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# Complete $^{13}\text{C}$ NMR Chemical Shifts Assignment for Cholesterol Crystals by Combined CP-MAS Spectral Editing and *ab Initio* GIPAW Calculations with Dispersion Forces

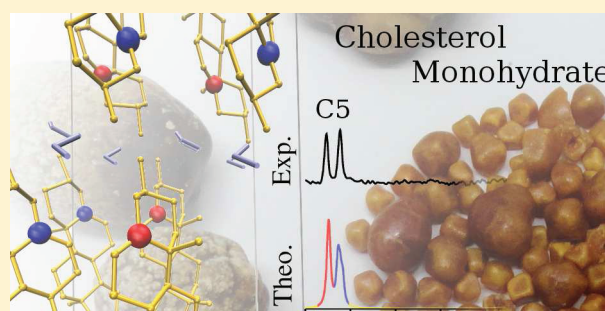
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## Supporting Information

**ABSTRACT:** We report here the first fully *ab initio* determination of  $^{13}\text{C}$  NMR spectra for several crystal structures of cholesterol, observed in various biomaterials. We combine Gauge-Including Projector Augmented Waves (GIPAW) calculations at relaxed structures, fully including dispersion forces, with Magic Angle Spinning Solid State NMR experiments and spectral editing to achieve a detailed interpretation of the complex NMR spectra of cholesterol crystals. By introducing an environment-dependent secondary referencing scheme in our calculations, not only do we reproduce the characteristic spectral features of the different crystalline polymorphs, thus clearly discriminating among them, but also closely represent the spectrum in the region of several highly overlapping peaks. This, in combination with spectral editing, allows us to provide a complete peak assignment for monohydrate (ChM) and low-temperature anhydrous (ChAl) crystal polymorphs. Our results show that the synergy between *ab initio* calculations and refined experimental techniques can be exploited for an accurate and efficient NMR crystallography of complex systems of great interest for biomaterial studies. The method is general in nature and can be applied for studies of various complex biomaterials.



## ■ INTRODUCTION

NMR has proven to be a powerful tool in crystallography, and especially in the case of biological systems, NMR-assisted structure determination is gaining importance rapidly.<sup>1</sup> The potential of first-principles calculations of chemical shifts in structural determination have been emphasized in several outstanding studies of systems ranging from minerals<sup>2,3</sup> and glasses<sup>4,5</sup> to organic/inorganic interfaces,<sup>6</sup> peptides,<sup>7</sup> and molecular crystals.<sup>8</sup> The greatest merit of these calculations is their simplicity and accuracy, as they are not affected by signal broadenings or low receptivity of isotopes and can provide valuable information complementary to experimental methods. The first implementation of combined  $^{13}\text{C}$  chemical shift tensor calculations and NMR experiments<sup>9</sup> showed great success in structure determination and various studies<sup>10–14</sup> combining the two approaches have been published since.

However, the use of first-principles calculations in biologically significant systems have been limited due to challenges regarding the treatment of the dispersion forces, intrinsic errors of widely used methods, and high computational demand of these calculations. In this work, we address these issues with the necessary tools and approximations we have developed, on the highly challenging biological system of cholesterol crystals.

Cholesterol molecules play key roles in biological processes in human cells, mainly as a precursor in synthesis of hormones

and biologically significant molecules,<sup>15,16</sup> and as a regulator of structural, functional, and dynamical properties of lipid membranes in eukaryotic cells.<sup>17</sup> Cholesterol crystals are formed upon crystallization of cholesterol molecules with or without water molecules incorporated in the final structure. Cholesterol crystals too are of great interest in biomedical studies, as they play an important role in human diseases such as gallstone formation<sup>18</sup> and atherosclerosis.<sup>19</sup> A recent solid state-nuclear magnetic resonance (SS-NMR) study<sup>20</sup> reports that different pathologies of gallbladder result in cholesterol gallstones with different polymorphs having distinct NMR features, and particularly, an unknown phase with a previously not observed NMR spectrum can be associated to gallbladder cancer. This finding sheds light on the much debated topic<sup>21–24</sup> of the existence of different cholesterol polymorphs in gallstones. Furthermore, it also suggests that determining the specific polymorph associated to a pathology can reveal valuable information on the prognosis of the disease and its effects on gallbladder environment. Therefore a precise knowledge of NMR spectra of all known phases of cholesterol crystals, and insight on structure determination of yet unknown

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phases is essential for further advancement in this line of research.

