Reviewer 3

Thank you for your positive evaluation and constructive comments. Below we copied our evaluation in black and we present our response is in red.

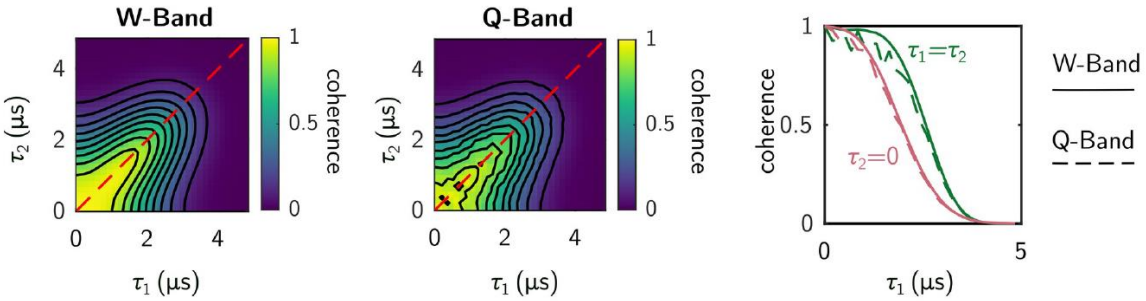
This is a very interesting study by the Goldfarb and Stoll labs demonstrating that the common assumption of short tau1 values leading to larger signals in DEER might not always be met. Tau2 will determine the distance range that can be retrieved from the DEER data and tau1 is commonly chosen short to minimize time for echo dephasing. The authors very clearly demonstrate that extending tau1 for a given tau2 can lead to increased sensitivity. This appears to be most relevant for samples with limited possibilities for deuterium exchange. Nevertheless, this is an important finding to report especially as optimizing tau1 for a given tau2 will likely be a very quick experiment in contrast to DEER averaging times that will often average for many hours if sensitivity is limiting. The authors further make an excellent effort to rationalize their findings in in terms of numeric simulations and conceptualization.

From the practitioner's point of view this has sparked a number of questions that might be worth commenting on in the final version of the manuscript. I am aware that some of the simulations or experiments that would be required to exhaust these questions will be beyond the scope of this work but I believe at least commenting on them will be of interest to the reader.

All experiments and simulations are performed at W-band. Considering that most reported DEER experiments have been measured at X- and Q-band how do these effects translate at lower fields. I suppose the non-zero transition amplitudes of the formally forbidden transitions will increase while the nuclear Larmor frequency will decrease. Is the overall effect field-independent? This should be straightforward to simulate. The title of the manuscript suggests a general treatment.

In our earlier work (Canarie et al, J.Phys.Chem.Lett., 2020), we have shown experimentally and by numerical simulations that the effect is field independent (no change between X and Q band). Also, theoretical considerations of a simple system of one electron spin and two spin-1/2 nuclei (see Lenz et al, ChemComm, 2017) shows that the effect is independent of the field and depends only on the ratio of the nucleus-nucleus coupling to the difference in the two hyperfine couplings.

Below are simulations for W-band versus Q-band. They show that the only difference is in the ESEEM modulations, and that the nuclear-spin-cluster driven dephasing is field independent:



Would softer pulses be expected to lead to decreased dephasing. This has been shown in the context of instantaneous diffusion (Jeschke and Polyhach, 2007) but in terms of forbidden transitions this might be relevant here as well.

Instantaneous diffusion is indeed an additional dephasing mechanism that needs to be considered. We checked for instantaneous diffusion by measuring the Hahn echo decay with softer pulses but we found the decay curves to be identical. This will be mentioned in p. 7 line 170. We will add: "We also checked that the contributions of instantaneous diffusion under these conditions was negligible by comparing the Hahn echo decay obtained with different pulse lengths (see Fig. S1c)".

When deuterating the solution of 3-maleimido-proxyl the data are interpreted as nuclear spin diffusion being suppressed and dipolar decoupling becoming ineffective as other dephasing mechanisms become dominating. Has this been explored using lower concentration or softer pulses? At sufficiently low concentration would dynamic decoupling become effective again in deuterated samples. Could deuterium nuclei be simulated using the same approach but potentially fewer nuclei?

We did not explore the relaxation mechanisms for the deuterated samples. The challenge is that the currently established CCE theory breaks down for a bath of spin-1 nuclei such as deuterium, because the CCE series expansion does not converge at low orders. Theoretical work is under way to work around this impasse, but hasn't been successful yet. In other words, accurately simulating a spin system with one electron spin and a few hundred deuterons is still out of reach.

