Recent directions in the solid-state NMR study of synthetic and natural calcium phosphates

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Christel Gervais,[1] Christian Bonhomme,[1] Danielle Laurencin[2,*]

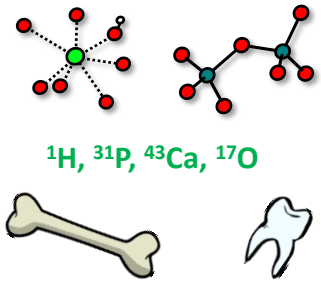
1/ Laboratoire de Chimie de la Matière Condensée de Paris (LCMCP), UMR 7574, Sorbonne Université, CNRS, 75005 Paris, France

2/ ICGM, Univ Montpellier, CNRS, ENSCM, Montpellier, France

* To whom correspondance should be addressed : danielle.laurencin@umontpellier.fr

Abstract

Materials containing a calcium phosphate component have been the subject of much interest to NMR spectroscopists, especially in view of understanding the structure and properties of mineralized tissues like bone and teeth, and of developing synthetic biomaterials for bone regeneration. Here, we present a selection of recent developments in their structural characterization using advanced solid state NMR experiments, highlighting the level of insight which can now be accessed.



- ✓ Ultra-high magnetic fields
- ✓ Dynamic Nuclear Polarization
- ✓ Micro-imaging under MAS
- ✓ Computational modeling
- ✓ Isotopic labeling

1. ***Introduction***

Calcium phosphates are widely studied in the field of biomaterials.[1-7] The main interest for these compounds originates from the fact that bone and teeth contain a calcium phosphate phase generally referred to as biological carbonated apatite.[2] While the first studies on calcium phosphates appear to date back to the 18th century,[8] numerous investigations have since been performed, crossing disciplines such as chemistry, biology and medicine. Indeed, the biocompatibility of calcium phosphate phases together with their capacity to host a wide variety of substituents make them highly attractive not only for bone and dental repair (as ceramic coatings on implants or as biocements), but also for applications as drug delivery systems or contrast agents for MRI.

From a chemist's perspective, calcium phosphates can come under a many different chemical compositions.[2, 6] First, the phosphate ions can vary in terms of degree of condensation and protonation state (Figure 1). Orthophosphates (PO_4^{3-}) are the most frequent. These anions can be *(i)* fully deprotonated, as in stoichiometric hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$) and β-tricalcium phosphate ($Ca_3(PO_4)_2$, β-TCP), *(ii)* protonated once, as in brushite $CaHPO_4.2H_2O$ and monetite $CaHPO_4$, or *(iii)* protonated twice, as in monocalcium phosphate monohydrate (MCPM, $Ca(H_2PO_4)_2.H_2O$). More condensed phosphate ions include pyrophosphates ($P_2O_7^{4-}$, as in α-$Ca_2P_2O_7$), metaphosphates and polyphosphates (with ~ 2 P-O-P bonds per phosphorous, as in γ-$Ca(PO_3)_2$), as well as ultraphosphates (with ~ 3 P-O-P bonds per phosphorous, as in $Ca_2P_6O_{17}$). While some calcium phosphate phases are anhydrous, others are hydrated. The Ca-pyrophosphate phases $Ca_2P_2O_7.nH_2O$ are a very good example of this, as mono, di, tri, and tetrahydrate phases have been reported, in addition to the anhydrous polymorphs.[9-12]

