Open Archive TOULOUSE Archive Ouverte (OATAO)
OATAO is an open access repository that collects the work of Toulouse researchers and makes it freely available over the web where possible.

This is an author-deposited version published in: http://oatao.univ-toulouse.fr/
Eprints ID: 18205

To link to this article: DOI: 10.1016/j.actbio.2015.10.016
URL: https://doi.org/10.1016/j.actbio.2015.10.016

To cite this version: Gras, Pierre and Baker, Annabelle and Combes, Christèle and Rey, Christian and Sarda, Stéphanie and Wright, Adrian J. and Smith, Mark E. and Hanna, John V. and Gervais, Christel and Laurencin, Danielle and Bonhomme, Christian

Any correspondence concerning this service should be sent to the repository administrator: staff-oatao@listes-diff.inp-toulouse.fr
From crystalline to amorphous calcium pyrophosphates: A solid state Nuclear Magnetic Resonance perspective

Pierre Gras a, Annabelle Baker b, Christèle Combes a, Christian Rey a, Stéphanie Sarda c, Adrian J. Wright b, Mark E. Smith d,e, John V. Hanna e, Christel Gervais f, Danielle Laurencin f, Christian Bonhomme f,⇑

⇑Corresponding author.
E-mail address: christian.bonhomme@upmc.fr (C. Bonhomme).

A B S T R A C T

Hydrated calcium pyrophosphates (CPP, Ca₂P₂O₇·nH₂O) are a fundamental family of materials among osteoarticular pathologic calcifications. In this contribution, a comprehensive multinuclear NMR (Nuclear Magnetic Resonance) study of four crystalline and two amorphous phases of this family is presented. ¹H, ³¹P and ⁴³Ca MAS (Magic Angle Spinning) NMR spectra were recorded, leading to informative fingerprints characterizing each compound. In particular, different ¹H and ⁴³Ca solid state NMR signatures were observed for the amorphous phases, depending on the synthetic procedure used. The NMR parameters of the crystalline phases were determined using the GIPAW (Gauge Including Projected Augmented Wave) DFT approach, based on first-principles calculations. In some cases, relaxed structures were found to improve the agreement between experimental and calculated values, demonstrating the importance of proton positions and pyrophosphate local geometry in this particular NMR crystallography approach. Such calculations serve as a basis for the future ab initio modeling of the amorphous CPP phases.

Statement of significance

The general concept of NMR crystallography is applied to the detailed study of calcium pyrophosphates (CPP), whether hydrated or not, and whether crystalline or amorphous. CPP are a fundamental family of materials among osteoarticular pathologic calcifications. Their prevalence increases with age, impacting on 17.5% of the population after the age of 80 [1]. Although often asymptomatic, they are frequently involved or associated with acute articular arthritis such as pseudogout, and, more rarely, with chronic polyarthritis and destructive arthropathy; current treatments are mainly directed at relieving the symptoms of joint inflammation but not at inhibiting CPP formation nor at dissolving these crystals [2–4]. CPP have been identified in vivo as two polymorphs of CPPD [5]: a triclinic form with a known structure [6], and a monoclinic form with a recently solved structure [7] (respectively denoted as t-CPPD and m-CPPD). Other crystalline forms of hydrated calcium pyrophosphates have been synthesized in vitro and characterized.

1. Introduction

Crystalline calcium pyrophosphate dihydrates (CPPD, Ca₂P₂O₇·2H₂O) are among the most common forms of pathologic articular minerals: their prevalence increases with age, impacting on 17.5% of the population after the age of 80 [1]. Although often asymptomatic, they are frequently involved or associated with acute articular arthritis such as pseudogout, and, more rarely, with chronic polyarthritis and destructive arthropathy; current treatments are mainly directed at relieving the symptoms of joint inflammation but not at inhibiting calcium pyrophosphate (CPP) formation nor at dissolving these crystals [2–4].

CPP have been identified in vivo as two polymorphs of CPPD [5]: a triclinic form with a known structure [6], and a monoclinic form with a recently solved structure [7] (respectively denoted as t-CPPD and m-CPPD). Other crystalline forms of hydrated calcium pyrophosphates have been synthesized in vitro and characterized.
including a dimorphic monoclinic tetrahydrate (CPPT: Ca$_2$P$_2$O$_7$·4H$_2$O, referred to as m-CPPT α and m-CPPT β [8,9]). Recently, Gras et al. [7] performed a systematic investigation of the synthesis of pure hydrated calcium pyrophosphates. They described the pH and temperature conditions leading to the formation of m-CPPT β, t-CPPD and m-CPPD [10], as well as the identification of a new monohydrated calcium pyrophosphate phase exhibiting monoclinic symmetry, referred to as m-CPPM (CPPM: Ca$_2$P$_2$O$_7$·H$_2$O) [11], and an unreported highly metastable trihydrated monoclinic calcium pyrophosphate phase derived from the structure of m-CPPT β [12]. The preparation of amorphous phases of biological interest, noted a-CP (Ca$_2$P$_2$O$_7$·nH$_2$O), has also been reported by Slater et al. [13] and Gras [10], and it was found that these phases are particularly stable compared to amorphous calcium orthophosphate and amorphous calcium carbonate.

