

## Comment 1

Below we copied the comments in black and our response is in red.

The authors of the manuscript “The decay of the refocused Hahn echo in DEER experiments” under discussion at Magnetic Resonance experimentally test optimal DEER observer sequence settings for two refocusing pulses (*i.e.* optimizing the 4- or 5-pulse DEER scheme) for a nitroxide, a trityl radical and a gadolinium(III) ion in frozen protonated and deuterated water-glycerol glass. CCE simulations for the nitroxide in a water-glycerol mixture rationalize the observed decoherence behavior for cases where proton-driven nuclear spin diffusion induces electron spin decoherence. Overall, the Results and Discussion section on the experimental data would benefit from a more careful and consistent discussion on contributing dephasing mechanisms. In this context, I would like to draw the authors’ attention to our own article called “*Dynamical decoupling in water-glycerol glasses: a comparison of nitroxides, trityl radicals and gadolinium complexes*” under review at another journal since January 6<sup>th</sup> 2021. We made this manuscript available as a preprint under <https://doi.org/10.26434/chemrxiv.13678447.v1> (hereinafter called DD-watergly2021) to facilitate the discussion here.

We were not aware of the results in this preprint (abbreviated henceforth as JS21), as it appeared on Chemrxiv after we submitted our manuscript to Magn.Reson., so unfortunately we could not refer to its findings in our initial submission. Indeed it is relevant to our manuscript, and we now cite it in the appropriate places.

We would like to emphasize that our work has a different scope than JS21. Our work does not aim at analyzing all dephasing mechanisms of paramagnetic centers in deuterated and protonated solvents, but rather focuses on the refocused echo decay in the context of DEER, and on how to optimize its parameters for the practically relevant cases where protons have a major contribution to the decoherence, due to their presence on proteins or on detergent molecules, which are practically expensive to deuterate.

For this, we present a theoretical analysis of the nuclear-spin-bath driven decoherence mechanism that is fully based on first principles, which is praised by the three reviewers. This is different from the approach used in JS21, which considers more mechanisms, but on a purely phenomenological basis.

### Comments regarding the discussion on dephasing mechanisms:

- **Fig. 3/157-159:** “While there is little difference between the refocused echo decay and the two-pulse echo decay for small  $\tau_2$  values.” I agree with this observation, though the authors could strengthen the interpretation of their results by also noting the progressive change of the maxima along  $\tau_1$  for increasing  $\tau_2$  in Fig. 3c. The

maximum at  $\tau_2 = 1 \mu\text{s}$  is particularly sharp and broadens for  $\tau_2 > 1 \mu\text{s}$ . This effect and the described deviation originate from the “fast” decoherence process driven by nitroxide methyl nuclei at low temperatures first demonstrated in glassy *o*-terphenyl (Soetbeer et al., 2018) which also contributes in water-glycerol glass (see DD-watergly2021: Fig. 4b) at the short time scale investigated here. Whereas our work stays in the DD condition e.g. of Carr-Purcell (CP)  $n = 2$ , the authors’ choice of  $\tau_2$  values in Fig. 3c acts as a filter to probe the two dephasing contributions arising either from nitroxide or solvent nuclei. Though this short time window is less relevant for DEER application work, understanding of this type of decoherence contributions are of fundamental interest. The authors should mention this contribution to also provide a more coherent discussion, as in the context of OX063 and Gd(III) data, dephasing mechanisms arising from the paramagnetic species itself are discussed (see next point).

Thank you for pointing this out. Our first-principle simulations indeed do not fully reproduce the cusp-like feature of the curves in Fig.3c for  $\tau_1 = 1 \mu\text{s}$ , indicating that there is a small contribution from another dephasing mechanism. However, this has no effect on the findings of our work, which show that the optimal  $\tau_1$  depends on the choice of  $\tau_2$ .