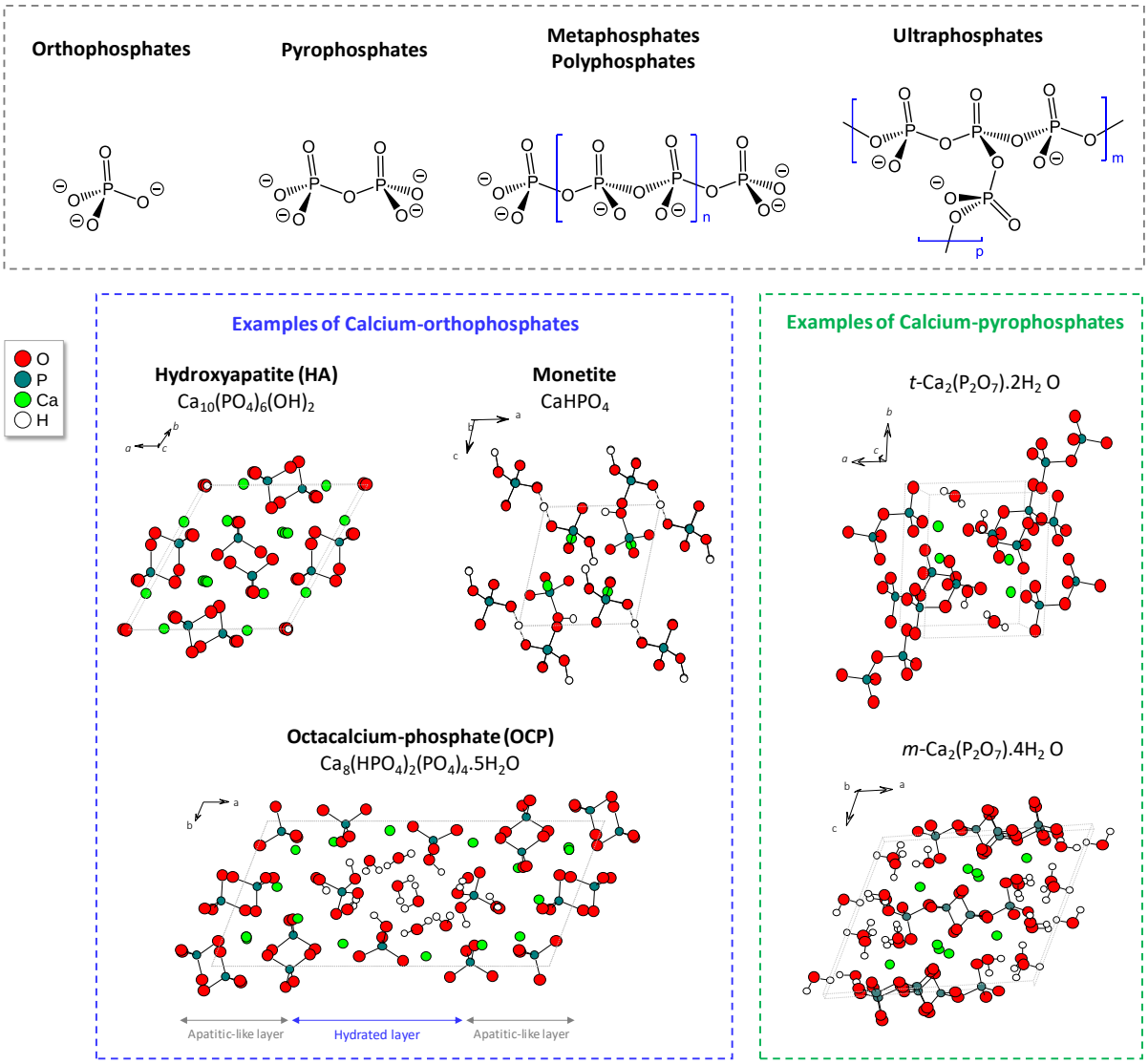


Figure 1. Molecular structure of different types of phosphate anions, and examples of calcium ortho- and pyro-phosphate crystal structures.

In addition to the structural complexity, synthetic and natural calcium phosphate phases can exhibit very different crystallinities, and several amorphous phases have also been described. Moreover, the degree of crystallinity/order can significantly vary within one mineral particle (e.g. from surface to bulk). They also frequently contain traces of other ions as substituents within their structures (e.g. CO_3^{2-}, F^-, SiO_4^{4-}, Na^+, Mg^{2+}…). Last but not least, a large proportion of these biomaterials also have an organic component (biopolymers, peptides…), which mainly consists of collagen in the case of native bone. In relation to this, many investigations have focused on the study of the structure

of bone mineral itself, and in finding synthetic models capable of mimicking some of its structural features. These have included carbonate-substituted nanocrystalline hydroxyapatite and octacalcium phosphate phases.

In view of gaining deeper insight into the structure of bone, and preparing calcium phosphate-based biomaterials with optimal properties, much effort has been made in the development of advanced characterization tools, capable of providing information on their structure at different scales. In this context, solid state NMR has played a significant role,[13, 14] with the first studies dating back to the 1960s (to the best of our knowledge).[15] Most of the early magic angle spinning (MAS) NMR work concerned ^{1}H and ^{31}P NMR analyses,[16-18] as both of these spin-1/2 isotopes have a very good receptivity (Table 1).[19-21] One of the highlights of these investigations was the study by Ackermann *et al* in 2003, who provided direct evidence of the presence of apatite-like hydroxyl groups in bone mineral using 2D ^{1}H-^{31}P HETCOR (heteronuclear correlation) experiments.[22] Other important landmarks subsequently followed between 2005 and 2010, including the ^{13}C{^{31}P} REDOR (Rotational Echo Double Resonance) studies initiated by Duer *et al* to elucidate the organic-mineral interface of bone,[23, 24] the first 2D NMR studies of substituted apatites aiming at locating precisely the position of ionic substituents,[25-27] and the first complete natural-abundance ^{43}Ca MAS NMR experiments of synthetic calcium phosphates and bone.[27-29] In the latter case, the main challenge was due to the very poor receptivity of calcium-43, which has a natural abundance of only 0.14% and a very low resonance frequency (Table 1).[30]