Several characteristics have been performed on all the hydrated calcium pyrophosphate phases mentioned above by several approaches, including powder XRD (X Ray diffraction) and vibrational spectroscopies, providing information on the configuration of the pyrophosphate groups [10]. It was observed that pyrophosphate ions in CPP phases have a wide range of P–O–P angles (between 123.1° and 134.1°) [8,9]. This angle is important in understanding the relationship between the various CPP forms and their stability and transformation ability. Developing complementary tools for the characterization of hydrated calcium pyrophosphates is of particular interest, especially to understand the structure of phases like m-CPPT, for which the positioning of protons may be very difficult based exclusively on X-ray powder diffraction data, particularly considering that single crystals suitable for diffraction structure resolution are not yet available. Indeed, this phase has the highest inflammatory potential of all CPP phases, and it would be of interest to determine its structure in detail in order to understand the inflammation mechanism, which is possibly based on rupture of lysosome phospholipid membranes induced by pyrophosphate groups on the surface of the crystals [14–17].

Solid state NMR is a technique which is attracting increasing attention for the study of synthetic and natural biomaterials [18] including calcium phosphate phases [19–27]. Indeed, solid state NMR can provide detailed atomic-scale information on the local structure around nuclei like $^{31}$P, even in disordered and amorphous phases, and is therefore highly complementary to other analytical tools like XRD and IR (Infra Red) or Raman spectroscopies. NMR studies of calcium pyrophosphate phases have been very limited to date. To the best of our knowledge, $^{31}$P NMR has only been applied to the characterization of the crystalline α- and β-Ca$_2$P$_2$O$_7$ anhydrous phases, and of a hydrated amorphous calcium pyrophosphate of composition $\sim$Ca$_2$P$_2$O$_7$·4H$_2$O [13,28]. In the latter case, the hydrosol of the P–O–P bridge upon heat treatment was demonstrated using $^1$H MAS. $^{31}$P MAS and $^1$H–$^{31}$P cross-polarization (CP) MAS NMR experiments. With regards to $^{42}$Ca NMR, only the anhydrous α-Ca$_2$P$_2$O$_7$ phase has been analyzed to date [29], showing that the two crystallographically-in-equivalent Ca sites can be unambiguously resolved at 14.1 T. Although $^{42}$Ca is a more challenging nucleus than $^{31}$P [30,31] given its quadrupolar nature [32], low natural abundance (0.14%) and small magnetic moment (leading it to be a member of the group of so-called low-$\gamma$ nuclei) [33], recent studies have shown that it can be very sensitive to subtle changes in Ca local environments [34–36]. Finally, even more challenging isotopes like oxygen-17 which usually require isotopic enrichment have been completely neglected so far.

The purpose of this study is to demonstrate, using a combined experimental–computational approach, how solid state NMR can be used for the structural investigation of calcium pyrophosphate phases, whether hydrated or anhydrous, and whether crystalline or amorphous. For this purpose, the $^{31}$P, $^{42}$Ca and $^1$H MAS NMR spectra of a series of crystalline CPP phases (m-CPPD, t-CPPD, m-CPPT β and m-CPPM) are first reported, followed by those of amorphous calcium pyrophosphates. Then, we report the results of first-principles calculations of the NMR parameters of the crystalline calcium pyrophosphate phases, which were carried out using the Gauge-Including Projector Augmented Wave (GIPAW) approach [37,38]. The comparison between experimental and calculated NMR parameters not only validates the structural models of each compound allowing the assignment of P and Ca sites in crystalline phases, but also helps determine what atomic-scale information can be determined by solid state NMR. Interpretations of the NMR spectra of amorphous calcium pyrophosphate are given, a phase that has recently been proposed as an interesting component of bone cements [39].

2. Materials and methods

2.1. Syntheses

Crystalline hydrated calcium pyrophosphates m-CPPD, t-CPPD and m-CPPT β were synthesized following the methods previously published by Gras [10], by double decomposition between a potassium pyrophosphate solution and a calcium nitrate solution mixed into a buffer solution at a controlled temperature. The crystalline m-CPPM phase was prepared starting from m-CPPT β crystals and heating them at 110 °C for 30 min as previously reported by Gras et al. [12].

The amorphous calcium pyrophosphate phases, of general formula Ca$_2$P$_2$O$_7$·xH$_2$O (with $x \sim 4$) [10,13], were prepared using two different synthetic procedures. According to Gras [10], precipitation at a controlled temperature (25 °C) and pH (5.8) was used (compound referred to as “sample A” thereafter). According to Slater et al. [13] a precipitation at room temperature without any specific control/monitoring of the pH was also performed (compound referred to as “sample B” thereafter). In the latter case, the amorphous phase was also heat treated to 140 and 220 °C in view of further solid state NMR characterizations of the transformations under temperature.

2.2. Characterization

2.2.1. General characterization

XRD measurements were performed using a Seifert XRD-3000TT diffractometer with a Cu Kα radiation (Cu Kα, λ = 1.54060 Å and Cu Kβ, λ = 1.54443 Å), and equipped with a graphite monochromator. The XRD patterns were obtained between 2θ = 5 and 70 ° (2θ) with a step size of 0.02° and a scan step time of 16 s at 298 K. The corresponding XRD powder patterns can be found in supporting information (Figure S1). The other characterization performed on the crystalline and amorphous synthesized phases, using notably vibrational spectroscopies, can be found in previous publications [10,13].