- **Line 212-214:** “This indicates that NSD, induced by the trityl OX063 protons themselves is still a contributing mechanism ...”. I note that our own work also identifies the OX063 protons as a source for NSD and we compare its decay under DD to the partially deuterated trityl radical OX071, demonstrating the DD is more efficient for short interpulse delays (DD-watergly2021: Section 3.3.3 in the main text, and Fig. Sa20 b/d in the SI part A) compared to partial trityl deuteration. Hence, we have experimental evidence for what is a speculation here.

We are glad that JS21 includes evidence for this. We now mention this and refer to JS21, see line 220. If you wish, you can also refer to our results in your paper when published.

- **Line 218-219:** “In protonated solvent, the shape of the 2D decay is similar to the ones observed for 3-maleimido-proxyl and trityl OX063”. I note that the echo maximum in Fig. 7a does not follow the  $\tau_1 = \tau_2$  line even at short delay times, whereas it does follow this line at early times for nitroxide/trityl in Fig. 3a/6a. Therefore, the behavior should not be called “similar”. It likely originates from a ZFS-driven dephasing contribution (see next point).

We will modify the sentence as follows “In protonated solvent (Fig. 7a), the shape of the 2D decay is generally similar to the ones observed for 3-maleimido-proxyl and trityl OX063, except for short  $\tau_1$  and  $\tau_2$ , where the slice-wise echo maxima detuned from the CPMG condition (red lines).”

- **Line 220-222:** “..., indicating that a second dephasing mechanism in addition to NSD is contributing, such as the transient zero field splitting mechanism. (Raitsimring et al., 2014)”

**Line 223-225:** “in deuterated solvents, ... nuclear spin diffusion plays a role [for Gd(III)] dephasing that is lower than in trityl OX063 but higher than in 3-maleimido-proxyl”

This discussion is inconsistent and requires further elaboration regarding the transient zero field splitting (tZFS) mechanism/Raitsimring et al., 2014 for the following reasons. First, the cited work introduces the tZFS for  $|m_S| > \frac{1}{2}$  transitions. Specifically, the Abstract of the article states “tZFS induced phase relaxation mechanism becomes dominant (or at least significant) when all other well-known phase relaxation mechanisms, such as spectral diffusion caused by nuclear spin diffusion, instantaneous and electron spin spectral diffusion, are significantly suppressed by matrix deuteration and low concentration”, and the cited article furthermore argues that the  $|m_S| = \frac{1}{2}$  transition behaves analogous to a  $S = \frac{1}{2}$  system, meaning that the dephasing at this field position is NSD-driven. Based on this citation alone, it is astonishing that the authors consider the tZFS mechanism in case of the protonated solvent but do not discuss its contribution in the deuterated case. Moreover, as the refocused Hahn echo decay was recorded at the maximum of the EPR spectrum, thus probing the  $|m_S| = \frac{1}{2}$  transition, the authors need to comment on this finding as it is in conflict with the statement in Raitsimring et al., 2014. In fact, our own DD study relies on three Gd(III) complexes with varying ZFS to demonstrate that in protonated water-glycerol glass a ZFS-driven dephasing mechanism contributes at the central Gd(III) field position (DD-watergly2021: Fig. 7a, Section 3.4.2). Second, the authors’ spin concentration choice of 100  $\mu\text{M}$  is highly likely to lead to ID contribution in deuterated water-glycerol glass. For this reason, the observed decoherence behavior (Line 223-225) cannot be interpreted as deuteron-driven NSD exclusively. On the one hand, because tZFS dominates the dephasing for Gd(III) (as our own DD data proves, DD-watergly2021: Fig. 7c). On the other hand, because at the same spin concentration and pulse excitation bandwidth, ID provides a more significant dephasing pathway for trityl radicals compared to nitroxides. We discuss this aspect in our own work (DD-watergly2021: section 3.3.1), stating that nitroxides are spectrally more diluted. To assess differences in deuteron-driven NSD, the authors would need to choose a lower spin concentration and demonstrate experimentally that ID is negligible.