Since 2010, materials chemists have continued to explore new frontiers to use NMR for gaining even deeper insight into these systems, by taking advantage of the progress made by the NMR community in terms of instrumentation, pulse sequence development, and data interpretation (using combined experimental-computational approaches). While some of these new achievements have been described in recent reviews,[13, 14, 31] the purpose of this manuscript is to highlight a selection of results and trends which have emerged over the past 5 years, to show what level of insight they are

likely to lead to in the future. The focus will first be set on studies relative to synthetic calcium phosphate biomaterials, before focussing on natural bone and dental tissues.

Isotope	Nuclear spin	Natural abundance (%)	Quadrupolar moment Q (fm^2)[32, 33]	Larmor frequency at 14.1 T (MHz)	Receptivity (rel. to ^{13}C)
^{1}H	1/2	99.98	/	600.1	5.87×10^{3}
^{31}P	1/2	100	/	242.9	3.91×10^{2}
^{17}O	5/2	0.04	-2.56	81.3	6.5×10^{-2}
^{43}Ca	7/2	0.14	-4.44	40.4	5.1×10^{-2}

Table 1. NMR properties of the main isotopes present in calcium phosphates.

2. Synthetic calcium phosphates and related biomaterials

2.1 Crystalline phases

As crystalline calcium phosphates are most frequently isolated as powders which are unsuitable for single-crystal X-ray diffraction, it is no surprise that approaches based on NMR-crystallography[34-39] are increasingly being used to help establish the details of their structure and local dynamics. In such investigations, high resolution NMR analyses are combined to powder X-ray and neutron diffraction data, and often complemented by *ab initio* calculations of NMR parameters (in the so-called « SMARTER crystallography » strategy),[40] in order to refine the atomic positions. Over the past 5 years, these approaches have clearly enabled to gain deeper insight into the bulk structure of monetite,[41, 42] hydrated calcium pyrophosphates,[43] and carbonated hydroxyapatite[44], as detailed below.

Concerning monetite ($CaHPO_4$), the main progress made concerns the higher resolution achieved in the ^{1}H, ^{31}P and ^{43}Ca NMR analyses, allowing a better assignment of the NMR resonances. On one hand, using fast-MAS ^{1}H NMR experiments (performed spinning at 66 kHz on a 600 MHz instrument), Edén and co-workers were able to not only resolve the signals from the three crystallographically inequivalent H1, H2, and H3 sites of monetite, but more importantly to

unambiguously attribute the peaks on the basis of 2Q–1Q ^{1}H correlation experiments (with [SR2$_2^1$] and [SR2$_4^1$] recoupling schemes).[41] Moreover, by performing fast MAS ^{31}P{^{1}H} HETCOR experiments and integrating the 2D NMR intensities of the resolved H_m–P_n signals, they derived the effective (average) distance within each of the six pairs of {P1, P2} and {H1, H2, H3} sites. Then, they proposed a strategy to deduce the actual interatomic distances, which led to a good agreement with previously published neutron-diffraction data. Concerning ^{43}Ca NMR, the main progress made concerned the significant gain in resolution achieved by performing analyses at ultra-high magnetic fields (35.2 T) on the series-connected hybrid (SCH) magnet of the US National High Magnetic Field Laboratory.[45] Indeed, while the different calcium environments of monetite systematically overlapped at lower magnetic fields,[29] clear resolution of two different types of Ca sites could be reached for the first time at 35.2 T (Figure 2), and a tentative assignment was subsequently proposed on the basis of DFT calculations[42] (performed using the Gauge Including Projector Augmented Wave (GIPAW) approach).[46-48]