2.2.2. $^{31}$P solid state NMR

All $^{31}$P MAS NMR data were recorded at 14.1 T using a VNMRS-600 (600 MHz $^1$H frequency) spectrometer operating at a $^{31}$P Larmor frequency of 242.81 MHz, using a Varian 3.2 mm HXY T3 MAS probe. MAS frequencies ranging from 2.8 to 10 kHz were used in order to extract the chemical shift anisotropy (CSA) parameters. Experiments were performed using a set of saturation $^{31}$P pulses, followed by a delay of 128 s, and a 90° excitation pulse of 2.5 μs. Spinal-64 $^1$H decoupling (100 kHz RF) was applied during acquisition [40]. A total of 4 transients were acquired for each spectrum. The $^{31}$P chemical shifts were referenced to Si$_4$O$_2$(PO$_4$)$_3$ as a
secondary reference (at –44.0 ppm with respect to an 85% H₃PO₄ solution) [41]. Temperature regulation was used during the experiments, to ensure that the temperature inside the rotor was ~10°C. Prior to all experiments, the magic angle was carefully set in order to obtain the best 31P MAS resolution, avoiding the reintroduction of any CSA or dipolar interaction which would broaden the spectra.

2.2.3. 43Ca solid state NMR

Natural abundance 43Ca MAS NMR data were acquired at 14.1 and 20.0 T using Varian V NMR-600 (600 MHz 1H frequency) and Bruker Avance III-850 (850 MHz 1H frequency) spectrometers operating at 43Ca Larmor frequencies of 40.37 and 57.22 MHz, respectively. All 43Ca chemical shifts were referenced to 0 ppm to a 1 mol L⁻¹ aqueous solution of CaCl₂ [29,30].

The 14.1 T experiments were performed using a Varian 7.5 mm HXY MAS probe enabling MAS frequencies of 5–6 kHz, and the temperature scheme was regulated at ~12°C throughout each measurement. The Double Frequency Sweep (DFS) [42,43] signal-enhancement scheme was applied for sensitivity enhancement prior to a 1.7 ms 90° solid pulse for the central transition. DFS parameters were optimized using 43Ca-enriched CaHPO₄, with a convergence sweep from 300 to 70 kHz (duration ~5.5 ms; RF ~8 kHz), leading to an enhancement factor of ~2. Only m-CPPPT β was recorded using a 9.5 mm Varian HX (for reasons unrelated to the nature of the sample); the spinning rate was set to 4 kHz, and the acquisition parameters for the DFS and excitation pulses were optimized in a similar fashion as described above.

At 850 MHz, a low-γ 7 mm Bruker MAS probe was used, spinning at 5 kHz. A RAPT (rotor-assisted population transfer) [44] enhancement scheme was applied prior to a 1.7 ms 90° solid pulse selective for the central transition. The RAPT pulses were first optimized on 43Ca-enriched CaHPO₄ with an offset of 150 kHz (RF ~ 9 kHz), leading to an enhancement factor of ~2. Details of the recycle delays, number of transients acquired, and total experimental times needed for each sample at both fields can be found in Table S1 (in supplementary information).

2.2.4. 1H solid state NMR

1H NMR spectra were recorded on a Varian VNMRS 600 MHz (14.1 T) NMR spectrometer at frequency of 599.82 MHz, using a 3.2 mm Varian T3 HXY MAS probe. Windowed-DUMBO (Decoupling Using Mind-Boggling Optimization) [45–47] 1H MAS experiments were carried out at a spinning speed of 10 kHz. A glycine sample was used for the optimization of the DUMBO experiment. The radio-frequency (RF) field strength was 100 kHz, the duration of one DUMBO element 34.4 μs, and the observation window 0.8 μs. 20 transients were acquired, with recycle delays ranging from 4 to 16 s (depending on the sample). The 1H chemical shifts were referenced externally to adamantane, used as a secondary reference (at 1.8 ppm with respect to tetramethylsilane, TMS). Temperature regulation was used during the experiments, to ensure that the temperature inside the rotor was ~10°C.

2.3. Calculations of NMR parameters

The first principles calculations based on the GIPAW [48] method were performed within Kohn–Sham DFT (Density Functional Theory) using the QUANTUM ESPRESSO code [49]. The crystalline structure is described as an infinite periodic system using periodic boundary conditions. The NMR calculations were performed as follows: for hydrated phases, proton positions geometry optimization was carried out, starting from the published experimental structures of t-CPPD [50], m-CPPPT β [51] and m-CPPPT [7] allowing the positions of protons to relax using the VASP (Vienna ab initio Simulation Package) code [52]. In the case of m-CPPPM [12], all atomic positions were relaxed to obtain a better agreement with the experimental data (see later in the text). The 1H-relaxed structures are named “Rel H” hereafter, and the fully relaxed structure of m-CPPPM is referred to as “Rel tot”. The α-Ca₂P₂O₇ [53] structure was calculated without further relaxation. For NMR calculations, the PBE generalized gradient approximation [54] was used and the valence electrons were described by norm conserving pseudopotentials [55] in the Kleinman–Bylander form [56].