Cholesterol crystals show a rich polymorphism with several molecules in the asymmetric unit cell ( $Z' > 1$ ). At room temperature, cholesterol has two stable crystal forms, cholesterol monohydrate (ChM)<sup>25</sup> and anhydrous cholesterol (ChAl),<sup>26,27</sup> both with  $Z' = 8$ . ChAl undergoes a phase transition at around 31.6 °C, a few degrees below body temperature, to the stable high temperature anhydrous form (ChAh),<sup>28,29</sup>  $Z' = 16$ . Because of the complex spectral features of these crystals, an unambiguous assignment of observed <sup>13</sup>C NMR chemical resonances to the distinct atoms by experimental means is not available. Moreover, these systems contain O(1000) atoms in the unit cell, and the nuances between spectral features belonging to each phase are as little as 2 ppm. Therefore even the *ab initio* determination of the chemical shifts of these crystals require state-of-the-art implementations.

In this article, we show that by combining cross-polarization magic angle spinning (CPMAS) NMR experiments with spectral editing and first-principles Gauge Including Projected Augmented Wave (GIPAW) calculations<sup>30,31</sup> with consistent PAW pseudopotentials and proper treatment of dispersion forces, a reliable peak assignment is achievable for the NMR spectra of cholesterol crystals.

## METHODS

**Experimental Methods.** The <sup>13</sup>C CPMAS experiments were performed for the three mentioned polymorphs of crystalline cholesterol. ChM and ChAl were recrystallized from cholesterol (>99% pure, purchased from Sigma Aldrich) using previously reported protocol,<sup>32</sup> whereas ChAh was obtained by heating ChAl at 50 °C. NMR spectra were recorded on Bruker Biospin Avance 600 MHz NMR spectrometer (Avance III, Bruker Biospin, Switzerland), operating at 600.15 MHz for <sup>1</sup>H frequency and 150.15 MHz for <sup>13</sup>C using Bruker 3.2 mm DVT probe. All spectra were recorded at 10.0 kHz Magic Angle Spinning (MAS) frequency. CPMAS experiments for ChM and ChAl were performed at 25 °C, whereas ChAh spectra were recorded at 50 °C. All <sup>13</sup>C CPMAS spectra were recorded with spectral width of 300 ppm, 2k data points, and 2k scans. For unambiguous assignment of the heavily overlapped region of <sup>13</sup>C spectra of cholesterol, spectral editing experiments<sup>33,34</sup> were also performed, which combine CPMAS experiments with depolarization and polarization inversion of <sup>13</sup>C spin.

**Theoretical Methods.** Our calculations have been performed in the framework of density functional theory (DFT) with codes in the Quantum Espresso (QE) distribution.<sup>35</sup> van der Waals (vdW) interactions were fully included using vdW-DF exchange-correlation functional by Dion et al.,<sup>36,37</sup> thus allowing both atomic positions and cell parameters to be relaxed starting from the information experimentally available via X-ray diffraction (XRD).<sup>25–28</sup> For all phases considered, the resulting unit cell volume deviate from the experimental one by less than +0.40% and the angular cell parameters show negligible difference. As reported earlier,<sup>25–28</sup> experimentally obtained bond lengths and angles vary within a large range of uncertainty due to the strong thermal motion and flexible nature of the aliphatic chain of the cholesterol molecules. The *ab initio* optimized bond lengths and angles, however, are more uniform within a molecule and throughout all the molecules of the cell and yet are well within

the experimental uncertainty. A detailed comparison between experimentally obtained and *ab initio* optimized geometries is given in Supporting Information.

