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Interactive comment on "Distance measurement between trityl radicals by pulse dressed electron paramagnetic resonance with phase modulation" *by* Nino Wili et al.

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Comments on 'Distance measurements between trityl radicals by pulse dressed electron paramagnetic resonance with phase modulation' by N. Wili, et al.

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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

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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.

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 $\sim$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.

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.

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 T2ïĄš 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 μs 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.

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 $\sim$100 MHz Rabi frequency (36 G B1) and this power could last for about 40 μs 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?

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

Figure S8 – legends need be corrected.

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