General comments

Thanks to a detailed comparative analysis of the relaxation data of two Gd(III) complexes with different zero-field splitting parameters, the authors rationalise their finding that deuterating the protein only results in a very limited enhancement of the phase memory time, a crucial parameter in pulse dipolar spectroscopy measurements.

The manuscript is clearly written. The adopted systematic multi-technique approach to isolate the various contribution to electron spin decoherence, the provided data and the data analysis fully support the conclusions.

Below are some comments for the authors.

Specific comments

Introduction

- Page 3, line 14: although the authors clearly specify what the terms "*direct spin-lattice relaxation*" and "*indirect T*₁" refer to, in the context of spin-lattice relaxation mechanisms "direct" may be misinterpreted as the contribution to $1/T_1$ which is proportional to the temperature ($(1/T_1)_{dir} = A_{dir}$ -T).
- Page 5, line 21: "*This showed that the protein's protons do affect phase relaxation*". I find it difficult to follow as the paragraph mostly refers to the deuteration of the solvent.
- Page 5, line 31: "the complexes were dissolved in $D_2O/glycerol-d_8$, thus serving as a reference for the longest possible T_m ". Was the deuteration of the solvent assessed?

Experimental

- Page 6, line 22: "*The synthesis of 4PS-5-Br-6PCA-(d_n)-DO3A-Gd(III) is described in detail in the Supporting Information*". As the corresponding structure is not reported in Figure 1, it may be useful to refer the readers to Figure 12.
- Page 8, lines 23-25: according to Raitsimring et al., 2014 *it was observed that in measurements which require a time base exceeding 12–13 µs the phase of the output echo signal sometimes varied substantially due to some features of the 'Quinstar' power amplifier. When a larger time base was required, the measurements were performed point by point using manual phase correction to achieve a maximum echo amplitude. Was a similar issue found in the measurements for this work?*
- Page 9, line 2: why was the inversion recovery sequence chosen over other methods less prone to spectral diffusion?
- Page 9, line 12: which version of the EasySpin program package was used?
- Page 9, lines 13-14: "The distributions of ZFS parameters were considered using a built-in EasySpin functionality (DStrain parameter)". Were the distributions of the ZFS parameters D and E considered to be uncorrelated or correlated?
- Page 9, lines 19-20: does the reported equation assume that the echo is generated by two pulses with same amplitude or same length? I went through the cited reference (Raitsimring et al., 2013) but I am afraid I didn't manage to locate the renormalisation equation in the form stated in the manuscript.

Results and discussions

- Page 11, figure 2: please, check the labels (D is missing and the figure next to A has none).
- Page 11, figure 2: "*small % of free*". What does this refer to? Does the component also appear in the inset of panel B?
- Page 12, lines 1 5: it may be worth specifying in the main text that the coupling constants have been converted into distances under the assumption of a purely dipolar coupling. This is already mentioned in the caption of Table S1.
- Page 12, line 17 (equation 1): I think that the correct form of the equation should read $y = 4 * ern \left[-\left(\frac{2\tau}{2}\right)^{\beta} \right]$

