Dear Dr Zweckstetter,

Two additional experts have re-evaluated your manuscript, with opposing views (as before). We therefore asked a third reviewer.

One reviewer raised several concerns, as you can read in report #1, but considers your contribution overall acceptable, but only after revision.

The other reviewer raised similar concerns as one of the reviewers before. This reviewer questions the general applicability and robustness of the model used in the P3D calculations and would not consider it an established method yet. This reviewer also finds the current manuscript no major advance vs your previous paper in Angew. Chemie and not presenting sufficient detail to reproduce the results.

The third reviewer is positive, stressed the advance in this manuscript vs your previous paper.

Considering the reports, I conclude that after a revision your contribution can be a worthy contribution for this Special Issue for Robert Kaptein in MR. I have attached all 3 reviews.

A new manuscript needs to address the concerns and comments raised by the reviewer #1 and #3 and repeating his words I would “urge you to cast your approach as a promising approach, tested on a single molecule, and thus as a preliminary but potentially feasible method to address the problem of determining the ensemble distribution of a flexible molecule by liquid crystal NMR”.
Please also consider reviewer #2, by presenting more detail to reproduce the results. Would it be possible to make the program P3D available via nmrbox.org or github, and present used parameters for the calculation in the supplement? This would also be in line with the comment by reviewer #3.

I hope that you will be able and willing to do so.

With kind regards, Rolf Boelens

mr-2020-32 Report #1

This manuscript describes a potentially very interesting extension to the recent introduction of the P3D method that predicts the alignment of small molecules in liquid crystals, somewhat analogous to the PALES program that has been widely used for proteins. It is perhaps surprising that P3D works as well as it does because the surface characteristic of the various alignment media is not accurately known at the atomic level, whereas this must play a role in aligning the solute (as highlighted by the brief discussion of (-)-IPC in PALF300 and PALF316). Considering that in many cases it does work fine, as recently published in Angew. Chemie, I have no problem with accepting that fact.

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. 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 long-standing problem of determining the ensemble distribution of a flexible molecule by liquid crystal NMR.

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.
2. The authors repeated point out that their highest energy conformer is close to the crystal 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.
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)?
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.
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)?

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!

The results for the two compounds from the first paper (strychnine and IPC) are given in Figure 2. The experimental RDCs in PBLG are given in column 2 and the calculated RDCs by P3D in column 1. The agreement of these values is not very good. Usually the results are presented as so-called D(calc) plots (experimental versus predicted RDCs). Furthermore, no quality factors or anything related are given. In the ACIE 2020 paper, they have given the R-values for strychnine (0.88) and IPC (0.84). It is surprising how the authors can even attempt a configurational assignment of both compounds given these large deviations for the correct configuration.

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!

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.

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.

Due to the many objections mentioned above, the manuscript should be rejected.

mr-2020-32 Report #3

Compared to the previous paper the in Angewandte Chemie, https://doi.org/10.1002/anie.202000311 this manuscript describes two new additions to the P3D method:

1) It assesses its applicability to other alignment media.

2) It extends the method to extract conformational ensembles in case on a mixture of conformations in solution.

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

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?

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.