

Final response

First and foremost, we want to thank referee #1 and #2 for their valuable insights. The – at times lively – discussion was appreciated. In this final response, we will shortly summarize some of the arguments laid out in the previous responses (mr-2021-35-AC1, mr-2021-35-AC2, mr-2021-35-AC3, mr-2021-35-AC4). We will mostly refer to the changes made to the manuscript, we will not recapitulate the previous exchanges in detail.

Referee #1

(1) “For example, in the case of an IDP where little residual structure is present and conformations are in rapid flux, and where diffusion anisotropy could well be present on a per-residue basis, will this method be able to detect this qualitatively? Will a per-residue “feel” (even qualitative) of the dynamics be attainable? Could the authors present some experimental data to try to answer these questions, or the general question of what insights we might be able to obtain practically.”

As discussed in our initial response, this is indeed a fair point. While we cannot provide detailed experimental results at this stage, we extended the Results & Discussion section by a qualitative discussion of possible scenarios to exemplify the attainable information content (page 11-12).

(2) “Can the authors comment on whether interactions involving other ^{13}C spins in a $U\text{-}^{13}\text{C}$ labeled IDP would be predicted to influence the extracted cross-correlation rates, especially in cases where rates are small.”

We included the considerations of our initial response in the Methods section. The expected CCR effects of remote carbons are discussed on page 9.

Referee #2

“(1) This study is totally predictive in nature. No validation, and/or examples illustrating actual applicability are provided.”

As previously stated in our two responses and in the response (1) to referee #1, this notion is warranted. Our study is indeed predictive and conceptual in nature. With the given deadline, the presented concepts and preliminary measurements represent the project’s current status. We deem it a worthwhile contribution to the Festschrift.

“(2) A cursory survey of the NMR-based studies of IDPs cited in mr-2021-35 shows that methods examining local features point to a random-coil situation (e.g., see Mantsyzov et al. 2014). CCR in $^{15}\text{N}\text{-}^1\text{H}$ and $^{13}\text{C}\text{'-}^{13}\text{C}^\alpha$ is a local feature. Obtaining information on long-range “order”, in particular “diffusion anisotropy”, requires empirical spectral densities comprising statistical elements, to be used in combination with coil libraries and molecular dynamics simulations (e.g., see Mantsyzov et al. 2014). These elements are absent in mr-2021-35 scheme.”

As previously discussed, the study of (Mantsyzov et al. 2014) invokes the concept of anisotropic diffusion of extended chain segments to rationalize unexpected variations in intraresidual and sequential $\text{H}^\alpha\text{H}^\text{N}$ NOEs, which are indeed local features. Clearly, long range “order” effects can

manifest in local spin probes. We agree that detailed mechanistic insights can only be obtained by including additional tools such as MD simulations. We are not implying that the spectral density – used to assess the information content of the CCR rates – can or should be used to “determine” a hypothetical diffusion tensor.

We adjusted the phrasing throughout the manuscript to clarify the general nature of the measure Q as means to detect the presence of anisotropic dynamics, emphasizing the sensitivity to slow segmental motions. We further clarify the toy nature of the tumbling symmetric top model and highlight the importance of MD simulations to rationalize experimental CCR rates in IDPs (e.g. page 11-12). As the concept of diffusion anisotropy might be too specific/evocative at times, the phrasing has been adjusted throughout.

“(3) It is indicated that anisotropic “segmental” motion is targeted. How are these “segments” defined? In other word, how is the second-rank diffusion tensor, D , defined?”

As described in the previous responses, we adopt the simplistic image of (Mantsyzov et al. 2014), assuming that the main axis of the axially symmetric diffusion tensor lies in the peptide plane. The orientation of the diffusion tensor with respect to the peptide plane is varied as described in the Methods section and covers the cases of idealized α -helices and extended chains as described by (Mantsyzov et al. 2014).

We now highlight the model of (Mantsyzov et al. 2014) in the introduction of the Theory section, page 4.

“(4) It is pointed out that NMR relaxation analysis methods applicable to folded proteins are not applicable to IDPs. The model-free (MF) spectral density, a variant of which is suggested here, refers to protein and probe as rigid bodies moving in a statistically independent (decoupled) manner. In IDPs the protein is not rigid and its motion is not decoupled from the motion of the probe. The spectral density suggested here does not reflect these features; rather, it is similar in character to the MF spectral density.”

As discussed in more detail in the previous responses, spectral densities of IDPs indeed cannot be expected to adhere to the simplified model underlying the MF-like spectral density. The spectral density is not intended to describe the dynamics of IDPs in any general sense, it is only used to assess the features previously asserted by (Mantsyzov et al. 2014). The experimental measure Q is sensitive to the presence of anisotropic dynamics independently of its source.

We further highlight the toy nature of the MF-like spectral density throughout the manuscript. The limited validity of the model and the importance of MD simulations for mechanistic interpretations are now further emphasized in the Results & Discussion section (page 11-12).

“(5) In the context of item (2) – please note that the “dynamics detectors” method (Smith et al. Angew. Chem. Int. Ed. 2017 56. 13590), shown to actually surpass MF, comprises statistical elements. Recently it was applied to proteins in solution (Smith et al. JCP 2019, 151, 034102). The authors might want to check applicability to IDPs.”

As previously discussed, this approach is indeed an interesting way to characterize/analyze the spectral densities in further detail. The references are now included and contextualized in the Theory and Results & Discussion sections.

Mathematical and physical objections regarding the MF-like spectral density

While the initial mathematical objections could apparently be clarified during the previous exchanges, the physical meaning of the order parameter and the use of an (apparent) auto-correlated TCF remained an open issue.

We reworked the Theory section to contextualize the proposed spectral density as a simplification of the formalism proposed by (Ghose et al. 1998) and generalized by (Vögeli and Yao 2009). Both the auto-correlated form of the TCF and the meaning of the generalized order parameters are now described in the newly added Appendix. The proposed spectral density is highlighted as a simplification. In the semi-qualitative context of Fig. 4., we consider the use of a single generalized order parameter as a sensible approximation. The more thorough way of handling internal motions is described in the Appendix as well. Parallels to the MF formalisms of (Lipari and Szabo 1982) and (Halle and Wennerström 1981) are illustrated.

References

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- Vögeli, B. & Yao, L. Correlated dynamics between protein HN and HC bonds observed by NMR cross relaxation. *Journal of the American Chemical Society* **131**, 3668–3678 (2009).
- Lipari, G. & Szabo, A. Model-Free Approach to the Interpretation of Nuclear Magnetic Resonance Relaxation in Macromolecules. 1. Theory and Range of Validity. *Journal of the American Chemical Society* **104**, 4546–4559 (1982).
- Halle, B. & Wennerström, H. Interpretation of magnetic resonance data from water nuclei in heterogeneous systems. *J. Chem. Phys.* **75**, 1928–1943 (1981).