Review in Black
Response in Red
Manuscript parts in blue

The manuscript "Distance measurements between trityl radicals by pulse dressed electron paramagnetic resonance with phase modulation" by N. Wili, et al. describes a novel PDS method based on clever evolving dipolar coupling in the spin-locked state, thereby improving the distance range for trityl spin labels. The manuscript is generally correct and should be published with the consideration of the comments that we made.

This work describes an interesting development as so far it has not been demonstrated in ESR that spin-locked electron spins could be used to evolve selectively electron-electron dipolar coupling. The manuscript is well organized and clearly written. Also, extensive effort to synthesize and characterize rigid trityl biradicals and to simulate the evolution of coherence is shown. In particular, we like the implementation of the pulse sequence refocusing the nutation phase and providing the dipolar evolution sandwich in the locked state followed by the readout sequence, which can in principle exclude unwanted dipolar evolution. A simple two-pulse echo sequence used for readout appears sufficient at this stage, and by using SIFTER and DQC it would be possible to approach the somewhat shorter distance range. However, the dead-time cannot be excluded. Maybe the authors need to apply SIFTER or DQC sequences in the locked state.

We thank you for the generally positive comments. Regarding the dead time and other sequences in the locked state, you are correct. We added a comment in the manuscript, after the discussion of the pulse sequence (starting from line 241 in the highlighted manuscript):

If the dead time becomes too large for the relevant dipolar oscillations, one could, in principle, apply the known dead-time free single-frequency pulse sequences also as a phase-pulse sequence in the nutating frame.

It is encouraging that despite all the limitations imposed by the interaction strengths, the method does work and notably improves the dipolar evolution time for trityls. This work suggests a set of possible factors limiting T2(rho), and hopefully future work may be able to mitigate the effects of some of these factors. We doubt, however, that TWTA amplitude or phase noise contributes to shorter T2(rho). This is certainly not the case for amplitude noise, which within the locking bandwidth is estimated to be in the~1-10 mW range or maybe even less for a typical tube (less than -10 dBm/MHz noise spectral density). The phase noise of a TWTA (which is likely made by Applied System Engineering) is expected to be rather low. At least, the phase-noise test data for all amplifiers built over 20 years for ACERT supports this notion. Introducing phase noise, while possible, would be a complicated matter. AM/PM conversion in a saturated tube may be a possible way to test the effect on T1(rho) and T2(rho) to provide some insights on the instrumentation-imposed limits.

We thank you for the comments regarding the TWTA noise and sharing your experience with the noise figures. We based our comment on (Cohen et al. 2016: https://doi.org/10.1002/prop.201600071). (Which we now also clearly state in the manuscript). While we agree that the noise is rather small compared to the wanted driving field, it is not clear to us that one can safely conclude that it is irrelevant for T2rho. 10 mW is roughly a factor of 10000 smaller than the nominal 150 W nominal output power. This amounts to a factor of roughly 1% in terms of Rabi frequency, i.e. around 1 MHz. At least on first sight, it seems to be possible that this might contribute to T2rho.

Unfortunately, the work gives no clue regarding what to expect at a different temperature for T1(rho) and T2(rho). The experimental setup allows for easy temperature change and it is highly desirable to see the ratio of Tm/T2(rho) for at least one more temperature.

Under normal circumstances, we would simply measure it at another temperature and put the data in the SI. Unfortunately, with the current situation regarding COVID-19, we will be unable to provide these measurements in the coming months. We do not think that finalization of the paper should be held up that long.

The reason we did not measure at a different temperature so far is because we thought that a single additional temperature is not relevant. When comparing different temperatures, we also want to compare different matrices and measure rotating frame relaxation times at room temperature, i.e. for immobilized samples. Unfortunately, all of this must wait for the time being. It might also be too much for the scope of this manuscript. We do work on a more extended study on trityl relaxation.

At the beginning of the project we did measure decay of the spin-locked echo at different temperatures. However, as seen in Fig S3 of the submitted SI, this decay corresponds neither to T1rho nor to T2rho.

We hope that you still regard our proof-of-principle as relevant, although we measured only at a single temperature.

There is no comparison with the existing pulse sequences such as DQC or SIFTER,but we agree this may be unnecessary for this work. There is sufficient data in the literature for them, and PDS heavily relies on nitroxides anyway.