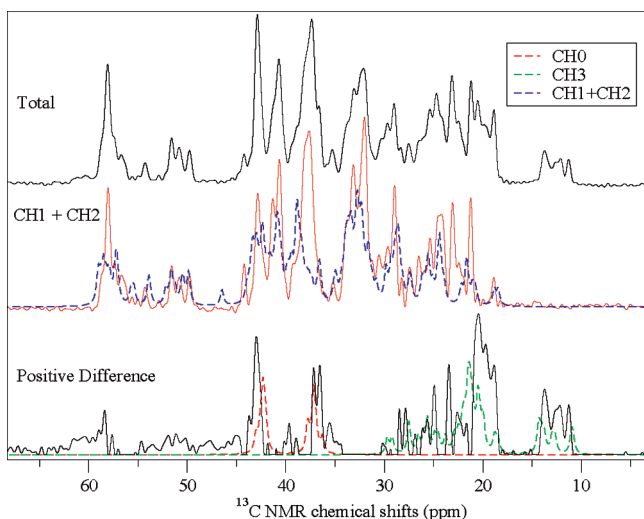
It should be stressed here that NMR chemical shifts are very sensitive to the underlying structure and that it is essential to use a full *ab initio* optimization for the cell and the geometry. For instance, in ChM phase, a pseudosymmetry inside the unit cell is well documented<sup>25</sup> such that molecules pair up forming two structurally unique groups (molecules A, B, G, and H as group I and molecules C, D, E, and F as group II following ref 25 notation). However, the uncertainty in the experimental atomic positions does not allow this pseudosymmetry to be clearly observed, and it can only be recovered by a full optimization process including vdW interactions, which then proves to have important consequences on the resulting calculated NMR spectra. NMR parameters are calculated using GIPAW method as implemented in QE, that we extended to PAW<sup>38</sup> formalism for increased efficiency and accuracy. *Ab initio* obtained isotropic chemical shieldings,  $\sigma_{\text{iso}} = \text{Tr}[\bar{\sigma}/3]$ , are compared to the experimental isotropic chemical shifts by using the standard expression:  $\delta_{\text{iso}} = \sigma_{\text{ref}} - \sigma_{\text{iso}}$ . In this work, we primarily take  $\sigma_{\text{ref}}$  as 167.5 ppm based on our results on gas phase cyclobutane.<sup>39</sup>

## RESULTS AND DISCUSSION

As previously reported,<sup>40</sup> GIPAW tends to over(under)-estimate the high(low)-ppm resonances with respect to the experiment. The error is, however, not a simple stretching of the spectrum, and this has hindered so far the interpretation of NMR spectra in complex materials. To remedy this error, an accepted practice in literature<sup>40</sup> is to use a different reference for each carbon atom, based on the experimental shift assigned to each carbon. This method, although it can be used to validate the experimental results, lacks in predictive power, as it requires a complete assignment of the peaks to the source atoms, which would not be feasible in complex spectra such as the one of cholesterol crystals.

We propose that the error in GIPAW calculations is environment dependent and we introduce a separate correction to our reference for each carbon group based on H-coordination: quaternary (CH0), single H (CH1), double H (CH2), and triple H (CH3) carbon groups are shifted with different references. We determine the correction values to be −4.0 ppm, −1.0 ppm, and +2.5 ppm based on the isolated peaks of C13, C9, and C18 in the ChM phase for CH0, CH1, and CH3 groups, respectively. As our original reference in cyclobutane is of CH2 type, we do not introduce any further correction for this group. For the isolated peaks of C5, C6, and C3 the corrections employed are −6.0 ppm, −3.1 ppm, and −5.8 ppm, respectively, still based on ChM phase. Note that all the carbon atoms in different groups based on H-coordination share the same hybridization,  $sp^3$ .

Using the synergy between spectral editing experiments and the reference correction based on H-coordination, we can test the validity of our approach. In Figure 1, we report the experimental and theoretical spectra for ChAl phase after the correction and find good agreement between the two. This agreement indicates that the referencing correction is phase independent, as the shift values were obtained from ChM phase but are valid for ChAl as well. Furthermore, the shift values were obtained using a small number of carbon shifts, but appear to apply to all carbons of the same group, pointing out the relevance of local environment groups. Therefore, environ-

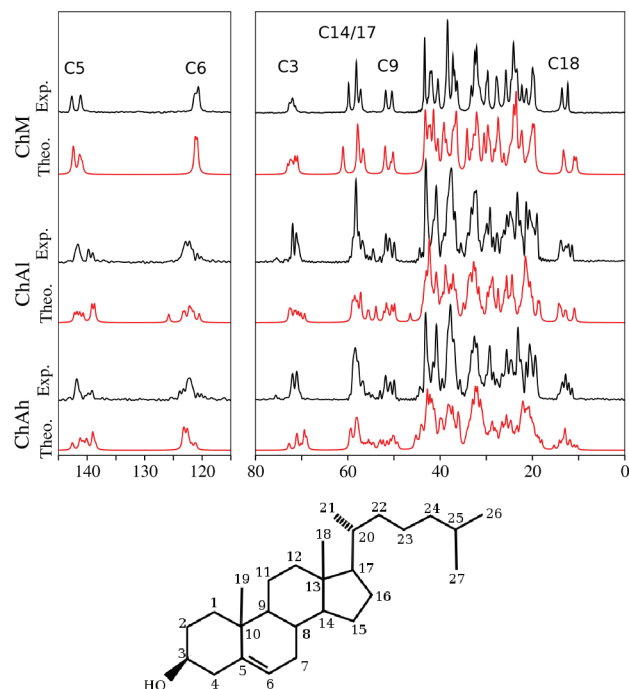


**Figure 1.** Results of NMR experiments (continuous lines) and theoretical calculations after reference correction is applied (dashed lines) for ChAl phase; given for the total (upper panel) and CH1+CH2-type edited (mid panel) spectra. Here, we also show the positive difference between the total and edited spectra (bottom panel), as it helps identify qualitatively the remaining CH0 and CH3 groups.