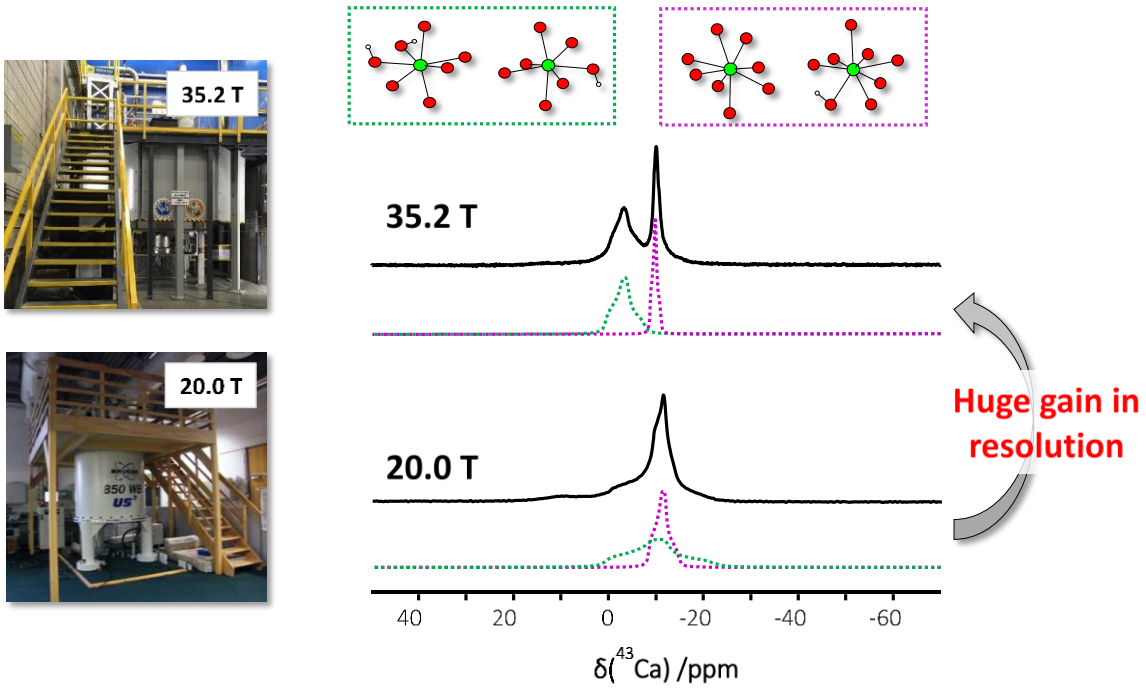


Figure 2. ^{43}Ca MAS NMR spectra of a monetite phase (enriched in ^{43}Ca), recorded at 20.0 and 35.2 T, showing the significant gain in resolution achieved in the latter case. The fits are shown below each spectrum (dashed green and purple lines). The local environments of calcium are represented above the spectra (Ca in green, O in red, H in white) ; these can be split into two sub-groups on the basis of the ultra-high field NMR experiments and GIPAW-DFT calculations of ^{43}Ca NMR parameters (the colours used for the two dashed boxes around the Ca-environments match those of the corresponding fits).[42] Adapted from Ref. [42] with permission from The Royal Society of Chemistry.

Crystalline calcium pyrophosphates $Ca_2P_2O_7.nH_2O$ (n = 0, 1, 2, 4) were extensively investigated by Gras *et al,* using high resolution ^{1}H, ^{31}P and ^{43}Ca NMR experiments and GIPAW-DFT calculations.[43] For each phase, ^{1}H MAS NMR spectra were recorded at 14.1 T using DUMBO homonuclear decoupling,[49] ^{31}P MAS NMR spectra were recorded at 14.1 T using different spinning speeds in order to extract the chemical shift anisotropy (CSA) parameters δ_{CSA} and η_{CSA}, and natural-abundance ^{43}Ca MAS NMR spectra were recorded at two fields (14.1 and 20.0 T) in order to extract the quadrupolar parameters C_Q and η_Q. The overlap between some of the resonances and similarity between their NMR parameters were found to be a challenge for identifying the different sites. GIPAW-DFT calculations were thus performed using as starting points the published crystallographic structures, and optimising either H atom positions only, or all atomic positions**.** This allowed not only the ^{31}P and ^{43}Ca NMR resonances of each phase to be assigned, but also the quality of the starting crystallographic data to be evaluated. The case of the monoclinic $Ca_2P_2O_7.2H_2O$ phase was in this context particularly interesting, because the two ^{31}P resonances could only be safely attributed after relaxation of H atom positions: this was the only way to have a consistent agreement between experimental and calculated δ_{iso}, δ_{CSA} and η_{CSA} parameters. The ^{1}H NMR analyses were also of interest, as the differences between experimental and calculated parameters were observed, which were interpreted as an indication of the presence of dynamics within the crystal structures. This point is currently being further investigated. Overall, this investigation clearly underscores the importance of combining high resolution NMR analyses to GIPAW-DFT calculations when studying hydrated calcium phosphates for which the position and dynamics of water molecules can have a significant impact on the spectra.

