Gd(III)−Gd(III) Relaxation-Induced Dipolar Modulation Enhancement for In-Cell Electron Paramagnetic Resonance Distance Determination

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Ⓢ *Supporting Information*

ABSTRACT: In-cell distance determination by electron paramagnetic resonance (EPR) spectroscopy reveals essential structural information about biomacromolecules under native conditions. We demonstrate that the pulsed EPR technique RIDME (relaxation induced dipolar modulation enhancement) can be utilized for such distance determination. The performance of in-cell RIDME has been assessed at Q-band using stiff molecular rulers labeled with Gd(III)-PyMTA and microinjected into *Xenopus laevis* oocytes. The overtone coefficients are determined to be the same for protonated aqueous solutions and inside cells. As compared to in-cell DEER (double electron−electron resonance, also abbreviated as PELDOR), in-cell RIDME features approximately 5 times larger modulation depth and does not show artificial broadening in the distance distributions due to the effect of pseudosecular terms.
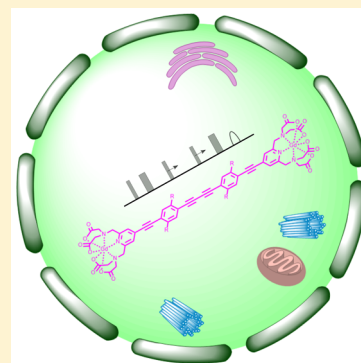
Electron paramagnetic resonance (EPR) spectroscopy provides the means to determine distances between magnetically coupled electron spins. By combination of pulsed EPR techniques with site-directed spin labeling, distances in the range up to 8 nm, and under the very special condition of a perdeuterated biomacromolecule up to 16 nm, can be determined.[1−10] Distance determination is based on the measurement of the dipolar coupling frequency, ω_{dd}, which is inversely proportional to the cube of the distance between two magnetically coupled spins and can be performed in any kind of environment, including living cells. Because most cell components are diamagnetic, EPR-based distance determination inside cells is virtually background-free. Double electron−electron resonance (DEER or PELDOR)[11,12] is the commonly used technique to perform such in-cell distance determination.[13−19] In-cell EPR distance determination puts tight requirements on spin labels in terms of toxicity and stability. Complexes of Gd(III) with chelating ligands are ideal candidates for in-cell EPR and feature low toxicity, high stability, suitable spin relaxation times, and no orientation selection.[18,20−25] Despite all these favorable properties of Gd(III)-based spin labels, conventional Gd(III)−Gd(III) DEER with rectangular pulses is still not the ideal technique for in-cell distance determination because of its low modulation depth and artifacts at short distances, e.g., below 3.4 nm if Gd(III)-PyMTA is used as the spin label.[17,18,26,27]

Though it is possible to increase the modulation depth and to reduce the artifacts at short distances by using chirp pulses[28] and a dual-mode cavity,[29] these improvements can be achieved only with an additional comprehensive and expensive equipment of the EPR spectrometer with a high-power microwave amplifier, an arbitrary-waveform generator, a broad-band, or a dual-mode cavity.

Alternatively, the downsides of conventional Gd(III)−Gd(III) DEER can be overcome in a related experiment abbreviated RIDME (relaxation-induced dipolar modulation enhancement).[30−32] RIDME is a single-frequency technique and makes use of relaxation-induced spin flips, whereas DEER is a two-frequency technique which detects at the observer frequency while flipping coupled spins in a controlled way by a π-pulse at the pump frequency. Thus, RIDME is technically less demanding than DEER and has no limitations with respect to the excitation bandwidth. As an example, consider a rectangular pump pulse of 24 ns (excitation bandwidth of 40 MHz), which flips only a small part of the spins out of the 2 GHz-broad spectrum of Gd(III). While the contribution to the DEER signal is determined by this part of excited Gd(III)