The wave functions were expanded on a plane wave basis set with a kinetic energy cut-off of 80 Ry. The integral over the first Brillouin zone was performed using a Monkhorst–Pack 2 × 2 × 2 k-point grid. The principal components Vₓₓ, Vᵧᵧ, and V₂₂ of the electric field gradient (EFG) tensor defined with |Vₓₓ| ≥ |Vᵧᵧ| ≥ |V₂₂| were obtained by diagonalisation of the tensor. The quadrupolar interaction was then characterized by the quadrupolar coupling constant CQ and the asymmetry parameter ηQ, which are defined as

\[ C_Q = \epsilon Q V_{zx}/h \] and \[ \eta_Q = (V_{yy} - V_{xx})/2V_{zz}. \]

Absolute shielding tensors were obtained. To fix the 43Ca scale, the calculated δiso (isotropic chemical shift) for a series of reference compounds were compared to experimental values [29] so that the average sum of experimental and calculated shifts coincide. Moreover, it should be noted that for the C2 (43Ca) calculation, an updated quadrupole moment of 44.4 mb was used, as recently recommended by Bryce et al. [57] In the case of 31P and 1H, external referencing with respect to crystalline berlinite [58] (δiso = 24.5 ppm) [59] for 31P and α-glycine for 1H [60] was chosen. Diagonalization of the symmetrical part of the calculated chemical shift tensor provides its principal components δ₁₁, δ₂₂, δ₃₃ from which the chemical shift components δ₁₁, δ₂₂, δ₃₃ can be calculated, δ₁₁, δ₂₂ and δ₃₃ are defined such as |δ₁₁ - δ₃₃| ≥ |δ₁₁ - δ₃₃| ≥ |δ₁₁ - δ₃₃|, and δiso = 1/3(δ₁₁ + δ₂₂ + δ₃₃). The CSA parameters are defined by δCSA = δ₃₃ - δiso (anisotropy) and ηCSA = (δ₁₁ - δ₁₁)/δCSA (asymmetry).

3. Results and discussion

3.1. 31P MAS NMR

31P MAS NMR spectroscopy is the most used NMR approach for the study of calcium phosphate and pyrophosphate phases, because phosphorus-31 is a highly sensitive NMR isotope (100% natural abundance, I = ½, high receptivity). In the particular case of hydrated calcium pyrophosphate phases, four NMR interactions have to be considered, namely the CSA, the heteronuclear 31P–1H and homonuclear 31P–31P dipolar interactions, and the 3JHP coupling. Under fast MAS and 1H decoupling, the corresponding anistropies are averaged efficiently leading to the determination of δiso (31P). Usually, the 3JHP couplings are not detected directly, although they can be revealed and measured using 2D (two dimensional) MAS experiments [26,61]. The number of isotropic lines is thus directly related to the number of inequivalent P sites in the asymmetric unit of a given structure.

31P MAS NMR spectra were recorded for m-CPPPT β, m-CPPPT, t-CPPPT and m-CPPPM and are shown in Fig. 1 (fast and slow MAS NMR spectra). At slow spinning frequency, numerous spinning sidebands are observed: their simulation by a CSA model [62] leads to the determination of the CSA parameters δCSA and ηCSA (as defined in the Materials and Methods section). Here, it is explicitly assumed that residual 31P–31P homonuclear dipolar couplings can be safely ignored in the simulations as they are much smaller (in Hz) than CSA effects. All simulations of the 31P MAS NMR spectra are presented in Figure S2. As expected from the crystal structures, two distinct 31P resonances are observed for each compound. For all spectra, the relative intensity of the two resonances differs from the expected 1:1 ratio, because the measurements were performed only for the purpose of determining the 31P NMR parameters δiso.
Concerning in agreement with data already published in the literature \[13,28\]. The case of \(\text{m-CPPD, m-CPPT}\) can act as constraints for the further refinement of a crystallo-
dense structure. In the case of \(\text{m-CPPD, m-CPPT}\), which means that the NMR data
responding structural data (obtained mainly by means of X-ray
determination). If not stated specifically, the exact rotation frequency, \(\gamma_s\), is specified in Figure S2 for each sample. For each spectrum,
the arrow indicates the region of the isotropic resonances (two in general, except in the case of \(\text{a-CPP}\)). All other lines correspond to spinning sidebands from which CSA parameters can be extracted. In the case of \(\text{a-CPP}\), the minor component at \(\delta_{31p} \sim 0\) ppm is assigned to orthophosphate species (see main text).