ment-dependent shifting can be used as a nonarbitrary tuning of theoretical results and can greatly increase the predictive power of GIPAW calculations, as only a small set of experimental information is necessary to yield chemical shifts that are highly compatible with the overall experimental spectrum. Throughout the rest of this article, theoretical spectra will be given with this referencing correction. The corresponding single reference results can be found in the Supporting Information.

In Figure 2, we report our theoretical and experimental results for the total NMR spectra of the three well-characterized cholesterol crystal structures. Our experimental results agree well with the previous experiments of ref 32. A number of isolated peaks can easily be assigned to specific atoms in cholesterol molecule. In ChM phase, double peaks of C5, C6, C9, C18 atoms, which have been attributed to the pseudosymmetrical nature of this structure, are well represented. Furthermore, the broad C3 peak in ref 32 is found here to be composed of three peaks with different intensity. The majority of carbon atoms, however, resonates in the region between 10 and 50 ppm and forms a complex line shape that prevents peak assignment solely based on experiments.

As can be seen in Figure 2, our calculations reproduce the experimental spectra satisfactorily. The similar yet distinct spectrum of each phase can be clearly distinguished, and the trends between phases obtained by our calculations follow closely the experimental ones: ChM phase is found to have highly symmetric double peaks for the majority of atoms including the ones in the 10–50 ppm region. From ChM to ChAh, the increase in number of inequivalent molecules can be observed in the increase of number of peaks for a given carbon. Considering also the previous experimental results of ref 32, we deduce that C18 has the most clear signal, which can be used as a fingerprint in distinguishing the phases. In addition to capturing the nuances between phases, we also successfully reproduce the experimental main features of the peak shape and positions for the isolated peaks assigned to C5, C6, C3, C14/



**Figure 2.** Experimental  $^{13}\text{C}$  CP-MAS NMR and theoretical GIPAW spectra for the three crystal phases of cholesterol. Theoretical carbon group references are corrected according to H-coordination (see text). The standard numbering of carbon atoms is also given.

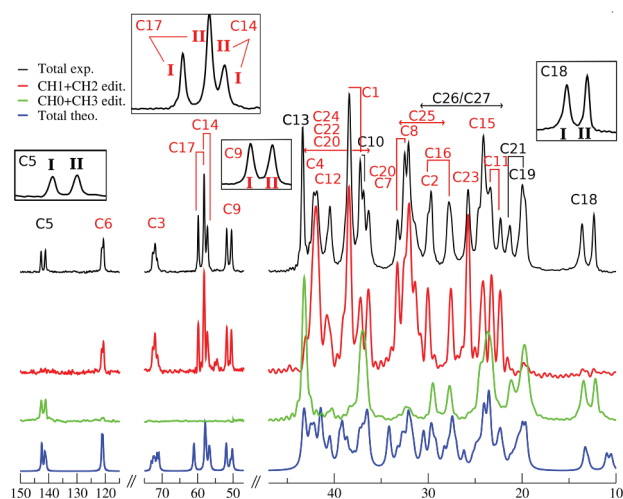
17, C9, and C18 individually for each phase. A close-up comparison for these features is given in the Supporting Information. For the highly symmetric ChM phase, the similarities between experiment and theory can be noticed even for the populated 10–50 ppm region.

We stress again the importance of a proper relaxation, consistently taking into account dispersion forces, to obtain these results. In fact, if the experimental positions for non-hydrogen atoms were directly used as input in the GIPAW calculation, the resulting chemical shifts associated to the isolated peaks would be scattered on a wide range up to 20 ppm and merge together with qualitatively wrong line-shapes.

