We thank our colleagues for their insightful feedback and comments which have been addressed as described below.

Blue: Answer to comment (*italic: changed/new text*)

Dr. Thomas Barbara:
Comment: This paper is a fine effort in the progression of "small volume NMR", a familiar topic I was introduced to during my days at Varian Associates. In 2009 I wrote up a small review on the history and progress for the eMagRes Journal: "NMR Probes for Small Sample Volumes" 2009 Thomas M. Barbara https://doi.org/10.1002/9780470034590.emrstm1084
Throughout that history, the issues of dielectric loss, background signals and magnetic susceptibility have always played critical roles. At one time, there was a great effort by a group to etch an approximately elliptical cavity into a glass capillary and this effort reminded me of that work. It would be of some interest to compare this effort with that one (I reference that effort in my article). Background signals in that case are much smaller. This is an aspect of 3D printing materials that can cause headaches for probe builders.
Section 2.1 might perhaps appear rather obscure to a neophyte. For those who are curious, "Cylindrical Demagnetization Fields and Microprobe Design in High Resolution NMR" Journal of Magnetic Resonance A109, 265-269 (1994) will bring satisfaction. It is a real advantage to recognize that magnetization can be viewed as equivalent to a current source whenever there is a gradient or a discontinuity. Back then, it was AOK to use Gaussian units in magnetostatics, but the conversions to SI are actually very simple.

Response: We thank Dr. Barbara for his helpful feedback and we apologize for not having been aware of the pertinent work he carried out related to our manuscript. We have inserted (line 51-53)*: The sample volume also can be reduced by inserting plugs, of a magnetic susceptibility close to that of the solvent, above and below the active volume of the sample {Barbara, 2009 #8044}*.

Comment: Section 2.1 might perhaps appear rather obscure to a neophyte. For those who are curious, "Cylindrical Demagnetization Fields and Microprobe Design in High Resolution NMR" Journal of Magnetic Resonance A109, 265-269 (1994) will bring satisfaction. It is a real advantage to recognize that magnetization can be viewed as equivalent to a current source whenever there is a gradient or a discontinuity. Back then, it was AOK to use Gaussian units in magnetostatics, but the conversions to SI are actually very simple.

Response: Agreed! We have inserted a reference to this insightful and rigorous analysis, both in line 126 and line 142.

Anonymous referee 1.
In the manuscript by Bax *et al.*, the authors presented a nice demonstration of 3D-printed microcell as an effective and inexpensive alternative for reducing the sample volume needed for protein NMR at high ionic strengths. Fully taking advantage of the versatility of SLA 3D printing, the authors created a 3D printed ellipsoidal polymer plug that could be inserted into regular NMR tubes, thus enabling high quality NMR acquisition without considering mismatching susceptibility. The authors further demonstrated the application of the 3D printed microcell for the observation of the tetramerization of melittin, which is challenging to perform in regular NMR tubes. The concept of employing 3D

printing to create freeform vessels for NMR is novel and the NMR experiments were conducted rigorously. This manuscript will be of great interest to the readers of *Magnetic Resonance* and this reviewer would like to recommend its publication. The following comments extend beyond the major findings of this work but may enhance the discussion section at the editor's discretion.
Response: We thank the referee for their positive feedback.

1. It is interesting that authors observed leaching impurities from the 3D-printed microcells as they noted in Figure 5. The authors were using Formlabs Clear V4 resin, which is mainly composed of methacrylate monomers, bismethacrylate crosslinker, and TPO (diphenyl(2,4,6-trimethylbenzoyl)phosphine oxide) photo-initiator. Telling from the chemical shifts of the leaching impurities, they are likely to be methacrylate (hydroxypropyl methacrylate, HPMA) monomers or oligomers. HPMA and its oligomers have reasonable water solubility and that would be problematic if this material were to be used for protein NMR.