While most biological apatites are known to be carbonated, the actual position of carbonate ions within the hydroxyapatite structure has been the subject of much discussion. Indeed, although these are known to be able to substitute for both hydroxyl groups (« A-type » substitution) and phosphates (« B-type » substitution), the question of order/disorder related to carbonate substitution, and the possible clustering of carbonate ions within the apatite lattice had long remained unanswered.[50] Using Dynamic Nuclear Polarization (DNP), Bonhomme and co-workers were able to

provide the very first direct evidence of carbonate clustering within synthetic carbonated nanocrystalline apatites.[44] By combining 2D $^{31}P \rightarrow {}^{13}C$ CP-HETCOR and ^{13}C double-quantum-single-quantum (DQ-SQ) MAS NMR experiments at 9.4 T, enhanced by indirect DNP using $^{1}H \rightarrow {}^{31}P$ and $^{1}H \rightarrow {}^{13}C$ CP transfers, respectively (Figure 3), it was possible to conclude on the presence of clustering of the carbonates, with a majority of clustered B/B substitutions, a minority of A/B substitutions, and only a small fraction of isolated B sites. Such analyses were only made possible thanks to the significant gain in sensitivity achieved using DNP, and appear as a major step forward to the study of carbonate substitutions within apatites, as the level of information achieved here could not have been extracted from X-ray or neutron diffraction data alone. On a more general perspective, such analyses are expected to be of great benefit to the study of other ionic substitutions in apatites and other calcium phosphates, which are still the subject of active NMR research.[51-53]

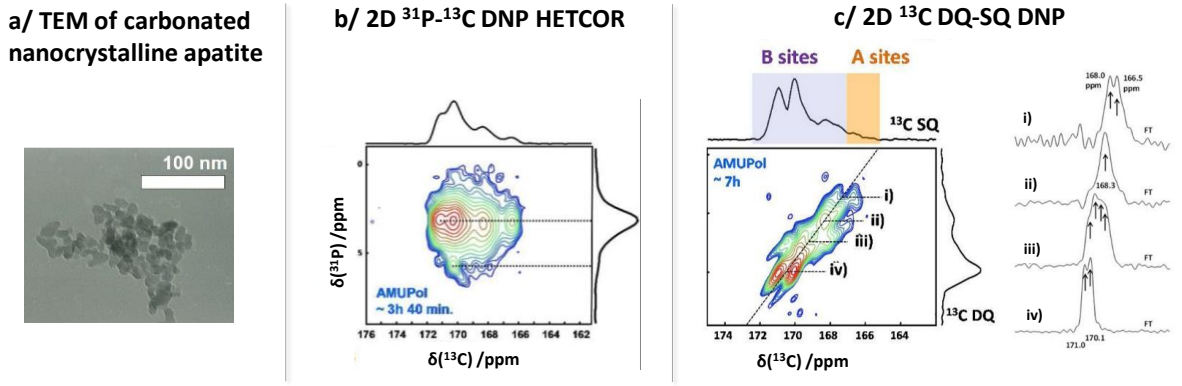


Figure 3. a/ Transmission Electron Microscopy (TEM) of a synthetic carbonated nanocrystalline apatite (enriched in ^{13}C) ; b/ 2D DNP-enhanced $^{31}P \rightarrow {}^{13}C$ DNP CP-HETCOR MAS NMR spectrum ; c/ 2D DNP-enhanced ^{13}C DQ-SQ MAS NMR spectrum, together with some of the 1D ^{13}C NMR slices (noted i) to iv)). Adapted with permission from Ref. [44]. Copyright 2017 American Chemical Society.

2.2 Amorphous phases

In parallel to the investigations on crystalline calcium phosphates, amorphous phases have also been the subject of active research, in relation with the increased evidence of their importance within mineralized tissues, and the need to understand their formation and possible evolution into more

crystalline forms. Amorphous calcium phosphates represent a real challenge in terms of structural characterization, due to the lack of long range order and significant distribution in local environments around the different ions. Advanced solid state NMR analyses have been shown to be particularly valuable in this context, as illustrated below in a selection of recent investigations.