spins, the contribution to the RIDME signal is determined by the spins from the entire spectrum, which flip because of relaxation. Consequently, the modulation depth of in vitro Gd(III)−Gd(III) RIDME is increased by a factor of 10 compared to in vitro Gd(III)−Gd(III) DEER.[26,33−35] These features of RIDME can also be beneficial for distance measurements inside cells. While the performance of Gd-(III)−Gd(III) RIDME has recently been demonstrated for model systems in deuterated solvents at Q- and W-band frequencies,[33−35] the in-cell application remained elusive. To the best of our knowledge, the only reported in-cell RIDME experiment was for trityl spin labels, which are $S = 1/2$ systems and have very different relaxation properties as compared to Gd(III) complexes.[36] Here, we utilize stiff molecular rulers spin labeled with a Gd(III)-PyMTA complex (Figure 1A,B) and demonstrate that the 5-pulse dead-time free RIDME sequence[32] (Figure 1C) is suitable for in-cell distance measurements between Gd(III)-based spin labels.
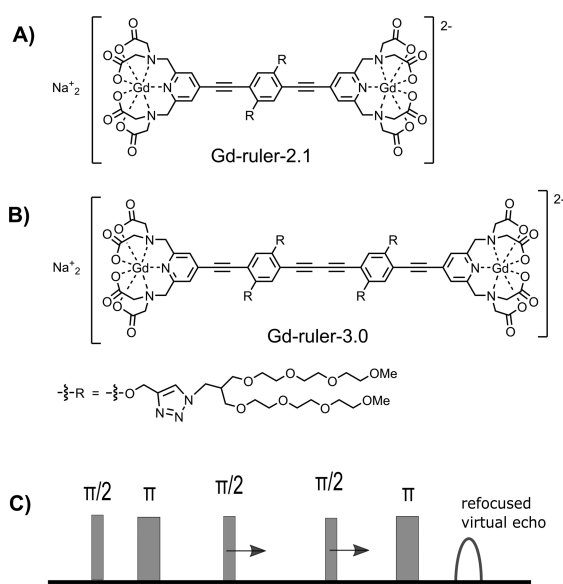

Figure 1. Structural formulas of Gd-ruler-2.1 (A) and Gd-ruler-3.0 (B). (C) Dead-time free RIDME pulse sequence.

In-cell RIDME implies that the measurement is performed in protonated media, which results in shorter relaxation times, as compared to deuterated solvents. Additionally, RIDME features a steeper signal decay than DEER because of the hyperfine spin diffusion. Therefore, accurate choice of experimental conditions with respect to relaxation and spin diffusion is crucial to realize in-cell RIDME. The measurement of relaxation times for Gd-ruler-3.0 in H_2O/glycerol (8/2 by volume) were performed in the temperature range between 10 and 30 K (Figure S1). The phase memory time decreases from 1.4 to 0.8 μs, and the ratio T_2/T_1 increases from 0.02 to 0.09 upon increasing the temperature. Though the highest ratio T_2/T_1 is favorable for RIDME, we performed all measurements at 10 K to have the longest phase-memory time. The pulse lengths were set at 8 and 16 ns for $\frac{\pi}{2}$ and π, respectively. The mixing time of 8 μs provided a reasonable trade-off between the steepness of the RIDME background and the dipolar modulation depth. No proton electron spin echo envelope modulation (ESEEM) was observed for RIDME in protonated aqueous solution (the ^1H nuclear frequency is approximately

48 MHz at 1.2 T). Neither was nuclear ESEEM observed from hyperfine and nuclear quadrupolar interactions with nitrogen atoms of the PyMTA ligand. Thus, no suppression of ESEEM effects was required. Consequently, the experimental RIDME data were processed as measured. Figure 2 shows the results of
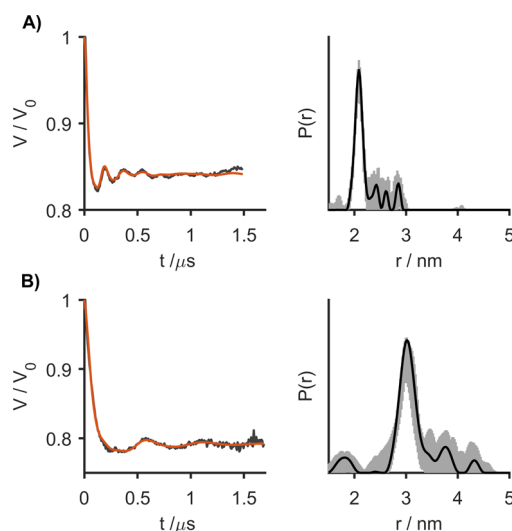

Figure 2. RIDME form factors (left) and corresponding distance distributions (right) obtained from frozen aqueous solutions of Gd-ruler-2.1 (A) and Gd-ruler-3.0 (B) in H_2O/glycerol (8/2 by volume). Gray areas in the right panels show the uncertainty range (as defined in the Supporting Information) in the distance distributions.

in vitro RIDME measurements in frozen protonated aqueous solutions (8/2 mixture of H_2O and glycerol) for two molecular rulers that bear Gd-PyMTA as the spin labels with distances of 2.1 and 3.0 nm between the two Gd(III) ions.[37−39] The RIDME form factors display modulation depths of 15% and 21% and visible oscillations, which indicate narrow distance distributions.

