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Reply on RC2

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Author comment on "Using delayed decoupling to attenuate residual signals in editing filters" by Kenneth A. Marincin et al., Magn. Reson. Discuss., https://doi.org/10.5194/mr-2021-24-AC2, 2021

We thank the referee for the thorough and thoughtful review of our manuscript. We agree with all points raised and made modifications and clarifications to address these concerns. Please find a response to every point raised below.

- **Regarding the model system (PCP1:pantatheinate covalent adduct) it would be useful if they could confirm whether the adduct interacts with the protein (i.e. tumbles at the macromolecular rate), or whether it is mobile relative to the protein (the latter might present an easier case for isotope-filtering because of the reduced relaxation penalty incurred by extra filter delays). Also, what is the size of the complex (about 20kDa?)**

Response: This is a good point, as we previously observed transient interactions between the prosthetic group and a different carrier protein (an aryl carrier protein). That is, the phosphopantetheine (PP) and its attached substrate sample both an undocked state and a docked state. However, we have not yet quantified this interaction equilibrium for PCP1. At this stage, we can only observe that the signals of PP and its substrate are broadened upon attachment, which may reflect a predominantly docked form or exchange line-broadening due to the docked/undocked equilibrium or both. Importantly, we plan to monitor the molecular response of the PP arm as PCP1 engages with partner domains when relaxation will be a challenge regardless of these equilibria. We have clarified that minimizing relaxation is a desired feature in general, rather than an immediate need for isolated PCP1. PCP1 loaded with its PP arm and substrate is 10 kDa. We have expanded on these points in the introduction, sample preparation, and future directions sections (Sects. 1, 3.2, and 5) by adding the following text:

In Sect. 1: "We and others have found that some CPs interact transiently with their tethered substrates […] such that the phosphopantetheine group and its attached substrate sample both an undocked state and a docked state."

And later:

"The NMR linewidths of the tethered moiety indicate that the arm does not tumble independently from the protein core but is also not rigidly docked onto the protein, in line with a transient interaction."

And:

"Our immediate objective is to attenuate these residual signals and mitigate sensitivity losses for the targeted signals of unlabeled moieties, which will be particularly important for future studies of PCP1 engaging with its larger partner domains."

In Sect. 5: "Further experiments using these improved filters will enable studies of interactions between the prosthetic arm and PCP1, in isolation and in presence of its catalytic partner domains."

In Sect. 3.2: "Briefly, PCP1 (9.6 kDa) is expressed as a His_6-GB1 fusion protein containing a Tobacco Etch Virus (TEV) cleavage site."

And:

"Upon confirmation of loading, purified Cys-loaded PCP1 (10 kDa with attached prosthetic group) was concentrated and buffer exchanged into NMR buffer containing 20 mM sodium phosphate pH 6.59 at 22 °C, 150 mM NaCl, 1 mM EDTA and 2 mM TCEP."

- **Although the authors point out the value of being able to combine the WATERGATE suppression block with the third (delayed-decoupling) tuned filter element, they are perhaps missing a trick in that earlier schemes as reviewed and described in (Breeze, 2000) already featured incorporation of WATERGATE into the second filter element. This approach already shortens the scheme relative to one in which the WATERGATE block is sequentially positioned after the double-tuned filter, so in that sense the comparison they present with their 'reference experiment' should reflect this fact (granted, their new scheme still has an advantage in filtering efficiency by introducing the third element with complementary J tuning – but it will suffer a slight sensitivity loss compared with the experiment with doubly-tuned filter incorporating WATERGATE into the second element).**

Response: We are extremely grateful for this comment as it revealed wording that led to confusion, in particular as we did not mention that WATERGATE elements could be included in filters. Indeed, we must modify our text to clarify what is compared with what and when, in particular when describing the advantages provided by our method. To assess the efficiency of our filter we needed a reference with the same pulse sequence length, as the filter would otherwise benefit from relaxation losses making the signals smaller not only due to the filter itself but also because of the duration of the filter. Thus, we started from a standard pulse sequence with a WATERGATE after the filters as a reference, and we incorporated our filter into that WATERGATE element. Here, when comparing our method with the reference, the attenuation of the residual protein core signals directly reports on the filter efficiency, without contamination by relaxation. However, when describing how our method improves on existing strategies, the "reference" (or point of comparison) does not need to be subject to this constraint, and indeed WATERGATE elements may already be combined with the last filters. To prevent confusion, we now avoid stating that we provide a filter without relaxation losses as this is only true if a stand-alone WATERGATE is available. Instead, we refer to a filter that mitigates relaxation losses through a shared evolution, or similar wording. While doing so, we also seize the opportunity to highlight that WATERGATE elements have already been incorporated within filters in previously published work. We apologize for this omission as we certainly knew we were not the first to do that.

We have added text in the manuscript to clarify the above points and credit the first uses of a repurposed WATERGATE – X half-filter as in (Breeze, 2000; Sattler, 1999):

In Sect. 4: "In reference experiments, the X_d block is replaced by a 3-9-19 water suppression scheme, thus keeping all pulse sequences the same length for comparison.