Amorphous calcium pyrophosphates have attracted the attention of several research groups, in view of : *(i)* trying to understand the formation of pathological calcifications composed of calcium pyrophosphates in the joints (a disease called « pseudo-gout »),[42, 43] or *(ii)* developing novel classes of phosphate-based biomaterials for bone substitution and regeneration.[54-56] Concerning the first point, precipitated amorphous phases of general formula $Ca_2P_2O_7.nH_2O$ ($n \sim 4$) were characterized by ^{1}H, ^{31}P and ^{43}Ca MAS NMR, and their spectra compared to those related to crystalline phases.[43] Differences in the ^{1}H and ^{43}Ca NMR spectra were observed depending on the precipitation procedure (i.e. with or without control or not of the pH), while ^{31}P MAS NMR spectra were essentially the same. This clearly demonstrates the importance of performing multinuclear NMR characterizations (including challenging nuclei like ^{43}Ca) when studying these amorphous materials. From a more materials perspective, it was concluded that the H-bond network of the water molecules within these amorphous phases depends on the synthetic protocol. Structural models were subsequently proposed, using a computational approach based on Monte Carlo (MC) simulations, structural relaxation using Ab Initio Molecular Dynamics (AIMD), and geometry optimization using DFT calculations.[42] For each model, GIPAW-DFT calculations of NMR parameters were then performed. Calculated ^{43}Ca MAS NMR spectra were compared to experimental ones, which had been recorded at natural abundance at 35.2 T in less than 4 hours (Figure 4a). Although the range of calculated ^{43}Ca NMR isotropic shifts was found to be overall consistent with the experimental data, discrepancies remained between experimental and calculated values, which may be due to dynamics of the water molecules within these systems (as also pointed out in section 2.1 when discussing the corresponding crystalline phases).[43]

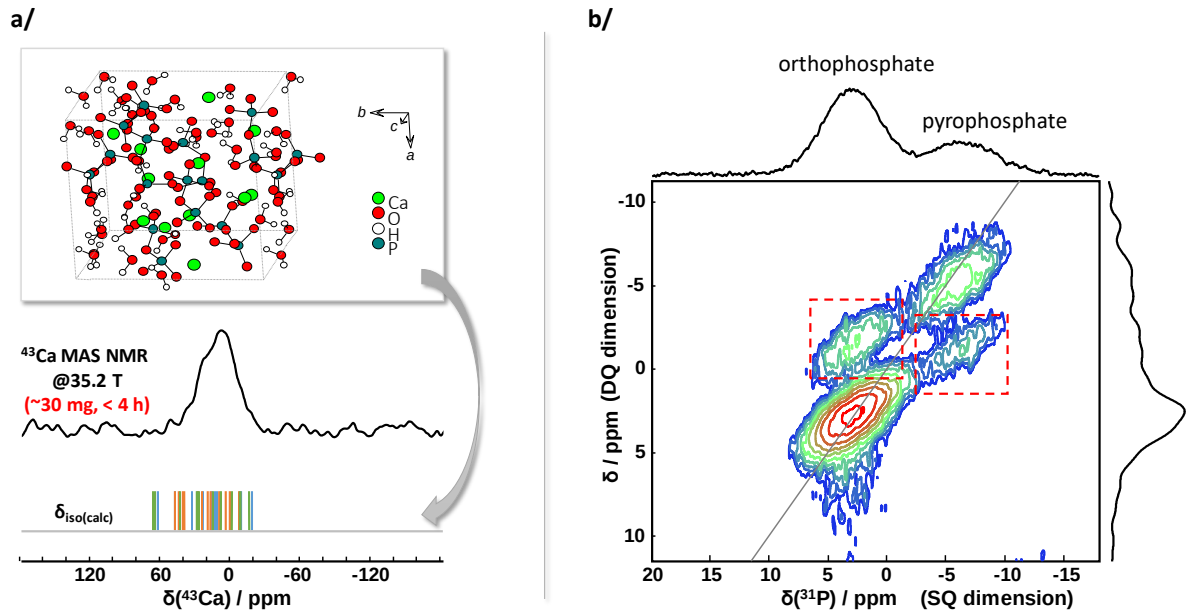


Figure 4. a/ Natural abundance ^{43}Ca MAS NMR spectrum of a precipitated amorphous Ca-pyrophosphate, recorded at 35.2 T, and comparison with DFT-calculated isotropic shifts of structural models of the material (as illustrated above in the insert). Adapted from Ref. [42] with permission from The Royal Society of Chemistry. b/ 2D ^{31}P DQ-SQ MAS NMR spectrum of an amorphous mixed ortho/pyrophosphate biomaterial, recorded at 16.4 T (using SPC5 recoupling and ^{1}H→^{31}P CP transfer to create the initial ^{31}P magnetization), showing clearly the proximity of ortho and pyrophosphate units (dashed red boxes). Adapted from Ref. [54] with permission from Elsevier.