For determination of the interspin distances from the RIDME data, it was taken into account that relaxation-induced spin flips with $\Delta m \geq 1$ are possible for Gd(III) ions which have $S = 7/2$. Thus, apart from the fundamental frequency of the dipolar coupling ω_{dd}, which corresponds to spontaneous spin flips with $\Delta m = 1$, overtones of this fundamental frequency occur, which originate from spin flips with $\Delta m > 1$. It has been shown for Gd(III) ions that only the first two overtones, i.e., $2\omega_{dd}$ and $3\omega_{dd}$, contribute significantly to the RIDME form factor and that the overtone fractions can be assumed constant for distances above 3 nm, but they may vary if the distance is shorter.[35]

The RIDME data were processed with the OvertoneAnalysis software package, which explicitly includes the overtone fractions into the kernel function to extract the distance distribution by the Tikhonov regularization procedure.[35] The fractions, which are represented by the coefficients P_1, P_2, and P_3 (for the fundamental frequency ω_{dd} and its overtones at $2\omega_{dd}$ and $3\omega_{dd}$, respectively), were determined by their systematic variations toward a narrow single-peak distance distribution, as is expected for the studied Gd-rulers.[26,35] We find the following overtone coefficients $[P_1 \ P_2 \ P_3]$: [0.68 0.21 0.11] for Gd-ruler-2.1 and [0.4 0.3 0.3] for Gd-ruler-3.0. The sets of coefficients for the Gd-rulers in the protonated solution are different from the ones determined in the deuterated solution (cf. [0.8 0.2 0.0] for Gd-ruler-2.1 and [0.51 0.40 0.09]

for Gd-ruler-3.0).[35] From the comparison of the overtone coefficients for different solutions and different Gd-rulers, two observations follow: (i) Upon a decrease of the Gd(III)−Gd(III) distance but staying with the same solvent, P_1 increases while P_2 and P_3 decrease.[35] (ii) For the same Gd(III)−Gd(III) distance but changing from a protonated to a deuterated solvent, P_1 increases and P_3 decreases (P_2 stays about the same). The distance distributions for both molecular rulers in aqueous solutions (Figure 2, right panel) were determined with the optimized overtone coefficients. The obtained distances are narrow and centered at 2.1 and 3.0 nm, in perfect agreement with the expectation.[35]

The origin of the different overtone coefficients in deuterated and protonated solutions still has to be investigated. For the moment, it is worth noting that some artifacts are possible in the RIDME experiment, which might affect the calibration of the overtone fractions. In particular, the echo crossing at zero time is very difficult to remove perfectly, and this might interfere with the calibration of the overtone fractions. Thus, the difference of these fractions between protonated and deuterated samples should be verified on a larger set of samples to be considered as an established fact.

After the proof-of-principle RIDME experiments in protonated media had been performed, Gd-ruler-3.0 was measured both in cell extract and inside oocytes of *Xenopus laevis*. The redox stability of Gd(III)-based spin labels allows for long incubation times.[18,39] Thus, Gd-ruler-3.0 was incubated in the cell extract and inside oocytes for 2.5 h prior to shock freezing the sample for distance determination by RIDME. The form factors and the corresponding distance distributions are shown in Figure 3. In both cases at least one oscillation is clearly
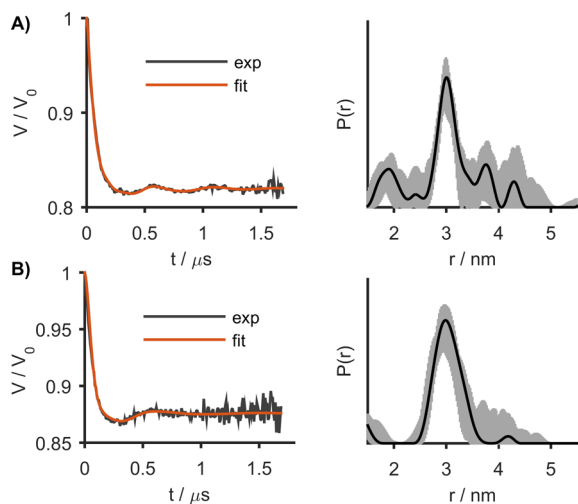