\(\delta_{\text{CSA}}\) and \(\eta_{\text{CSA}}\), and thus in conditions which do not necessarily ensure full relaxation of the different \(\text{P}\) resonances. The observed
range for \(\delta_{\text{iso}} (\text{P})\) is \(-12\) to \(-5\) ppm (average value \(-\text{7 ppm})
, in agreement with data already published in the literature \[13,28\]. Concerning \(\delta_{\text{CSA}}\), the observed range is \(-59\) to \(-86\) ppm, and the average
genesis for all sites is \(-77\) ppm. The average values of \(\delta_{\text{iso}} (\text{P})\) and \(\delta_{\text{CSA}}\) are fully compatible with the data obtained for \(\text{a-CPP}\). In
the case of \(\text{a-CPP}\) (Fig. 1), a minor resonance centered at \(-0\) ppm is
observed. As suggested by Slater et al. \[13\], such a contribution can be safely assigned to \(\text{PO}_4^{3-}\) or \(\text{HPO}_4^{2-}\) moieties, caused by the
presence of traces of orthophosphates in the precursors used to prepare CPP phases, and/or resulting from the partial hydrolysis
of pyrophosphates under ageing \[10,13\]. As a matter of fact, the relative intensity of this particular resonance was found to increase
with time (see supporting information, Figure S3).

Studying calcium pyrophosphates represents a challenge in
terms of first principles calculations of \(\text{P}\) chemical shifts, as the
experimental resonances are closely separated (\(-1\) ppm in the case of \(\text{t-CPPD}\), see Table 1). The accuracy of the GIPAW method applied
to \(\text{P}\) has been reviewed previously \[63\]. Generally, the precision
of such calculations depends explicitly on the accuracy of the corresponding structural data (obtained mainly by means of X-ray
rotation or neutron diffraction) \[38\], which means that the NMR data
can act as constraints for the further refinement of a crystallo-
graphic structure. In the case of \(\alpha-\text{Ca}_3\text{P}_2\text{O}_7\), t-CPPD and \(\text{m-CPPD}\)
and \(\text{m-CPPT}\), good agreement is obtained between the experimental and calculated values both in terms of \(\delta_{\text{iso}}\) and \(\delta_{\text{CSA}}\). We notice that the calculated isotropic value for \(\text{P1}\) in \(\text{m-CPPD}\) is underestimated. Nevertheless, \(\text{P1}\) and \(\text{P2}\) can unequivocally be assigned.

Table 1: Experimental and calculated \(\text{P}\) chemical shift tensor (CSA) data for \(\text{m-CPPD, m-CPPM, t-CPPD, m-CPPT}\). The definitions of \(\delta_{\text{CSA}}\) and \(\eta_{\text{CSA}}\) are given in the
experimental section. In the case of \(\text{m-CPPD}\), results obtained before and after
relaxation of the proton positions (“Rel H”) are presented. In order to validate the
combined experimental/computational approach, both sets of data were added for
\(\alpha-\text{Ca}_3\text{P}_2\text{O}_7\) (anhydrous phase). The maximum error on experimental \(\delta_{\text{iso}}\) values was estimated to 0.15 ppm.

<table>
<thead>
<tr>
<th>(\alpha-\text{Ca}_3\text{P}_2\text{O}_7)</th>
<th>(\text{P1})</th>
<th>-7.70</th>
<th>-9.38</th>
<th>59.4</th>
<th>65.58</th>
<th>0.62</th>
<th>0.53</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{m-CPPD})</td>
<td>(\text{P1})</td>
<td>-9.70</td>
<td>-8.66</td>
<td>82.6</td>
<td>59.55</td>
<td>0.34</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td>(\text{P2})</td>
<td>-5.90</td>
<td>-6.44</td>
<td>72.5</td>
<td>97.70</td>
<td>0.41</td>
<td>0.24</td>
</tr>
<tr>
<td>(\text{m-CPPM})</td>
<td>(\text{P1})</td>
<td>-5.90</td>
<td>-7.42</td>
<td>72.5</td>
<td>61.59</td>
<td>0.41</td>
<td>0.60</td>
</tr>
<tr>
<td>(\text{Rel H})</td>
<td>(\text{P2})</td>
<td>-9.70</td>
<td>-7.80</td>
<td>82.6</td>
<td>96.17</td>
<td>0.34</td>
<td>0.18</td>
</tr>
<tr>
<td>(\text{t-CPPD})</td>
<td>(\text{P1})</td>
<td>-5.94</td>
<td>-5.22</td>
<td>76.6</td>
<td>79.95</td>
<td>0.56</td>
<td>0.57</td>
</tr>
<tr>
<td>(\text{Rel H})</td>
<td>(\text{P2})</td>
<td>-4.97</td>
<td>-4.58</td>
<td>75.6</td>
<td>79.28</td>
<td>0.27</td>
<td>0.26</td>
</tr>
<tr>
<td>(\text{m-CPPT})</td>
<td>(\text{P1})</td>
<td>-9.31</td>
<td>-15.80</td>
<td>84.5</td>
<td>85.16</td>
<td>0.21</td>
<td>0.11</td>
</tr>
<tr>
<td>(\text{Rel H})</td>
<td>(\text{P2})</td>
<td>-7.33</td>
<td>-6.90</td>
<td>71.6</td>
<td>78.50</td>
<td>0.41</td>
<td>0.54</td>
</tr>
<tr>
<td>(\text{m-CPPM})</td>
<td>(\text{P1})</td>
<td>-11.27</td>
<td>-3.4</td>
<td>64.0</td>
<td>68.78</td>
<td>0.80</td>
<td>0.70</td>
</tr>
<tr>
<td>(\text{P2})</td>
<td>-7.34</td>
<td>0.2</td>
<td>85.7</td>
<td>93.66</td>
<td>0.48</td>
<td>0.56</td>
<td></td>
</tr>
<tr>
<td>(\alpha-\text{CPP})</td>
<td>(\text{P})</td>
<td>-6.7</td>
<td>78.2</td>
<td>0.80</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The case of \(\text{m-CPPD}\) is an interesting one as it demonstrates the
importance of proton relaxation. Indeed, starting from the published structure \[10\], the assignment of \(\text{P1}\) and \(\text{P2}\) is not straightforward, because when assigning the sites based on the relative
values of \(\delta_{\text{iso}}\), there is a contradiction with the relative order of the \(\delta_{\text{CSA}}\) values (and vice versa). In contrast, starting from the
\(\text{m-CPPD}\) relaxed structure (Rel H in Table 1, see Section 2.3), the