$$y = A * exp\left[-\left(\frac{2\tau}{T_m}\right)^r\right]$$

- Page 15, figure 5: especially considering the number of points, it may be good to have the corresponding data in the SI.
- Page 15, figure 5: what are the corresponding β values?
- Page 16, lines 18-19: "while holding the time between the first $\pi/2$ pulse and the last echo constant equal to 2τ ". It may be better to reformulate this as τ is not constant throughout the experiment.
- Page 16, lines 26-27: "β exhibits a monotonic decrease from n=1 to n=3 and levels off at n≥3, where it reaches a value of 1". β for 200 μM Gd-PyMTA seems to be consistently above 1 (Fig. 7D) whereas β for Gd-TPMTA seems to reach an asymptotic value < 1 (Fig. 7E).
- Page 18, Figure 7: it may be better to report the concentration of the Gd-TPMTA sample.
- Page 20, line 2 (caption of Figure 8): the reported parameters do not seem to be related to Equation 2 (A_f + A ≠ 1, no reference to A_f in Equation 2).
- Page 20, line 16: "This might be why full CP train refocuses the SD contributions better". What does "This" refer to? E.g., $\tau = \tau_{min}$ in the full CP train experiment? Were full CP train traces recorded for τ values larger than τ_{min} to check that indeed $\tau = \tau_{min}$ yields the best refocussing conditions?
- Page 22, Figure 10: what were the used concentrations?
- Page 22, Figure 10: why is there no datapoint for Gd-TPMTA at 20 K if this experiment has been reported in Fig. 9B (bottom right)?
- Page 22, lines 5-6: "For the Hahn echo, we observed a clear enhancement of the decay rate outside the CT". Is it worth referring the reader to Fig. 4 C/F?
- Page 23, Figure 11: what do the colours and the filled/empty symbols refer to?
- Page 23, Figure 11: were the pulse amplitudes adjusted for the individual field positions? (see *e.g.* the reported equation in Section 2.4).
- Page 25, lines 10-11: "the contribution of the protonated HEPES is small, but that of the lower amount of glycerol is significant". How was the effect of the lower amount of glycerol interpreted? Enhanced instantaneous diffusion due to a poorer quality of the glass?
- Page 26, Figure 12: to enable direct comparison, it may be useful to include the reference values (unconjugated tag) to this plot.
- Page 26, line 4 (caption of Figure 12): "*Gd-PyMTA, the results for the CPMG slow component have also been added*". I am afraid I can't follow what this statement refers to: CP data for Gd-TPMTA have been reported as well. Moreover, from what I understood the Meiboom-Gill variant of the CP sequence was not performed.

Supplementary information

- Page S15, Figure S9B: why were the $1/T_m$ data plot against t_{π} ? $(1/T_m)_{ID}$ is expected to be proportional to $\sin^2(\theta_2/2)$, see Equation S1. As a related point, are the experimental results consistent with the theoretical prediction?
- Page S21: "As shown by Mitrikas and co-workers". Does this reference to Mitrikas 2023?
- Page S24, caption of Figure 20: it would be beneficial to specify in the caption what the sample labels (*e.g.*, Gd-PyMTA HEPES 8:2) refer to.

Technical corrections

- Page 9, line 1: " T_1 measurements **for** were performed using the inversion recovery sequence, $\pi t_{wait} \pi/2 \tau \pi \tau echo$, with varying t_{wait} ". Please, remove the "for".
- Page 9, line 29: *10K → 10 K (add a space between the figure and the units). This occurs
 in other places throughout the text.
- Page 11, line 14: **phenyl rings* \rightarrow phenyl ring (GdPyMTA has only one phenyl ring)
- Page 13, line 15: where the central transition exhibits a longer T_m ; a characteristic of the *tZFS mechanism*. It may be better to replace the semicolon with a comma.
- Page 14, line 7 (caption of Figure 4): $(\mu s, \mu M)^{-1} \rightarrow (\mu s \cdot \mu M)^{-1}$ or $\mu s^{-1}/\mu M$.
- Page 14, line 9 (caption of Figure 4): in the equation for the dotted lines in panels B and E the *x* axis is a concentration; the parameter *T* should have the same units.
- Page 15, line 6: the sentence "*a linear correlation was observed only for the 5-15K*;" is incomplete.
- Page 21, Figure 9B, bottom right: do the numbers in brackets refer to the different deuteration conditions?
- Page 24, line 14: *for Gd-TPMTA labelled ubiquitin, a significant effect was noticed. I would consider removing the comma.
- SI, page S14, notes to Table S1: *Gd-F distance \rightarrow Gd-H distance.
- SI, page S15, Figure S10: for the sake of a better presentation it may be more convenient to estimate the magnetisation at the thermal equilibrium by averaging a few points at the end of each trace; this would ensure that all the traces have 1 as their asymptotic value.
- SI, page S16, Figure S11: as both T_1^{-1} and T_m^{-1} are reported on the same plot, it may be better to use a logarithmic *y* scale.
- SI, page S16, beginning of section 7: **the contributions of* $T_{ID} \rightarrow$ the contribution of T_{ID} .
- SI, page S20: multiple spacing in **pulses are* so close.
- SI, page S23, caption of Figure S19: $*A_f \rightarrow A$ to keep consistency with Equation 2 in the main text.