After emphasizing the power of major PDS methods in the introduction, the authors demonstrated that the sequence works at least for trityl labels, but they make no comment on whether it could be extended to any other known spin-label. The labels that are commonly used in biomedical research and are subjects of most of the key works cited are nitroxides, which demonstrate Tm's longer than the trityl's T2(rho) of this work. It is well known in this field that nitroxide labels quite often destabilize and precipitate proteins, the issue being even more critical with trityl labels, which are by no means mainstream. This is a significant limitation to the scope of this complex novel PDS method. Assuming that trityl labels were to have progressed to comparable use, there are other challenges that need to be addressed. The protein and lipid dynamics leading to Tm's in the low microsecond range as well as high local concentrations in the case of membrane proteins may contribute a set of problems in achieving T2ï Aš anywhere close to that observed in this work in dOTP glass. Note that the T2(rho)'s obtained are considerably shorter than for nitroxides in this glass. We have (unpublished) data that demonstrate $40\mu s$ evolution time in this glass using the DEER-5 method; 4-pulse DEER is also not very far from this mark. We also found very long Tm's for (partly) deuterated proteins (Georgieva et al., J. Biol. Chem., 2010). We think this work should be cited in the context of deuterated proteins.

We stressed in the article that the Rabi frequencies must be larger than the offsets. This is of course not possible for most labels other than trityls with current hardware. We now emphasize more that nitroxides are the most commonly used labels and that there are cases where nitroxide Tm is longer than the T2rho that we observe for trityls.

Changes:

Introduction (line 63 in highlighted manuscript): "Note that the sequence presented in this work relies on the narrow spectrum of the trityl radicals. We do not expect it to work with the much more commonly used nitroxide radicals."

Conclusion(line 415): "Note that in dOTP, the Tm values of the slow relaxing component of nitroxides (the relaxation of nitroxides in dOTP can be described by a sum of two stretched exponentials) can still be bigger than the T2rho times measured here for trityl radicals (Soetbeer2018)."

The mentioned paper by Georgieva et al. is now cited. We were unaware that it preceded the work by Ward et al. in 2010, and it is indeed relevant when discussing protein deuteration and PDS. We apologize for the oversight.

Regarding all the other issues: We agree that there are most likely other hurdles. Lipid dynamics might indeed contribute to shorter T2rho values – or it may not (some dynamics can indeed be decoupled in rotating frame experiments in NMR). Only experiment will tell. We publish our findings early to encourage other interested researchers to help answer these questions.

We clearly state in the conclusions that the sequence is not yet ready for application work. We believe that this statement is sufficiently strong in pointing out current limitations.

The special technical requirements of this experiment to provide long intense locking pulses need to be described in greater detail. Such pulses are not normally used in pulse ESR. A 150 W TWTA was employed to achieve~100 MHz Rabi frequency (36 G B1) and this power could last for about 40μs periods limited by the amplifier. In the first place it is a lot of power that can cause heating, arcing, and a damage to the receiver, thus limiting the repetition frequency. How was the receiver protected?

No special measures beyond the ones reported in (Doll & Jeschke, 2017) (now cited) were taken to protect the receiver – we simply made sure we had a spare switch and low-noise amplifier ready in case we destroy them. However, this has not happened so far (We started doing the experiments in August 2019). We also let it run overnight. We found no reduction in performance so far.

However, your point is now emphasized in the manuscript, in order to alert researchers who want to use this sequence or similar sequences that their hardware might be at risk.

We added the following paragraph in the Materials and Methods section (line 255):

Note that the long spin-lock pulses with full power can be dangerous for the receiver, since much of the power is reflected by the overcoupled resonator. We did not take any special measures beyond the receiver protection switch (Doll & Jeschke 2017). However, we are planning to install an additional limiter or a slow switch that could take more power. Since the spin-lock pulses are rather long, a slow switch could be used for most of the time, while the fast switch could be used for the transient times of the pulses to still provide the small dead time.

The origins of the baseline and of modulation depth need to be discussed.

We added the following lines in the results section:

Note that not only intermolecular dipolar couplings from remote spins contribute to the background. Transverse relaxation of dressed spins with time constant T2rho also contributes because we do not perform a constant time experiment. Regarding modulation depth we would have expected it to be unity, which is clearly not seen in our experiments. We suspect that imperfections in the dressed spin pi-pulse lead to an unmodulated background, which cannot be

removed by phase-cycling. The phenomenon is similar to reduced instantaneous diffusion for a Hahn Echo if the flip angle of the pi-pulse is reduced.

Figure S8 – legends need be corrected.

Thank you for pointing this out. We corrected the legend that was mixed up.