Response: We thank the referee for this insightful remark. We showed the presence of water-soluble material that leaches out of the sample wall only as an alert that such material can be observed if the sample is not extensively rinsed. These signals effectively disappear if the wash with water is extended to 1 week, although they then very slowly reappear over the course of multiple days at concentrations much lower than seen in Fig.5. Considering that the concentration of these unlabeled contaminants, even after days, remains below the mid-micromolar range, in our experience they do not interfere with protein NMR. As the referee points out, the leached impurities correspond to small oligomers. To show this, we have added a supporting information spectrum of an aqueous sample that was briefly vortexed in the presence of liquid Formlabs Clear V4 resin (Figure A3). We have inserted the following sentences in the main text (lines 282-292):
Comparison of the NMR spectrum of these impurities with those in a sample obtained by briefly vortexing a mixture of unpolymerized resin and water followed by entering the aqueous phase into an NMR sample tube (Figure A3) suggests that these impurities consist of residual, polymerized methacrylate components that apparently can slowly diffuse from the cell walls into the aqueous contents of the cell. Leaving the sample cells filled with H_2O for a week prior to a final rinse reduces the impurity levels further, but in our experience is not necessary considering we have not observed any interaction between these very low impurity concentrations and isotopically enriched proteins. For applications to samples in D_2O, leaving the microcell filled with D_2O for 24 h, prior to using it, reduces the intensity of a weak, very broad (~1 kHz) signal at ~3.8 ppm, that results from H_2O diffusing into the resin.

2. To remove water-soluble components, this reviewer noticed that the authors used rigorous post-printing processing, including IPA wash, UV curing, and Milli-Q water wash at an elevated temperature. Water-soluble polymers with hydrophobic nature like poly(HPMA) tend to exhibit lower critical solution temperature (LCST) behaviors (*Macromolecules* **2008**, *41*(14), 5132-5140) meaning they become less soluble in water as temperature increases. Therefore, washing with cold water rather than hot water would likely be more effective at removing these impurities, as they would remain dissolved at lower temperatures. This reviewer suggests the authors try cold water washing of the printed microcells, which might help reduce the leaching problems observed in the NMR experiments.

Response: We thank the referee for this insightful suggestion. However, comparison of sample cells washed at 60 C and at room temperature did not reveal any significant impact of temperature. It appears likely that faster diffusion from the resin into the aqueous phase at 60 C offsets the lower aqueous solubility at the elevated temperature. We also note that the impurity intensity observed does not appear to be limited by solubility. Impurity levels in an unwashed cell are nearly two orders of magnitude higher than shown in Fig. 5. We also apologize for not having pointed out that the cell used for the spectra of Fig.5 deliberately was only washed for 2 h, such that the reader can see what to expect when rinsing is incomplete. As we now point out (lines 463-472), these impurities are much lower when the sample is washed for 1 week.

3. If poly- or oligo- HPMA is indeed the leaching impurities, another phenomenon the authors might have observed is when the ionic strength is increased, the resonances of the impurities might broaden and disappear. This reviewer would be interested, and it would be much appreciated if the authors could comment on that.

Response: The referee raises the interesting point that at high ionic strength the solubility of these hydrophobic HPMA derivates is likely to be strongly reduced (salted out). However, the concentration of the impurities in Figure 5 is so far below the solubility limit that we were unable to see any difference in their intensity between 0 M and 2 M salt.

4. In Method Section, a different resin, ELEGOO ABS-like Resin V3 was used for doping study. However, experiments using V3 were not mentioned elsewhere in the manuscript. It would be valuable if the authors could include results from these experiments and comment on the NMR performance of this material compared to Formlabs Clear V4 resin. Based on the material specifications, ELEGOO ABS-like Resin V3 is relatively more hydrophobic than Clear V4, which might result in less leaching of water-soluble components. However, this potential advantage might be offset by its high titanium oxide content, which could potentially interfere with magnetic susceptibility matching despite the optimized shape design. Including those comparative results would be helpful in understanding material selections.

Response: The referee is correct that other more hydrophobic resins like ELEGOO ABS-like Resin V3 shows less leaching of impurities from the plugs into the solution. However, we were unable to use this LCD-based printer for correctly shaping the sample cells, in particular the narrow “neck” of the sample. We therefore were forced to use the slower Formlabs laser printer instead, with the Clear V4 resin being the only one allowing printing at 25-um resolution.

