Answers to the reviewers

mr-2020-32 Report #1

A significant fraction of the current manuscript focuses on a very long-standing problem: Can liquid crystal or otherwise anisotropic NMR define the ensemble distribution of flexible molecules? This question has been studied for nearly 50 years, including by some of the giants of the magnetic resonance field (incl. Luz, Pines, Jim Emsley), but with mixed success (nicely reviewed by J. Emsley in the Encyclopedia of NMR). P3D now changes the approach because it uses the shape of the conformer to directly predict its alignment, thereby removing the key problem that the number of adjustable parameters steeply increases with the number of conformers considered.

Reply: We thank the reviewer for highlighting the importance of our work.

The flipside, however, is that P3D predictions are quantitatively not all that precise. I also note that the agreement is provided in terms of an RQ parameter that does not account for the alignment strength, which is most definitely needed when calculating the ensemble populations. It also is not clear how sensitive the method is to the accuracy at which P3D predicts the alignment of a given conformer, and therefore, whether it is statistically warranted to select individual conformers, and their populations, from an effectively infinite ensemble. Although I believe the current manuscript is suitable for publication in Magnetic Resonance, I strongly urge the authors to cast these efforts as a promising approach, tested on a single molecule, and therefore a preliminary but potentially feasible method to address the longstanding problem of determining the ensemble distribution of a flexible molecule by liquid crystal NMR.

Reply: We modified the final conclusion in accordance with the reviewer comment: "*As tested with the example of sucrose, P3D is also a promising approach and a preliminary but potentially feasible method for the determination of conformational ensembles of flexible small molecules*."

A few minor points: 1. For sucrose, the authors decided to focus only on the one-bond C-H RDCs. However, I note that for the glucose ring, four of the CH-bonds are effectively collinear, reducing the number of independent observables to five.

Reply: In order to solve the question about the reduced number of RDCs we included a sentence with the results obtained using all RDCs: "When all RDCs are considered we get R=0.926 for the average with 0.806, 0.615 and 0.838 respectively for each conformer. The obtained populations (26/20/54%) are very similar to the ones obtained with the one-bond CH RDCs only."

2. The authors repeated point out that their highest energy conformer is close to the cristal structure, perhaps suggesting it is a reasonable conformation despite being 2.7 kcal higher in energy than their lowest energy conformer. Some more discussion on why the actual energies are only rough estimates may be appropriate.

Reply: The free energies of the conformers come from the Ndukwe et al. reference, from DFT calculations. Even for them, the energies do not fit exactly with the obtained

populations, but can discriminate the conformers when the energy difference is big. The step of energy calculations is needed to reduce the number of conformers to be considered, excluding the ones with too big energies, but that doesn't mean that, in the experimental conditions, the calculations are exact.

3. To what extent are the sucrose conformers compatible with other parameters (e.g. JHH, NOEs, RDCs) of this extensively studied molecule (see e.g. Freedberg JACS 124, 2358, 2002 and Carb. Res. 340, 863, 2005 and references cited therein)?

Reply: The aim of our work is to investigate the ability of P3D to characterize multiple conformations in small molecules. For that we took a published paper with data obtained using our selected alignment media (i.e. PBLG) and compared it with our simulations. Comparing with other results (including RDCs in different conditions) is beyond the scope of the current work. However, the current analysis demonstrates that the crystal structure of sucrose fits well to the P3D predicted RDCs.

4. Strain-induced alignment in a gel (SAG) was first (simultaneously) introduced by Tycko (JACS) and by Grzesiek (J Biomol NMR), and that perhaps should be clarified in the referencing.

Reply: We included the suggested references, thanks.

5. Is it possible to show by cross validation that indeed the 3-member ensemble provides an improved fit (treating the 4 parallel Glc RDCs as 1)?

Reply: As indicated in the first point, we decided to solve this question including the results for all RDCs.

mr-2020-32 Report #2

The manuscript by Ibáñez de Opakua and Zweckstetter describes the assignment of the relative configuration of three compounds (strychnine, IPC, and sucrose) using anisotropic NMR parameters. The anisotropic NMR parameters used in this investigation are one-bond RDCs. The RDCs (literature data) are translated into structural informational by the author's own computer program P3D. This is a continuation report of a previous publication in ACIE (acie 2020, 59, 6172-6176). The problem for the reader is that the authors do not give enough information about their method (P3D). This was already true for their first paper in ACIE on this topic (acie 2020, 59, 6172-6176). It needs to be further mentioned that two out of the six compounds from the ACIE paper were used again (strychnine and IPC). Therefore, the title of the manuscript ("Extending the applicability of P3D for structure determination of small molecules") seems to be totally exaggerated. The three known compounds of the manuscript were already studied as model systems many times and are by no means an extension of the applicability!