---

**Fig. 1.** \(\text{P}\) MAS NMR spectra (decoupled from \(\text{H}\) during the acquisition time) of \(\text{t-CPPD, m-CPPD, m-CPPT}\) \(\gamma_s\), \(\text{m-CPPM}\) and \(\alpha-\text{CPP}\) (sample A) \((14.1 \text{ T}, 242.81 \text{ MHz}, \text{spinal} 64 \text{ }\text{H}
decking, relaxation delay: 128 s, number of scans: 4, regulation of the temperature: 10 \degree\text{C}, saturation pulses on the \(\text{P}\) channel before the first 90\degree\ pulse). MAS rotation
frequency: 10.0 kHz (left) and 2.8 to 5.0 kHz (right). For the slow MAS spectra, the exact rotation frequency, \(\gamma_s\), is specified in Figure S2 for each sample. For each spectrum,
the arrow indicates the region of the isotropic resonances (two in general, except in the case of \(\alpha-\text{CPP}\)). All other lines correspond to spinning sidebands from which CSA parameters can be extracted. In the case of \(\alpha-\text{CPP}\), the minor component at \(\delta_{31p} \sim 0\) ppm is assigned to orthophosphate species (see main text).
relative orders of \( \delta_{\text{iso}} \) (\(^{31}\)P) and \( \delta_{\text{CSA}} \) are correctly described by the GIPAW calculations. This point emphasizes the crucial importance of the H positions in the starting structure, in agreement with recent studies in the literature \([38,64]\). Moreover, we note that for m-CPPD, both P sites have similar proximities to protons (\(\sim 2.7–2.8\) Å), meaning that cross-polarization experiments cannot be used to distinguish them based on differences of CP dipolar dynamics. Finally, in the case of m-CPPM, even after relaxation of all atomic positions, the agreement with the experimental values remains poor but nonetheless allows a relatively safe assignment of the P1 and P2 sites since the relative order of the \( \delta_{\text{iso}} \) (\(^{31}\)P) and \( \delta_{\text{CSA}} \) values is consistent.

The geometric parameters of pyrophosphate ions (especially the P–O–P angle) have been used to explain the spectroscopic parameters on the dynamics. Several tests were performed to evaluate the influence of these parameters on the relative order of the P1 and P2 sites since the relative order of the \( \delta_{\text{iso}} \) (\(^{31}\)P) and \( \delta_{\text{CSA}} \) values is consistent.

Fig. 2. Configuration of the pyrophosphate anions in the crystalline CPP phases studied (as shown along two different viewing directions). O and P atoms are in red and gray, respectively.
range of all resonances observed for the crystalline CPP phases, and is more specifically centered around the resonances of m-CPPT, in agreement with elemental analyses which suggests the presence of an average of 4 water molecules [13]. Nonetheless, at this stage, no chemical shift and/or quadrupolar distribution could be extracted for a-CPP.

High field $^{43}$Ca NMR was actually found to be a highly relevant tool of analysis of other related a-CPP phases. Indeed, depending on the synthetic protocol (control or not of the pH during the precipitation of a-CPP), $^{43}$Ca MAS NMR spectra presented subtle differences (see Figure S6 for the comparison of samples A and B), showing that the structures of these materials actually slightly differ, despite the similarities in the $^{31}$P MAS NMR data (see Figure S4).

Table 2
Experimental and calculated $^{43}$Ca chemical shift and quadrupolar parameters for $\alpha$-Ca$_2$P$_2$O$_{7}$, m-CPPD, t-CPPD, m-CPPT $\beta$ and m-CPPM. The definitions of $C_Q$ and $\eta_Q$ are given in the experimental section. In the case of m-CPPD, results obtained before and after relaxation of the proton positions (Rel H) are presented. The spectra from which the experimental values were determined are shown in Figure S5, together with their simulation. Errors were estimated to $\pm 2$–3 ppm on $\delta_{iso}$ and $\pm 0.2$–0.4 MHz on $C_Q$, except for the m-CPPM phase for which the fitting was performed at only one magnetic field (thus leading to larger errors).