Another important family of amorphous phases involving both ortho- and pyrophosphate anions are the monolithic glass-like materials which have been developed by Soulié, Combes and co-workers,[54, 55] as a means to propose alternatives to silicate bioactive glasses. The idea behind the synthesis of these materials is that by controlling the ortho/pyrophosphate ratio it should be possible to tune the kinetics of biomaterial degradation and bone mineral formation *in vivo*. These materials are of high complexity, as they contain a variety of anions (ortho and pyrophosphates, which can be protonated or not), a variety of cations (Ca^{2+} as well as Na^{+} or K^{+}, depending on the pyrophosphate precursor used in the synthesis), and water molecules. Hence, many questions deserved to be answered, regarding *(i)* the ortho/pyrophosphate ratio in the isolated material (in comparison to the one used in the starting solution), *(ii)* the relative protonation of ortho and pyrophosphate anions, and *(iii)* the possible segregation between ortho and pyrophosphate species within the precipitated

material. To address these questions, a variety of different NMR experiments were used, which included *(i)* ^{31}P INADEQUATE analyses (Incredible Natural Abundance DoublE QUAntum Transfer Experiment), as some overlap between the ^{31}P ortho and pyrophosphate resonances can occur), *(ii)* ^{31}P{^{1}H} HETCOR studies at short contact time, which revealed that the orthophosphates are mainly protonated, and *(iii)* ^{31}P DQ-SQ analyses (using SPC5 recoupling and ^{1}H→^{31}P CP transfer to create the initial ^{31}P magnetization), which confirmed the intimate mixing of both anions (Figure 4b).[54] Such information is currently being used, in conjunction with additional high resolution ^{43}Ca and ^{23}Na data, and pair distribution function (PDF) analyses, to construct computational models of the materials.

Concerning amorphous calcium orthophosphates (ACPs), several recent investigations have also been reported.[57-61] One important study by the group of Azaïs, Nassif and co-workers, is the NMR analysis performed in view of deciphering between two representations of bone mineral : *(i)* nanoparticles formed of an apatitic crystalline core surrounded by an ACP-like surface layer; and/or *(ii)* phase-separated particles of ACP and apatite. To distinguish both cases, a series of solid-state NMR analyses were carried out on ACP, synthetic biomimetic apatites, and physical mixtures of apatite and ACP, and spectra were compared to those of sheep bone mineral. NMR experiments consisted of *(i)* a double cross polarization (CP) ^{1}H → ^{31}P → ^{1}H pulse sequence, in order to select the ^{1}H resonances belonging to calcium phosphate environments, followed by *(ii)* a ^{1}H spin diffusion experiment (EXchange SpectroscopY, EXSY) in order to look into the relative proximity of the different calcium phosphate domains.[58] These NMR analyses were able to provide unambiguous evidence that bone mineral particles have a core-shell organisation, with a crystalline apatitic core, surrounded by an ACP coating. Such information is particularly important, considering the complexity of bone mineral and the ongoing discussions regarding its structure and formation.

2.3 Calcium phosphate surfaces & interfaces

In addition to investigating the bulk structure of crystalline and amorphous calcium phosphate phases, a number of NMR studies have also concerned characterizing the surface of these particles,

and how they can interact at the interface with organic functions (such as those found in bone) or other inorganic materials (such as bioactive glasses).

While experiments such as those described just above can provide evidence of an interface between the crystalline and amorphous calcium phosphate components in biomimetic apatites and bone,[58] the obvious next step is to determine what ionic environments are present at the surface of the particles. This was actually one of the goals of the DNP studies recently reported by Lee *et al*.[62] Thanks to the significant gain in sensitivity provided by DNP, it was possible to identify not only surface carbonate ions (Figure 5a), but also surface calcium sites (Figure 5b). The latter result is all the more noteworthy when considering the challenges related to performing 2D ^{1}H-^{43}Ca HETCOR analyses at natural abundance (0.14 %), due to the unfavorable NMR properties of calcium-43 (Table 1).