Reply: Please note that the point is not that we used again two out of the six compounds from our ACIE paper, but that we address two new aspects. The first one is if the P3D-PBLG simulation is applicable to other alignment media. This of course can only be

tested for small molecules, for which RDCs in several different alignment media are available (i.e. strychnine and IPC). The second new aspect, which is evaluated in the current manuscript, is if it is possible to use P3D for the conformational analysis of flexible small molecules. This is an important question as stated by reviewer #1 *("A significant fraction of the current manuscript focuses on a very long-standing problem: Can liquid crystal or otherwise anisotropic NMR define the ensemble distribution of flexible molecules? This question has been studied for nearly 50 years, including by some of the giants of the magnetic resonance field (incl. Luz, Pines, Jim Emsley), but with mixed success (nicely reviewed by J. Emsley in the Encyclopedia of NMR).")*.

It remains unclear for the reader why the authors have added eight columns for strychnine and IPC (further alignment media). The values do not contribute anything to the manuscript (although a long discussion is included here) because you cannot compare experimental RDCs from alignment medium B with the calculated RDCs from alignment medium A. This comparison is invalid!

Reply: In fact, the comparison was one of the aims of the study. We compared the RDCs obtained from simulations with experimental RDCs from different alignment media to identify those media that have similar alignment properties as PBLG and those which don't (and thus are either amendable to P3D prediction or not).

For the third molecule (sucrose), the authors used the RDC data of Ndukwe et al. (cc 2019, 55, 4327-4330). They applied only eight out of the 23 originally described RDCs ("one-bond CH RDCs"). Given the limited number of RDCs (in respect to the assignment of the relative configuration, the conformation, and the conformational ensembles), additional fitting parameters (molar fractions) must necessarily lead to an increase in the fitting quality between the experimental and simulated data.

Reply: To address this issue, we included the results obtained using all RDCs: "When all RDCs are considered we get R=0.926 for the average with 0.806, 0.615 and 0.838 respectively for each conformer. The obtained populations (26/20/54%) are very similar to the ones obtained with the one-bond CH RDCs only."

In total, the P3D method is a very rough and coarse-grained model that reduces the interactions of the alignment medium with the molecules under investigation to purely static van der Waals and electrostatic interactions. All dynamic aspects of the polymer in a condensed LLC phase are discarded.

Reply: What is pointed out here is in fact one important conclusion of our work: the dynamic aspects and specific interactions are less important for the alignment in PBLG and the alignment of small molecules (at least those tested here and in our Angew. Chem. Paper) is dominated by ("coarse-grained") steric and electrostatic effects.

mr-2020-32 Report #3

Related to 1), did the authors tried to parametrize other alignment media in their P3D-PALES software? I would think that the electrostatic component might vary in some cases?

Reply: While this is an interesting suggestion it is currently not possible to implement. The chosen alignment medium, PBLG, has a defined alpha-helical structure, i.e. a prerequisite for structure-based alignment prediction. In order to implement other alignment media, high quality structural models would be required.

Related to 2), the authors chose to test their method using three conformations of sucrose previously shown to be representative of what is found in solution based on previous NMR experiments. But what if they were to use more conformations? Would they still converge to three and to the same ones?

Reply: Using too many conformations is dangerous because of the overfitting, so it is possible to get a good fitting even with wrong conformers if too many conformers are being used. That's why an additional filter, like energy calculations, should be included.

In the current era of open science, I find the statement about data availability rather strange. The authors should provide all data and scripts to allow to repeat the current work, ideally in e.g. a GitHub repository. If the authors have different policies about distributing the software itself, this is fine. But the data for this work should be made public, together with the scripts to reproduce it. Only then can other easily use this new tool.

Reply: Thanks for the suggestion. We added to the methods section the command used to run our simulations with a brief description of the involved files. The P3D algorithm is available as part of the PALES program, which can be downloaded from the PALES webpage (https://www3.mpibpc.mpg.de/groups/zweckstetter/_links/software_pales.htm).