Thank you for this detailed discussion. Our intent is not to provide an in-depth discussion of all relaxation mechanism in all samples but rather our focus is on (partially) protonated samples and the fact that the optimal setting of tau1 and tau2 deviates from the CP condition if tau2 is not too short.

We clearly show the proton NSD is very active for Gd(III) in protonated solvents. It was not our intent to make specific statements about deuteron NSD, since we don’t have it

isolated well experimentally and also because we don't have a first-principles theoretical handle on it.

To clarify this, we will add in line 235 "As evident from Fig. 7c, proton nuclear spin diffusion, arising from protons on the Gd(III) chelate, plays a role in dephasing that is lower than in trityl OXO63 (Fig.6c) but higher than in 3-maleimido-proxyl (Fig. 4c)."

#### Comments regarding the data analysis/CCE simulations:

- **Fig. 3a/b** seem to display a small asymmetry with respect to fixed  $\tau_2$ , variable  $\tau_1$  compared to fixed  $\tau_1$ , variable  $\tau_2$ . The same appears in Fig. 6a for OXO63. The authors should comment whether this is an artefact arising from the data analysis or reflects a true asymmetry.

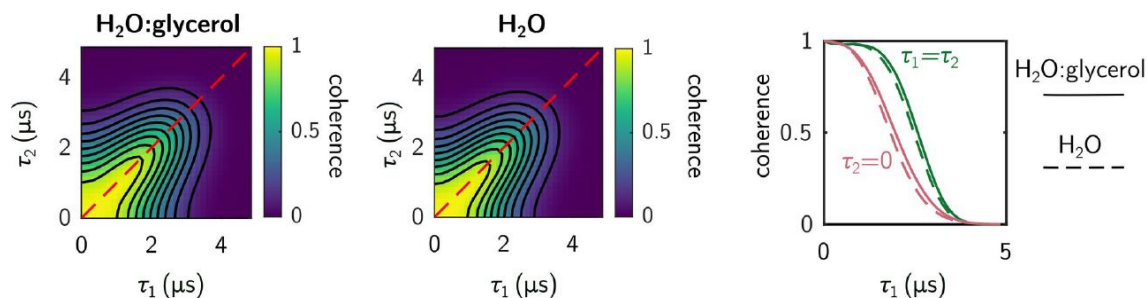
This is an experimental imperfection. Theoretically, in the high-temperature limit (which is applicable here), with ideal pulses (neglecting intra-pulse evolution), and for  $T_1 \gg T_M$  (applicable here), one can show that  $V(\tau_1, \tau_2) = V(\tau_2, \tau_1)^*$ .

- **Figure 5a/b:** The location of the maxima along  $\tau_1/\tau_2$  (red lines) display many irregularities in particular for small interpulse delays. The authors should comment on their origin. Potentially, these stem from  $^2\text{H}$  ESEEM, if this is the case, the authors should specify in the Methods section how these modulations are treated during the normalization.

These wiggles are a consequence of noise in the experimental data. For small interpulse delays, the echo decay along  $\tau_1$  for a given  $\tau_2$  (and vice versa) are relatively flat, so that experimental noise can generate larger apparent scatter of the ridge points.

- **Line 260:** The presented CCE simulations are performed in a water-glycerol mixture, though previous published CCE results were obtained in pure water (Canarie et al., 2020), reasoning that "since MD simulations in pure water are well calibrated, whereas water-glycerol mixtures are significantly less tested against experiment, particularly in the solid phase." How did the authors ensure that the calibration of the water-glycerol glass is appropriate?