The original manuscript inaccurately described that the plug-doping experiments were carried out on an ELEGOO printer using ABS resin. However, the measurements were actually carried out on using the Formlabs printer with doped Clear V4 resin after we had determined (on the ELEGOO printer) how much doping was required. We have changed the corresponding paragraph to describe the preparation of the plugs that were actually used (lines 517-520).

5. The observations regarding different resin materials lead to a more general consideration that is beyond the scope of this work but worth noting. Commercial 3D printing resins are formulated in a way that try to meet various performance specifications, such as material strength, printing resolution, and printing speed. As such, they typically contain multiple components and are not necessarily suitable for high precision work such as protein NMR. This work provided a great proof of

concept and would motivate material chemists to develop specialized resins for analytical applications.
Response: We whole-heartedly agree with the referee, and we have inserted a few sentences in the Concluding Remarks section (lines 463-468)

Anonymous referee 2
General comments:
The manuscript describes the construction of NMR microcells using 3D printing. The authors thoroughly measured the magnetic susceptibility of water containing NaCl, D_2O and cobalt-doped Clear V4 resin. The authors also constructed an ellipsoidal-shaped microcell using Clear V4 resin and successfully observed HSQC signals of N-acetylated alpha-synuclein in PBS buffer and tetramerized melittin at 2M NaCl using the microcell. As the experimental magnetic susceptibility data are useful for designing NMR microcells with a wide variety of sample chamber shapes, I recommend publication of this manuscript.
Response: We thank the referee for their positive feedback.

Specific comments:
When an NMR tube made of glass is used, there is a case where a protein adsorbs to the surface of glass. Was such adsorption to the surface of the Clear V4 resin observed for synuclein and melittin? The authors state that H_2O appears to diffuse into the Clear V4 resin (in Line 260). If a protein sample is placed in the microcell and left it for a long time, will the protein leach from the sample space into the printer resin?
Response: The referee raises an interesting point. Indeed, it is conceivable that hydrophobic macromolecules could "stick" to the cell surface. However, we have seen no evidence for such behavior when using lipophilic solutes such as detergents, perhaps because the methacrylate-based Clear-V4 resin is relatively polar.
The referee also asks whether, analogous to water, it is possible for polypeptides or proteins to diffuse into the polymerized Clear V4 resin. Although strictly speaking this appears to be possible for small solutes, we note that even for water the fraction of water that diffuses into the cell wall is only ~0.1%. Unless a solute has high affinity for methacrylate polymers and is sufficiently small to diffuse through the molecular-size pores, it appears unlikely that a detectable amount of protein could diffuse into the cell wall. We have inserted the following sentences in the Concluding Remarks section (lines 469-472):
While a small amount of solvent (<0.1%) can diffuse into cavities of the polymerized resin, no detectable loss of solute signal was observed. For example, the intensity of the sucrose NMR resonances remained unchanged, to within ±0.2%, over a duration of more than one week.

Technical correction:
Figures 1 to 4: While each panel is labeled in lowercase letters in the artwork, capitals are used in the figure legends.
Response: Thanks! Now fixed.

Editorial comments by Dr. Gottfried Otting:

