimized for the nitroxide (S = 1/2) it is very inefficient for Mn^{2+} and therefore the contribution from the Mn^{2+}–Gd^{3+} distances to the DEER trace is expected to be negligible as well. This is confirmed by the large modulation depth of 5.5%, which is much larger than the depth expected for Mn^{2+}–Gd^{3+} DEER when the pump pulse frequency is set outside the central transition of the Gd^{3+} spectrum. For this optimized setup we obtained a Gd^{3+}–nitroxide distance distribution with a maximum at 2.8 nm. Although this distance is closely similar to the Mn^{2+}–Gd^{3+} distance, our experimental conditions allow unambiguous discrimination between the two. Measurements with the pump pulse set to different positions within the nitroxide EPR spectrum to test for orientation selection are shown in Fig. S5.

To measure the Mn^{2+}–nitroxide distance distribution we positioned the pump pulse on the maximum of the nitroxide spectrum and placed the observe pulses 140 MHz upfield (See Fig. 2a). At this observe position we expect the signal to have contributions from all three spin labels. Therefore, the distance distribution should have contributions from both Mn^{2+}–nitroxide and Gd^{3+}–nitroxide dipolar interactions, but we can exclude contributions from the Mn^{2+}–Gd^{3+} interactions because the pump pulse length was optimized for the nitroxide. Indeed, the distance distribution

shows two distances, the first at about 2.8 nm, which is very similar to the Gd^{3+}–nitroxide distance measured using the dual-mode cavity, and the second distance at 4.4 nm, which we attribute to the Mn^{2+}–nitroxide distance (see green traces in Fig. 3).

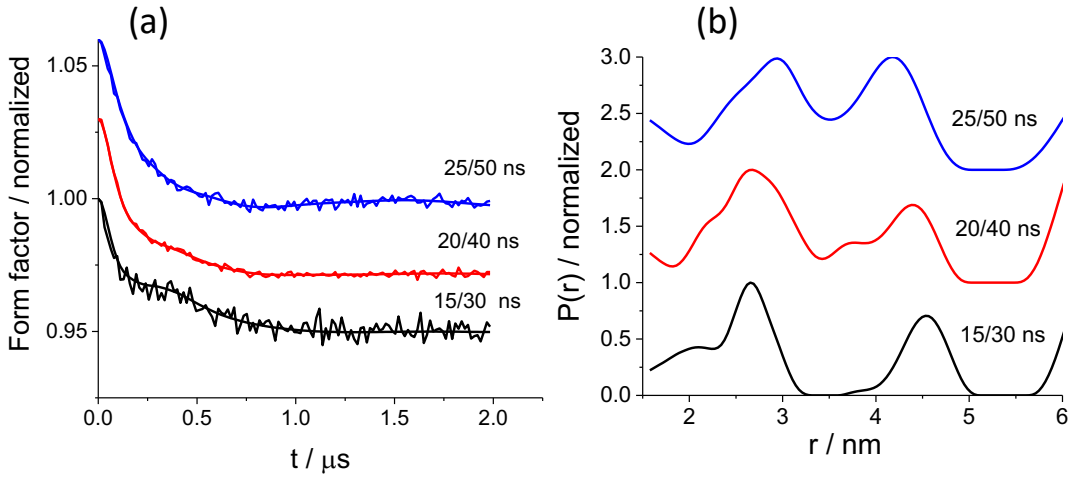


Figure 4. DEER measurements on the *Antennapedia* homeodomain-DNA complex using different observe pulse durations and same microwave power. The pump pulse was set on the nitroxide maximum and the observe pulses on the overlapping signals from Gd^{3+} and Mn^{2+} (see Fig. 2a) (a) DEER traces after background subtraction, including the fit based on the distance distributions shown in (b). (b) Distance distributions obtained from the data in (a).

To substantiate this assignment we repeated the DEER measurements with different observe pulse lengths (same microwave power) to differentiate between Gd^{3+} and Mn^{2+}. At this observe frequency the major contribution to the Gd^{3+} spectrum comes from the M_s = -3/2 to M_s = -1/2 transition, while that of the Mn^{2+} spectrum is mostly from the central transition, M_s = -1/2 to M_s = 1/2. Accordingly, the optimal nutation frequency of Gd^{3+} is expected to be larger by a factor of at least $\sqrt{15/9} = 1.29$ due to its higher spin. For a constant power setup with observed pulses of 15 and 30 ns, optimized for the central transition of Gd^{3+}, which has a nutation frequency very similar to that of the Gd^{3+} observe transition ($\sqrt{15/16} = 1.03$), we thus expect longer observe

pulses to reduce the Gd^{3+} contribution and increase the Mn^{2+} contribution to the echo and, consequently, increase the relative contribution of the Mn^{2+}–nitroxide distances to the distance distribution. Figure 4 shows the result of such an experiment. While for the 15/30 and 20/40 ns set up the observed difference in the relative intensity of the peaks is within experimental error, for the 25/50 ns the relative weight of the 4.4 nm peak clearly grows. Here we observe a shift of the maxima of two peaks from 4.4-4.45 nm to 4.2 nm and from 2.7 to 2.9 nm, the origin of which is currently unknown. (See Fig. S6 for the primary time domain DEER data along with the ED-EPR spectra recorded with the observe pulses indicated in Fig. 4, and Figs. S7 and S8 for uncertainties in the distance distribution as obtained from the validation process using DeerAnalysis for the data shown in Figs. 3 and 4). To confirm this assignment we repeated the nitroxide-Mn^{2+} DEER experiment on a sample where the homeodomain was labeled only with Mn^{2+}. As expected, this experiment yielded only one peak in the distance distribution, at 4.3 nm (magenta curve in Figure 3).

Finally, we compared the distances we obtained from the DEER experiments to distances predicted from the structure of the homeodomain-DNA complex.[39] The results are shown as dashed traces in Figure 3b. The experimentally determined Mn^{2+}–nitroxide and Gd^{3+}–Mn^{2+} distance distributions agree well with the calculated ones except for the additional small peak at 4.5 nm in the Gd^{3+}–Mn^{2+} distribution, which may arise from a small amount of Mn^{2+} that migrated to the DNA (see Fig. S1). The calculated Gd^{3+}–nitroxide distance is shifted by ~0.5 nm towards a longer distance.

To conclude, we have demonstrated for the first time DEER distance measurements between three different spin labels in a single biological sample and obtained three independent distance constraints. Specifically, a protein was labeled orthogonally with a Gd^{3+} tag inserted through the

use of an unnatural amino acid and a Mn^{2+} tag attached to a cysteine residue. The third spin label, a nitroxide, was introduced in the DNA part of the complex. This approach reduced the number of samples needed to obtain the distance information from three to one. This new approach is particularly useful for studying complex formation in bio-macromolecules.

Supporting Information. Experimental details, T_1 measurements, ED-EPR spectra of Antennapedia homeodomain with and without DNA, primary DEER data, Gd^{3+}-nitroxide DEER, validation of distance distributions.

AUTHOR INFORMATION

The authors declare no competing financial interests.

ACKNOWLEDGMENT

D. G. and G. O. acknowledge the support of the Israel Science Foundation (ISF grant No. 334/14) and the Australian Research Council, respectively. This research was made possible in part by the historic generosity of the Harold Perlman Family (D. G.). D. G. holds the Erich Klieger Professorial Chair in Chemical Physics.

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