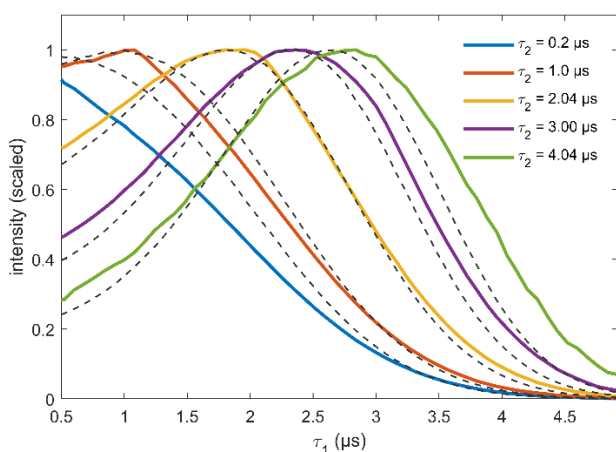
Water and water/glycerol have very similar proton concentrations, so the decays are expected to be similar. We now have simulations that compare water and water/glycerol mixtures that show that the two matrices give very similar results. In the below figure, the water/glycerol simulation is the 3-CCE simulation from Fig.10. The water simulation has the same parameters. There are 494 1-clusters, 4124 2-clusters, and 49178 3-clusters. We will mention this in the text and add it as Fig. S3 to the SI.



- **Line 275:** “Remarkably, [the simulated refocused-echo decay] matches the experimental result both in shape and time scale...”

The reader would benefit from adding the experimental data trace of CP  $n = 2$  in Fig. 9c so that the time scale and shape as well as CCE convergence can be judged more easily (e.g. as done in Canarie et al., 2020). This display is likely to reveal a mismatch for short  $\tau_1/\tau_2$  as evident from comparing the normalized slices (red lines) in Fig. 3a-b with the ones in Fig. 9 a-b. I also expect this from my own experimental results (DD-watergly2021: SI, Fig. Sa12 CP  $n = 2$  for protonated nitroxide in protonated water-glycerol at 40 K – according to Fig. Sa5 comparable to decay behavior at 20-30 K used in the article under discussion). This contribution originates from the methyl protons of the nitroxide.

The figure below shows the suggested plot (color: experiment; dashed black: first-principle simulation). It shows the first-principle simulations are overall in remarkable agreement with experiment. It also illustrates the mentioned deviation of the theory from experiment at short  $\tau_2$ . We will mention this in the text and add this Figure to the SI as S4.



- **Line 281-282:** “Including two-nucleus clusters in the simulation yields an echo decay that has the correct shape and an almost correct time scale. Adding three-nucleus clusters improves the time scale slightly...” Considering Fig. 9c 2-CCE is  $\sim 1 \mu\text{s}$  off from

3-CCE decayed at 4  $\mu$ s. This deviation is relatively large and convergence appears to be reached for 3-CCE so that the authors should reconsider their somewhat misleading wording here.

We will reword this from “almost correct time scale” to “time scale of the correct order of magnitude.”

**Comment regarding the sample choice:**

The article presents the refocused echo decay as a function of  $\tau_1$  and  $\tau_2$  for a nitroxide, a trityl radical and GdCl<sub>3</sub> in protonated and deuterated water-glycerol glass as specified in the Introduction (Line 78-79). The authors should justify the additional sample choice/discussion of the Gd-C2-labeled MdfA protein solubilized in detergent (DDM) micelle without providing the full data with  $\tau_1$  and  $\tau_2$  variation. First, because this sample varies many experimental parameters at once, namely

- Gd-C2-complex instead of Gd(III) ion, altering the ZFS
- additional HF field arising from the protein’s protons (which appears to be the variable of interest and thus should be the only varied parameter)
- micelle environment instead of aqueous water-glycerol mixture
- two labeling sites which may be exposed to different local environments

Second, compared to frozen water-glycerol solvents the micelle environment is known to accelerate the electron spin dephasing strongly (*e.g.* see Dastvan et al., 2010). For both reasons, it is not clear to me how the reader benefits from this somewhat unconnected “application example”.

The point of this application example is to show that there are cases where even when the solvent is fully deuterated, there are decoherence contributions from the remaining proton bath, and one can choose a long tau1 for collecting the data. So to our opinion, this is a practically relevant example that complements the more fundamental exploration of the other samples. See also our response to a similar comment from Reviewer 3.