The bioengineering group of Prof. Adam Perriman at the ANU happens to have the 3D printer and printing material described in this article, and his postdoc Dr Mark Shannon kindly printed a few ellipsoidal shaped microcells. Our preliminary observations:
- printing using the STL file provided was very easy;
- washing with isopropanol needs to be thorough to avoid non-smooth inner surfaces of the microcells from residual resin (perhaps the authors can give more details on the extent of washing);
- degassing in vacuum made a significant difference to field homogeneity, even when air bubbles are not visible to the naked eye;
- the microcells are designed for thin-wall NMR tubes (and don't fit into inexpensive tubes with slightly thicker glass walls) but scaling by 5% in the x-y plane would be easy; as they are, they glide easily down and back out the NMR tube (if not, they can probably be fished out with some chicken wire);
- the microcells tend to bend slightly during UV curing at 60 oC (the Perriman group proposes to cure by leaving in sunlight at room temperature first, but this has not been tested for this application).
It would be nice to know, whether 3-axis pulse field gradient probes are a significant advantage over single-axis gradient probes. Preliminary experiments suggest that the non-axis shims can be quite different from the z-axis ones.
Bruker also sells shaped tubes with an approximately rectangular cross-section that are claimed to provide 86% of the sensitivity of a 5 mm tube in the absence of salt, equivalent sensitivity at 20 mM salt, and 20% better sensitivity at 100 mM salt. A direct comparison may be impossible as it requires access to a specifically equipped probe head, but a comment would be welcome. Certainly, the micro-cell proposed is far less costly.

Response: We are pleased that Dr. Otting and co-workers were able to print the sample cells on their own Formlabs printer and we thank him for the useful feedback.
Regarding the washing procedure, we used a syringe needle connected to an FPLC running at 5 mL/min for 3 minutes. We have added this info to the Methods section (lines 505-507).
Indeed, the cell diameter was designed for 4.2-mm ID mid-quality NMR sample tubes and not compatible with the discardable cheaper tubes. Considering that the cell is easily removed from the NMR sample tube, allowing the NMR tube to be recycled without cleaning, we believe this is the preferred sample tube to use.
We have not observed any sagging/bending of the 3D printed cells but we kept the cells vertical at all times till curing was completed. This information is now included in the Methods section (line 507)
The 3-axis pulsed field gradient probe was only used for recording the 3D image. All other spectra were recorded on a single-axis PFG probe. Indeed, adjustment transverse shim settings deviate somewhat from those obtained for a regular reference sample, and adjustment of the transverse shims is needed to obtain high resolution (line 442-444).
Indeed, other microcells such as the “slit-Shigemi” sample tube can also be used to reduce detuning of the 1H channel of the probe by NMR samples, while reducing the sample quantity needed. We lack the accessory needed to correctly position such a tube into the probe and were unable to compare performance. We also note that the tubes are expensive and not that easily washed. We prefer not to comment on the use of such tubes as we do not have hands-on experience with their use.

We have expanded/modified Methods section 4.2, which now reads:
Cells were kept vertical during printing and subsequent UV-curing using Form Cure for 16 hours at 60 °C. Subsequently, cells were filled with Milli-Q water and immersed in a water-filled falcon tube that was heated at 60 °C for 12 hours to remove water-soluble components. After this cleaning, to further minimize the amounts of resin-derived impurities from leaching from the cell wall into the solvent,

which occurs at a rate that decreases steadily with time, cells can be stored filled with and immersed in water for a week at room temperature. After removal of the water from the cells by pipetting followed by centrifugation upside down in Eppendorf tubes, they were briefly dried under vacuum. Subsequent use of such a cell showed strongly reduced intensities of these impurity signals (Figure A3).

Referee 3 (Marcel Utz):

Kakeshpour et al present a study of susceptibility matching for aqueous samples. They show an ingeniously simple method for measuring the susceptibility difference between a solid material (eg, glass) and a liquid, by comparing the observed chemical shift of signals stemming from the solid/liquid interface to those observed for a glass/liquid interface of the same geometry. They also show that 3d printed inserts can be susceptibility-matched by doping of the 3d printing resin with a suitable paramagnetic dopant. Finally, they demonstrate the use of a 3d printed insert with an ellipsoidal sample chamber to reduce the impact of susceptibility differences. The resulting lower filling factor also makes the probe tuning less sensitive to sample conductivity, and therefore enables the study of samples that require high salt concentrations.

The manuscript is well written, with figures that clearly illustrate the concepts and results. This is a strong contribution to the field, and should definitely be published in Magnetic Resonance.

Response: We much appreciate the reviewer's positive feedback.

