Answers to the reviewers

mr-2020-32 Report #1

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

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).