Figure 3. RIDME form factors (left) and the corresponding distance distributions (right) for Gd-ruler-3.0 in cell extract (A) and in oocytes (B). Gray areas in the right panels show the uncertainty range (as defined in the Supporting Information) in the distance distributions.

visible in the form factor. The accurate measurement of the second and further weak oscillations in the in-cell RIDME data is difficult because of the lower signal-to-noise ratio. The modulation depth of the in-cell measurement (13%, Figure 3B) is smaller than in the aqueous solution (21%, Figure 2B) and in cell extract (17%, Figure 3A), which is most likely due to the endogenous Mn(II) present in the cells (approximately 10 μM).[18] Because endogenous Mn(II) ions contribute to the

intensity of the RIDME signal but not to the modulation depth, the latter becomes diminished in in-cell measurements as compared with measurements in H_2O/glycerol and in cell extract, in full analogy with in-cell DEER.[18] The in-cell RIDME data for Gd-ruler-3.0 was analyzed using the same overtone coefficients as found for the protonated aqueous solution (vide supra). The resulting distance distribution is in agreement with the distance distribution determined in aqueous solution. This suggests that the overtone coefficients determined with a protonated aqueous solution can be used to determine Gd(III)−Gd(III) distance distributions for both in-extract and in-cell RIDME measurements.

In general, in vitro RIDME measurements applied to Gd(III) spin-labeled compounds provide larger modulation depths and more accurate distance distributions than in vitro DEER measurements.[34,35] In-cell measurements are performed under the challenging conditions of a protonated environment. In the presence of protons, the relaxation processes become faster and the hyperfine spin diffusion stronger. This results in a faster decay of the RIDME signal and shortens the length of the dipolar evolution time trace. If the experimental settings are chosen to maximize the length of the dipolar evolution time trace, i.e., low temperature and short mixing time, a RIDME time trace of at least 2 μs can be recorded, be it at the cost of the modulation depth. In this case, the modulation depths of in-cell and in vitro RIDME are in the range of 13−21%, which is still less than the modulation depth of 50% for RIDME measurements in deuterated solutions[33] but is a significant improvement over an in-cell DEER measurement using the same Gd-ruler-3.0 (a modulation depth of about 4%).[18]

The overtone coefficients in a protonated aqueous solution and inside cells are the same for Gd-PyMTA spin labels at a given interspin distance. The distance distributions extracted from RIDME do not contain artifacts caused by pseudosecular terms, as opposed to DEER.[20,26] We find that the overtone coefficients for aqueous solutions are significantly different for Gd(III)−Gd(III) distances of 2.1 and 3.0 nm, in agreement with what was reported for deuterated solvents.[35] This implies that for broad distance distributions, which span distances below and above 3 nm, the dependence of the overtone coefficients on the interspin distance has to be included in the processing of the RIDME data.

In conclusion, we demonstrated for the first time the performance of Gd(III)−Gd(III) RIDME for distance measurements inside cells. It is suggested that the overtone fractions remain the same for samples in aqueous solution and inside cells, being primarily determined by the presence of protons in the medium. In the current state, Gd(III)−Gd(III) RIDME can be applied only to distance distributions where the overtone fractions can be assumed constant. Further methodological developments are required to account for variable overtone fractions during data processing and also to slow the decay of the RIDME signal during data acquisition. Along these lines, current developments of other Gd(III)-based spin labels[40−45] and the application of shaped pulses[46] are very promising. Giving its inherent advantages, i.e., precise distance determination and large modulation depth, RIDME has the potential to become a method of choice for in-cell distance measurements.

ASSOCIATED CONTENT

Ⓢ Supporting Information

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Experimental details and additional figures (PDF)

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The authors declare no competing financial interest.

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