<table>
<thead>
<tr>
<th></th>
<th>$\delta_{iso}$ (ppm)</th>
<th>$C_Q$ (MHz)</th>
<th>$\eta_Q$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exp</td>
<td>Calc</td>
<td>Exp</td>
</tr>
<tr>
<td>$\alpha$-Ca$_2$P$<em>2$O$</em>{7}$</td>
<td>Ca1</td>
<td>-18.0</td>
<td>-22.1</td>
</tr>
<tr>
<td></td>
<td>Ca2</td>
<td>12.0</td>
<td>6.4</td>
</tr>
<tr>
<td>m-CPPD</td>
<td>Ca1</td>
<td>14.0</td>
<td>12.5</td>
</tr>
<tr>
<td></td>
<td>Ca2</td>
<td>29.0</td>
<td>18.0</td>
</tr>
<tr>
<td>m-CPPD</td>
<td>Ca1</td>
<td>14.0</td>
<td>14.9</td>
</tr>
<tr>
<td></td>
<td>Ca2</td>
<td>12.0</td>
<td>12.0</td>
</tr>
<tr>
<td>Rel H</td>
<td>Ca1</td>
<td>11.0</td>
<td>11.9</td>
</tr>
<tr>
<td></td>
<td>Ca2</td>
<td>7.5</td>
<td>11.4</td>
</tr>
<tr>
<td>m-CPPT $\beta$</td>
<td>Ca1</td>
<td>33.0</td>
<td>15.2</td>
</tr>
<tr>
<td></td>
<td>Ca2</td>
<td>20.0</td>
<td>5.3</td>
</tr>
<tr>
<td>Rel tot</td>
<td>Ca1</td>
<td>20.0</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td>Ca2</td>
<td>7.5</td>
<td>11.4</td>
</tr>
</tbody>
</table>

For $\alpha$-Ca$_2$P$_2$O$_{7}$, the calculated values reported here differ from those previously published [29], due to the difference in computational code used (PARATEC vs Quantum Espresso).

For $\alpha$-Ca$_2$P$_2$O$_{7}$, only the quadrupolar parameter $P_Q$ had been reported [29].

The sign of $C_Q$ cannot be determined by solid state NMR experiments at room temperature.

Fig. 3. Natural abundance $^{43}$Ca MAS NMR spectra of t-CPPD, m-CPPD, m-CPPT $\beta$ and m-CPPM, recorded at 14.1 T [$\nu_0(^{43}$Ca) = 40.37 MHz], and 20.0 T [$\nu_0(^{43}$Ca) = 57.22 MHz]. MAS rotation frequency: between 4 and 6 kHz. For details on the relaxation delay and number of scans for each sample, see Table S1. The fitting of the different MAS NMR spectra can be found in Figure S5.

Fig. 4. Correlation between the computed $^{43}$Ca isotropic chemical shifts and the mean Ca...O bond distance in m-CPPD, t-CPPD, m-CPPT $\beta$, m-CPPM and $\alpha$-Ca$_2$P$_2$O$_{7}$, depending on the Ca coordination number (CN) and the number of water molecules in the coordination sphere. The values are taken from Table S2.
Finally, it is worth noting that the effect of heat-treatment of a-CPP could also be followed by $^{43}$Ca MAS NMR (Figure S7). Indeed, despite rather symmetrical lineshapes at 20.0 T, a broadening of the signal was clearly observed after having heated the a-CPP phase at 220 °C, indicating an increase of the distribution of the $^{43}$Ca NMR parameters, possibly due to the formation of hydrogen-phosphate anions within the material, as previously evidenced by $^1$H and $^{31}$P MAS NMR [13]. All in all, $^{43}$Ca NMR appears as a valuable tool for investigation of hydrated calcium pyrophosphates, which would deserve to be looked into more systematically, as it can provide complementary information about their structure at the atomic scale.

3.3. $^1$H MAS NMR

Finally, $^1$H MAS NMR spectra were recorded using homonuclear decoupling (DUMBO) during acquisition (Fig. 6). Despite the use of advanced decoupling techniques, it was not possible to fully resolve the different $^1$H resonances related to inequivalent water molecules in the structures. For all crystalline structures an average value of ~5–6 ppm was observed. Such a range of isotropic chemical shifts is compatible with those obtained for protons involved in H-bond networks [19].

A comparison with GIPAW calculated $^1$H chemical shifts (Table S3) is also presented in Fig. 6 for t-CPPD, m-CPPD, m-CPPT $\beta$, m-CPPT $\alpha$, m-CPPM and a-CPP (samples A and B – see section 2.1) using windowed-DUMBO homonuclear decoupling during the acquisition (14.1 T, 599.82 MHz, relaxation delay: 4–16 s depending on the sample, number of scans: 20, regulation of the temperature: 10° C), MAS rotation frequency: 10.0 kHz. All observed resonances correspond to isotropic lines. In red: GIPAW calculations (see Table S3).

![Fig. 6. $^1$H MAS NMR spectra of t-CPPD, m-CPPD, m-CPPT $\beta$, m-CPPT $\alpha$, m-CPPM and a-CPP (samples A and B – see section 2.1) using windowed-DUMBO homonuclear decoupling during the acquisition (14.1 T, 599.82 MHz, relaxation delay: 4–16 s depending on the sample, number of scans: 20, regulation of the temperature: 10° C), MAS rotation frequency: 10.0 kHz. All observed resonances correspond to isotropic lines. In red: GIPAW calculations (see Table S3).](image-url)
ability of these materials at the atomic scale, by providing information which is not necessarily accessible from more commonly used techniques such as IR spectroscopy. Low temperature experiments (down to ~100 K) would also be of high interest for a more detailed comparison of experiment and GIPAW computed parameters. Indeed, water molecules must experience intrinsic dynamics in the various structures (at least at room temperature), as demonstrated by the relative lack of resolution of the $^1$H MAS spectra. It follows that the $^1$H isotropic chemical shifts observed at room temperature correspond to averages. Freezing local dynamics would lead to determine “static” isotropic chemical shifts which could be compared safely to GIPAW predictions and act therefore as pertinent constraints for structure refinement.