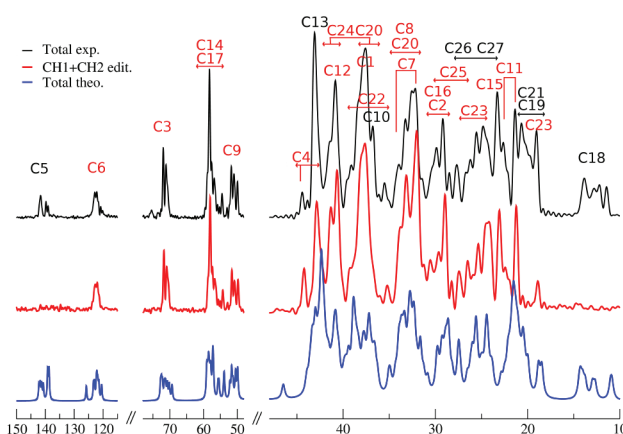
Although the agreement between theory and experiment is remarkable, for some carbons the discrepancies are not negligible. One reason behind this is that the error-bar of our calculations (0.5 ppm) can significantly modify the details of complex lineshapes such as the one of C5. A second contribution to differences between experimental and theoretical results comes from finite temperature effects as experiments are performed at room temperature where dynamical narrowing of peaks occurs. For a thorough understanding of this currently debated topic,<sup>41</sup> extensive NMR calculations should be carried out at room temperature. However, from our results, we can conclude that, in the case of cholesterol crystals, 0 K vdW-aware DFT calculations are precise enough to distinguish different phases solely based on their *ab initio* spectra, and we leave this important issue for further studies.

With the confidence gained from this display of high-precision and accuracy, we use the calculated peak positions and shapes in combination with the edited spectra to make a detailed peak assignment for ChM and ChAl phases. The resulting peak assignments are given in Figures 3 (ChM) and 4 (ChAl). In the symmetric ChM phase, the assignment could be performed with high confidence due to the clarity of the





**Figure 3.** Peak assignment for ChM phase. Sharp single peaks, double/triple peaks, and scattered peaks are indicated separately. The results of the CH1+CH2-type spectral editing is also given. Carbon labels are color coded (red for CH1–CH2, black otherwise) to easily validate the quality of the peak assignment performed. In separate close-up panels, the isolated doublets are assigned to the individual pseudosymmetric molecular groups:<sup>25</sup> group I (molecules A, B, G, and H) and group II (molecules C, D, E, and F).



**Figure 4.** Peak assignment for ChAl phase. The multiplicity of the peaks and H-coordination of carbons are indicated as before (see caption of Figure 3).

spectrum originating from the pseudosymmetry among the molecules in this phase.<sup>25</sup> For isolated doublets, further assignment of the peaks to the pseudosymmetric molecular groups is possible and is also given in Figure 3. We note that the sharp peaks in this spectrum can be assigned to the corresponding carbon atoms straightforwardly, whereas the carbons in the flexible tail region, such as the C26/C27 pair, show scattered peaks both theoretically and experimentally and are assigned to a range in the spectrum. We confirm our peak assignment by making use of spectral editing experiments and see that it holds even for very fine scales, e.g., the peak assigned to C10 (group CH0) disappears in the CH1+CH2 edited experiment, as expected.

For the ChAl phase, the multiplicity of the peaks increases; therefore, spectral editing experiments become necessary to make peak assignments with higher confidence. Following the lack of highly obeyed pseudosymmetry in this phase, resonance peaks show a distribution of values rather than doublets;

therefore, they are mostly assigned to the source carbons in terms of ranges. It can be seen experimentally that the high temperature ChAl phase spectrum is very similar to the one of low temperature phase ChAl, implying similarities also in peak assignment. However, because of the complexity of the ChAl crystal, higher precision spectral editing experiments would be needed for a more detailed peak assignment.

## CONCLUSIONS

In summary, we demonstrated that state-of-the-art first-principles structural determinations and GIPAW calculations are precise, accurate, and efficient enough to well reproduce the experimental NMR spectrum of cholesterol crystals, the largest systems that have been investigated with ab initio NMR methods so far. We also showed that the good agreement between experiment and theory can be further improved with the introduced local-environment based referencing correction. This improved agreement in combination with spectral editing experiments allowed us to perform peak assignment for ChM and ChAl phases, indicating that ab initio calculations hold a promising potential for unambiguous peak assignment even for large systems with complex spectral features.

## ASSOCIATED CONTENT

### Supporting Information

Details of spectral editing experiments and resulting spectra, details of structural relaxation, resulting spectra with and without relaxation, spectrum with different exchange-correlation functionals, theoretical chemical shifts with single reference, and notes on peak assignment. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

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