There are a few minor issues to address, as listed below:

1. L120: the approach based on eq. 4 implicitly neglects all other interfaces. However, the experimental setup has a glass/air interface at the bottom end of the shigemi tube that, while further away, represents a much larger step in susceptibility (of order 10ppm). I suspect that it is correct to ignore it due to the initial shimming on a filled NMR tube, which has the same interface. This should be more clearly explained in the text.

   Response: Yes, the reviewer is fully correct. We have inserted the following sentences (line 147-151): *The above analysis does not take into account the B_0 gradient inside the NMR tube that is caused by the large magnetic susceptibility mismatch at the bottom of the tube. However, when assuming that the magnetic susceptibility of the Shigemi glass is much closer to that of water than air, shimming on a water-filled sample tube inserted to the same depth into the probe can be used to remove this B_0 gradient, to first order.*

2. Figure 1: the method relies on the shift difference between the maximum of the h=0 slice spectrum and one that has been taken at h=13mm. While the latter is a single peak, the former has a strong downfield shoulder due to the lateral inhomogeneity. To what extent does this affect the precision of the resulting shift difference? An error estimate based on the accuracy with which the peak positions can be determined should be carried out.

   Response: Agreed. We were initially also worried about the accuracy at which the frequency just above the interface can be determined, but it proved better than expected. We have added the following sentences to clarify (line 192-199):

   The precision at which the frequency at the glass-water interface can be measured is impacted by the slice thickness of the selected solvent layer, which is subject to lateral gradients that increase with slice layer thickness and towards the edges of the slice. By

varying the position of the center of the slice from ~0.2 mm below the interface to ~0.2 mm above the interface, increased total intensity with a strong downfield shoulder is observed. The upfield edge of this line shape (Figure 1b, blue) remains invariant to the precise position of the selected slice and represents the true $(\chi_g + \chi_s)/2$ value, which can be determined at a precision of ~0.02 ppm.

3. L 151: the extensive work of Wapler et al (10.1016/j.jmr.2014.02.005) to determine the susceptibility of a range of construction and microfabrication materials should be cited. Their method was based on susceptibility-induced MR image distortions in water, and therefore measured susceptibility differences with respect to water.

Response: We apologize for having been unaware of this beautiful study and now have inserted a sentence referencing this work (line 96-99): *Magnetic susceptibilities of materials with application to magnetic resonance imaging (MRI) as well as microfluidic NMR technology commonly have employed MRI scanner equipment, and elegant methods applied to a wide range of materials were presented by Wapler et al. {Wapler, 2014 #8046}.*

4. L197: the units are not clean in this expression. the concentration needs to be divided by 1M, and it should be -9.01 ppm rather than -9.01

Response: We have added that molarity is defined in units of moles per liter (line 223)

5. We have proposed a tangentially related approach to compensate for solvent/container susceptibility differences in the context of microfluidic NMR. (Ryan et al, 10.1039/C3LC51431E, Hale et al, 10.1039/C8LC00712H) Maybe the authors could consider citing this work for the benefit of the interested reader?

Response: Thanks for drawing our attention to this very interesting work, now referred to in a newly inserted paragraph (lines 74-81)

We note that susceptibility mismatching for non-ellipsoidal shapes can also be obtained by introducing nearby suitably shaped small "compensation structures" of different magnetic susceptibility, that effectively shim the sample to homogeneity for microfluidic applications {Ryan, 2014 #8047}. Alternatively, the magnetic susceptibility of an aqueous solution can be increased by addition of Eu^{3+}-complexed diethyl-triamine pentaacetate (DTPA), which due to the short Eu^{3+} electron T_1 has minimal broadening effects on other solutes {Hale, 2018 #8048} but is restricted to cases where the solvent susceptibility is more negative than the surrounding material, which does not apply for 3D printer resin.

Editorial Comment by Gottfried Otting:

Looking forward to the revised version! When preparing the revision, please go through the references with a fine comb to check for completeness (e.g., doi, page numbers), correctness (Copernicus does not have the means to check for typos in references) and format (it is recommended that titles of articles do add a capital to every noun).

Response: We've made an effort to insert the correct doi links in a homogeneous fashion and to change the capitalization issue.