Different scenarios of residual proton content will likely lead to different outcomes. 25% of protons already have a significant effect but there are no experimental points up to full deuteration. Is it feasible to thin out the protons in the simulation until the dephasing effect will vanish when proton clusters with sizeable nuclear couplings become improbable.

We agree that this would indeed be of theoretical interest. As mentioned in the previous point, however, accurate theoretical simulations for situations of very low proton concentration are currently not possible, since then the deuterium bath starts co-determining the decoherence time scale, and the CCE expansion for a spin-1 bath has

convergence problems. We hope to perform these simulations, and the associated experiments, once we have overcome the theoretical hurdles.

For non-homogeneous distributions of protons that will be most relevant practically (El Mkami et al., 2014) it will be very interesting to see the influence of the proximity of protons. The full effect was recovered with protons in 1.2 nm. This suggests the dephasing of a spin label well solvated in deuterated solution away from the protein will be substantially slower than when buried in the fold of a protonated protein or membrane. Will the simulation approach be applicable to inhomogeneous distributions of protons?

Yes, the simulation approach is fully general and applies to any spatial distribution of protons. There is a very large spread of T_M for nitroxides label on different sites of the same protein. However, accurate comparison of simulation and experiment is more challenging for proteins due to the large structural modeling uncertainty of the solvated protein structure, in particular the side chain conformation of the spin label. In addition, another important relaxation mechanism in proteins is mediated by tunneling in protein methyl groups. This is currently not quantitatively understood, preventing a successful comparison of experiment and simulation for protein samples.

The MdfA double mutant V44C/V307C doubly labelled with Gd-C2 is measured in detergent micelles. Without further knowledge of structure and labelling positions the effect of non-exchangeable protons is hard to predict. An earlier report by Dastvan et al. (https://doi.org/10.1021/jp1060039) suggests the increased proton density in lipids in comparison to aqueous solution leads to increased dephasing. This might also be relevant for detergent. In this light, this might not be the most relevant protein system to demonstrate these results from homogeneous solutions of free spin labels.

Our intent with the MdfA example was precisely to show this. There are cases where even when the solvent is fully deuterated, there are protons that drive decoherence. In such situations it is advantageous to choose a long tau1 for collecting the data, as shown in Fig. 8. Therefore, in our opinion, this is a real practical and relevant example, more so than a soluble protein with surface-exposed spin labels in fully deuterated buffer. To clarify this better, we will reword the corresponding section (see line 256) and also cite the paper by Dastvan et al.

Without having done the simulations, is my extrapolation that a larger number of proton clusters and larger couplings between protons expected for media with increased proton density will lead to faster dephasing consistent with the findings here?

This is indeed correct. The dephasing rate i1/T_M is indeed roughly proportional to bulk proton concentration. This was already found experimentally by Zecevic et al in 1998, by canvassing a range of solvents with different bulk proton densities. For low proton concentrations, the geometric details of the proton clusters will likely have a strong

impact on T_M. However, from our numerical simulations we have not been able to identify simple intuitive rules.

Further points

The introduction of the 3 and 4 pulse DEER sequences seems to suggest they were initially reported in 1984 and 2000, respectively. I suggest changing the wording or adding the original references.

Thank you for noting this. We will update the reference for 3-pulse DEER to Milov 1981, and the reference for 4-pulse DEER to Martin 1998.

(Jeschke and Polyhach, 2007) set the S/N ~ exp(-2tmax/T2) and this still holds in the approximation that even with an optimized tau1 the refocused echo will decay exponentially with tau2.

We referred to eq. 3.16 and 3.17, which are more general. Yes, with a fixed tau1 the echo will decay with tau2, but not necessarily exponentially, but with a stretched exponent.

The discussion of dephasing by electron-electron dipolar interaction is confusing. An increased concentration will lead to larger signal and faster dephasing. As shown in (Jeschke and Polyhach, 2007) there will be an optimal concentration depending on the required trace length. If dilution lead to longer averaging times dilution it was overdone.

We fully agree. We should have made this clearer in our discussion. To address this, we will add: “However, this concentration reduction leads to a loss in absolute signal intensity and may significantly prolong the experiment run time and therefore there is an optimal concentration for best SNR.”

In line 74 it is said short tau1 values minimize phase relaxation” but considering instantaneous diffusion I suggest “minimize dephasing”.

We will adjust this.