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 may 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.
- 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.
- 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?

- 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?

- 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?

- 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.

- 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?

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.

(Jeschke and Polyhach, 2007) set the S/N $\sim \exp(-2t_{\text{max}}/T2)$ and this still holds in the approximation that even with an optimized $\tau_1$ the refocused echo will decay exponentially with $\tau_2$.

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.

In line 74 it is said short $\tau_1$ values minimize phase relaxation” but considering instantaneous diffusion I suggest “minimize dephasing”. 