For a stronger discussion, the authors should consider to compare a single-labeled water-soluble protein with the chosen spin label in the same solvent environment *e.g.* at best in a deuterated water-glycerol mixture to be sensitive to the protein’s protons. Our own DD study in water-glycerol took exactly this approach for Gd-DOTA-M (DD-watergly2021: Fig. 7c-d and section 3.4.2), demonstrating the decoupling effect for proton-driven NSD arising from the protein’s backbone.

While we think this is an interesting and worthwhile comparison, it is not within the scope of this manuscript. We will cite JS21 in this regard.

### General comments:

- Line 250: “range of 3-4  $\mu\text{s}$ ” (blue trace)? This should refer to the yellow and purple trace.

Fixed.

- Due to the eight-step phase cycle your experiments do not correspond to a CPMG but instead to a CP sequence.

We will change all CPMG occurrences to CP in our manuscript when referring to our measurements.

### Comments regarding citations:

- **Line 45/Eq 1:** Zecevic et al., 1998 uses the stretched exponential model, but the cited equation cannot be found in this work.

The equation is a combination of Zecevic 1998 (which omits  $T_1$ ,  $V_0$ , and  $\lambda$ ) and Jeschke/Polyhach 2007 (which omit the stretched exponential and  $\lambda$  and  $V_0$ ), and our addition of  $V_0$  and  $\lambda$ , which are generally known prefactors.

We will add Jeschke/Polyhach to reference the  $T_1$  factor.

- **Line 50:** In Jeschke and Polyhach, 2007 the approximation reads  $\tau = \tau_2$  if  $\tau_2 \gg \tau_1$  and in this limit the Hahn decay approximates the refocused echo decay well (as visible in Fig. 3c, Fig. 4c, Fig. 6c). If the statement in Line 72-75 “In the context of DEER, it is usually assumed that the refocused echo decays monotonically as a function of the overall pulse sequence length  $2(\tau_1 + \tau_2)$ , similar to the two-pulse echo.” refers to the above approximation, it should be rephrased.

See corresponding comment to reviewer 3.

- **Line 132-133:** Technically, Harbridge et al., 2003 determined the CPMG time constant, which corresponds to the decay of the  $n$  refocused echoes between  $n$  refocusing pulses. The work under discussion observes the decay of the refocused Hahn echo, more closely related to our dynamical decoupling (DD) study in OTP (Soetbeer et al., 2018) as well as our recent DD study in water-glycerol glass (DD-watergly2021). Both works systematically address the effect of DD for nuclear spin diffusion (NSD) for organic radicals (and gadolinium complexes) “dilute frozen solutions at cryogenic temperature” (Line 131-132) for both protonated and deuterated matrices, the authors should cite.

We now added Soetbeer, 2018 as well as JS21.

- **Line 177-179:** “It is apparent that NSD is suppressed here and DD is ineffective. The decay is dominated by other dephasing mechanisms such as instantaneous diffusion (ID) ...” We demonstrated this effect for 20 compared to 100  $\mu\text{M}$  protonated nitroxide in deuterated OTP (Soetbeer et al., 2018, Fig. 8d-f). The latter matches the used concentration choice in the present work, so that a citation would be appropriate.

The effect of concentration on the instantaneous diffusion rate has been known for a long time. For completeness, we will add the mentioned reference.

## References

DD-waterygly2021 <https://doi.org/10.26434/chemrxiv.13678447.v1>

Canarie et al., 2020 <https://doi.org/10.1021/acs.jpcclett.0c00768>

Soetbeer et al., 2018 <https://doi.org/10.1039/C7CP07074H>

Raitsimring et al., 2014 <https://doi.org/10.1016/j.jmr.2014.09.012>

Dastvan et al., 2010 <https://doi.org/10.1021/jp1060039>