4. Conclusion

In this contribution, several hydrated calcium pyrophosphate phases (both crystalline and amorphous) were characterized by multinuclear MAS NMR. It was demonstrated that $^{31}$P and $^{43}$Ca MAS NMR spectroscopies are suitable for the clear distinction of the various phases. Even in the case of amorphous samples, subtle variations of the resonance lines were observed by $^{43}$Ca MAS NMR depending on the synthetic protocol and heat treatment temperature. Moreover, $^1$H NMR was found to be informative about differences in the H-bond networks within these phases.

All crystalline structures were then studied in the context of NMR crystallography, using GIPAW as a theoretical bridge between experimental and computed data. All in all, a fairly good agreement was observed for the three studied nuclei, provided that a relaxation of the structures (focusing especially on proton positions) was carried out. Nonetheless, discrepancies remained for the hydrated phases, which may be due to the fact that NMR calculations do not take into account any temperature/local motion effects. Previous studies have indeed shown that these factors could induce significant differences between experimental and computational data [38].

Concerning a-CPP phases, important structural features have been derived from multinuclear solid state NMR analyses. First of all, the $^{31}$P MAS spectra are rather insensitive to the synthetic protocols: it demonstrates that the $^{31}$P NMR parameters are mostly determined by the local geometry of the $\text{P}_2\text{O}_7^{2-}$ species (angle, bond lengths) and not by the localization of the calcium cations and the water molecules. This is clearly not the case when considering $^{43}$Ca and $^1$H NMR parameters. In particular, $^1$H MAS spectra seem suitable to distinguish local environments comparable to those observed in m-CPPT $\beta$ and m-CPPM crystalline phases, though contents in water molecules are comparable from one sample to another one.

Slater et al. [13] already pointed out structural similarity between m-CPPT $\beta$ and a-CPP by pair distribution functions (PDF) analysis. This structural similarity was furthermore linked to comparable behavior regarding hydrolysis reactions. Thus it has been observed that m-CPT $\beta$, m-CPPM and a-CPP are hydrolyzed at high temperature but this is not the case for t-CPPD and m-CPPD samples [10,12].

Based on the elemental composition of the amorphous calcium pyrophosphate phases, and on the structural information gathered here by $^1$H, $^{43}$Ca and $^{31}$P MAS NMR, realistic structural models for these amorphous phases are currently being developed. Following the pioneering approach initiated by Charpentier [37] and very recent results presented by the same author [69], the idea is to compare the calculated NMR values for computational models of this material with the experimental ones, in order to propose a realistic model. This study will be presented in a forthcoming publication.

Finally, the contribution of this study in structural refinement of poorly known hydrated CPP phases is a first step towards the understanding of in vivo phenomena related to osteoarthritis: structure-inflammatory response relationships, potential precursor phase formation and evolution, role of trace elements in CPP crystal formation occurring in associated diseases like hypomagnesaemia [70], Wilson’s disease (copper excess) [71] and haemochromatosis (iron excess) [72]. In addition, some applications of CPP compounds in the biomaterial field can be envisioned [39].

Acknowledgments

The French/UK CNRS PICS project QAMAT is acknowledged. D. Laurencin and E. M. Smith thank the Royal Society for funding collaborative research (Warwick-Montpellier JP090313 partnership). The UK 850 MHz solid-state NMR Facility used in this research was funded by EPSRC and BBSRC, as well as the University of Warwick including via part funding through Birmingham Science City Advanced Materials Projects 1 and 2 supported by Advantage West Midlands (AWM) and the European Regional Development Fund (ERDF). We thank Dr Dinu Iuga for his help at the 850 MHz Facility. NMR spectroscopic calculations were performed on the IDRIS supercomputer centre of the CNRS (Project: 091461). Finally, the authors thank the French Agence Nationale de la Recherche (CAPYROSIS project – ANR-12-B508-0022-01) and the Institut National Polytechnique de Toulouse (PRECYPICA project – BQR INPT 2011) for supporting part of this research work.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2015.10.016.

References


C. Roiland, F. Fayon, P. Simon, D. Massiot, Characterization of the disordered phosphonate network in Ca$_3$P$_2$O$_7$: see ICSD 22225.


D.W.J. Cruickshank, The role of 3d-orbitals in π-bonds between (a) silicon, phosphorus, sulphur, or chlorine and (b) oxygen or nitrogen, J. Chem. Soc. (1961) 5486–5504.


T Balic Zunic, I. Vickovic, IVTON – a program for the calculation of geometrical aspects of crystal structures and some crystal chemical applications, J. Appl. Cryst. 29 (1996) 305–306.