This consideration ensures that attenuations in signal intensities report exclusively on the efficiency of the filter and not on relaxation. The 3-9-19 scheme simply omits the inversion pulses on ^{13}C and ^{15}N shown in the $X_{d,J3}$ block, as well as the delayed composite pulse decoupling sequences."

And:

"Incorporation of water suppression schemes in X-half filters has already been described (Breeze, 2000; Sattler et al., 1999). Briefly, inversion pulses are applied on ^{13}C and ^{15}N concomitantly with the existing proton inversion, here in the form of a 3-9-19 sequence, to enable evolution under scalar couplings. In our strategy, composite pulse decoupling is then delayed until coherences have become antiphase during detection." Including the new reference to:

Sattler, M., Schleucher, J. and Griesinger, C.: Heteronuclear multidimensional NMR experiments for the structure determination of proteins in solution employing pulsed field gradients, Prog. Nucl. Magn. Reson. Spectrosc., 34(2), 93–158, https://doi.org/10.1016/S0079-6565(98)00025-9, 1999.

In Fig. 4 Caption: "The pulse sequences used to obtain all spectra that are compared have the same lengths, and the comparisons report exclusively on the efficiency of the filters."

In all places where the wording was referring to general advantages of our method, we made sure to use terms such as "mitigate" or "minimize" when describing relaxation losses.

e.g:

Abstract: "[…] can be attenuated with mitigated sensitivity losses […]"

Sect. 1: "Our immediate objective is to attenuate these residual signals and mitigate sensitivity losses […]"

"[…] a method to attenuate undesired signals that escaped traditional filters with minimal increase in the length of the pulse sequence."

"[…] to attenuate residual signals from coupled spins that have escaped filters with minimal or no increase in the lengths of pulse sequences"

Sect. 2:

"[…] at reduced costs in sensitivity for the signals of unlabeled moieties"

Sect. 4:

"[…], thus mitigating relaxation losses"

- **2 is not as clear as it might be. It's unclear (needs to be stated) (i) that these are simulations (ii) what the solid grey and dotted lines are, and what the value of tau is in every case (i.e. ratio to J)**

Response: We have updated the caption to Figure 2 to clarify the meaning of solid, grey, and dashed/dotted lines in the simulations. The new caption to Figure 2 is now:

"**Figure 2. Principles of editing through delayed decoupling.** (a) Applying decoupling once coherences are antiphase truncates their FID and attenuates their signals (dashed

line), as shown here for the isolated component of a doublet. (b) The two components combine into a broadened and attenuated shape (dashed line). The analytical expressions of Eqs. (2) (solid grey line) and (4) (dashed black line) were used in (a) and (b). (c) Further attenuation is obtained when evolution into antiphase coherences is shared between a preparation period and detection as shown through simulations. The total evolution, D, was set to 1/2J, with evolutions during detection t = 1/2J (dashed line), 1/4J (dotted line), and 1/8J (solid line). In (a)-(c), spectra without delayed decoupling are shown in grey for reference. (d) Simulation where the duration D is arrayed for a fixed preparation period D_{prep} = 1/4J, and t ranges from zero to 3/4J leading to D = 1/J in ten increments Dt of 3/40 J. This simulation predicts the results seen in Fig. 4(b). In (a)-(d), J is set to 120 Hz. (e) A delayed decoupling targeting 150 Hz leads to residual positive in-phase signals for spins with couplings at 120 Hz. (f) A delayed decoupling targeting 120 Hz leads to negative residual in-phase signals for couplings at 150 Hz. In (e) and (f), D_{prep} = 1/4J and t is set to 1/4J for the targeted J, i.e. half of the total duration D."

- **Near the end of p5, it would be helpful to use consistent nomenclature to describe sinc function convolution (they use Sa function).**

Response: We have replaced the reference to a Sa function on page 5 with a sinc function for consistency:

"This description is reminiscent of discussions of truncation artefacts, which, in the frequency domain, lead to the convolution of Lorentzian signals with a sinc function. "

- **Minor points to do with sample preparation: (i) why so much (presumably unlabelled) EDTA in a filtered experiment? (ii) Use of TCEP not advisable in phosphate buffer.**

Response: We thank the reviewer for pointing out that the concentration of EDTA was unnecessarily high. We will lower this concentration in future studies. Regarding TCEP, unfortunately, we cannot use DTT or similar thiols as reducing agents as we have observed disulfide-thiol exchange leading to DTT adducts on the PP arm and/or dimerization of cysteine-loaded PCP1 through disulfide bond formation. Addition of TCEP successfully restored the sample to the original form. Indeed, TCEP is much more rapidly oxidized in phosphate buffer than other buffers, and we buffer exchange our NMR samples with fresh buffer and degas the NMR tube before adding argon at the final stage of sample preparation. We use phosphate buffer because our first application will be to compare data of loaded PCP1 with that of apo-PCP1, which had been collected in phosphate buffer (Harden and Frueh, 2017).