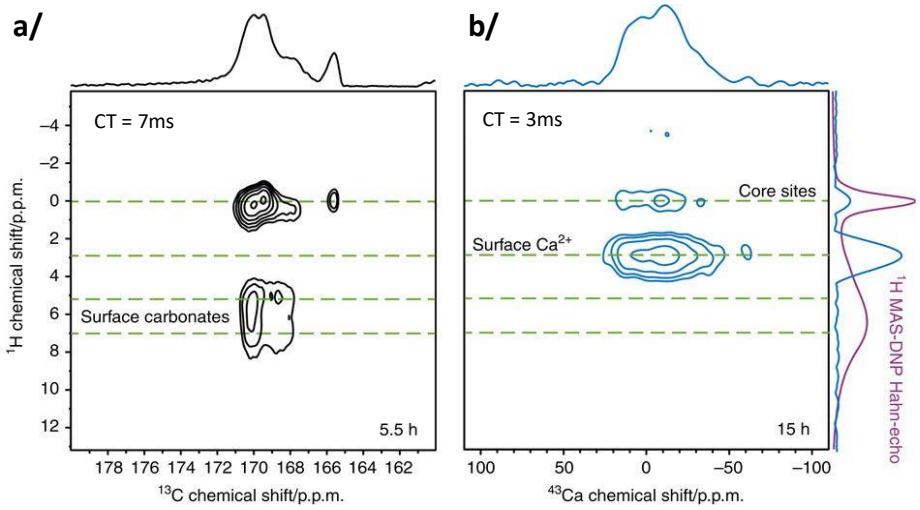


Figure 5. DNP studies of nanocrystalline apatite at 9.4 T and 100 K. a/ ^{1}H–^{13}C HETCOR spectrum with associated ^{13}C skyline projection (top). b/ ^{1}H–^{43}Ca HETCOR spectrum with associated ^{43}Ca (top) and ^{1}H (right) skyline projections (in blue), and the DNP-enhanced ^{1}H Hahn-echo MAS NMR spectrum (in purple). Reproduced with permission from Ref. [62] (Copyright © 2017, Springer Nature).

The study of organic-mineral interfaces in synthetic hybrid materials involving a calcium-phosphate component has also been the subject of active research. While some of the recent studies have been performed in order to establish the structure of apatite-based drug formulations and composite bioceramics for bone repair,[63, 64] others were carried out to further investigate the

structure of bone (by looking at octacalcium phosphate derived phases,[65, 66] or at the adsorption of non-collagenous bone proteins like osteocalcin on hydroxyapatite [67]), or to help understand how ACPs can be stabilized in presence of proteins.[68] In the latter case, Gésan-Guiziou and co-workers looked at the structure of ACPs in presence of high concentrations of casein (used as a model phosphorylated protein). Using a combination of static and MAS ^{1}H and ^{31}P NMR experiments, the signatures of bound and free phosphoserine moieties were distinguished, and evidence of the interaction of non-phosphorylated amino acids with the amorphous mineral surface was obtained from a series of 2D ^{1}H-^{31}P HETCOR experiments. Moreover, measurements were also performed at higher temperatures (45°C), demonstrating how highly concentrated casein continues to stabilize the ACP particles.

Inorganic-inorganic interfaces involving a calcium phosphate component have also been looked into. In particular, the formation of hydroxyapatite-like phases at the surface of bioactive glasses upon immersion in simulated body fluids (SBF) has been the subject of much attention, in view of optimizing the synthesis of biomaterials for bone regeneration. In this context, several recent studies underscore how solid state NMR can be used as a qualitative and/or quantitative technique for understanding the reactivity of a variety of bioglasses, prepared according to different synthetic procedures (melt-quench or sol-gel syntheses), and displaying different chemical compositions (« CaO-SiO_2 », « CaO−SiO_2–P_2O_5 », and « Na_2O-CaO-SiO_2-P_2O_5 » families).[69-72] For example, Edén and co-workers demonstrated how ^{1}H MAS NMR spectra edited by $^{1}H \rightarrow {}^{31}P$ CP transfer are more sensitive than powder X-ray diffraction and than simple ^{31}P MAS NMR experiments for detecting the beginning of apatite formation at the surface of the materials.[71] Moreover, by performing NMR analyses on model ^{17}O-labeled CaO-SiO_2 glasses, Smith and co-workers showed how the quick release of calcium from the bulk-glass structure (which promotes the subsequent precipitation of hydroxyapatite) can be made evident by the loss of the ^{17}O NMR resonance of non-bridging oxygens.[69] The latter study underscores the interest of developing methodologies to analyze some of the more challenging

isotopes, like oxygen-17 (Table 1), which despite their unfavourable NMR properties are likely to provide valuable information.

3. Natural calcium phosphate phases

3.1 Considerations on sample preparation for NMR and DNP analyses

While the NMR study of synthetic calcium phosphates is relatively straightforward in terms of sample preparation, as these can generally easily be reduced to a powdered form and packed inside rotors, analyzing biological tissues raises a number of questions. First, the grinding of bone and teeth can be problematic, because *(i)* the grinding conditions can lead to changes in the organic and mineral components, or in the way in which they are associated to each other,[73-75] *(ii)* the grinding leads to a loss of integrity of the sample, thereby preventing further analyses to be performed on it (e.g. mechanical studies), which can be a problem if the sample is « precious », as is the case for bone and dental tissues coming from genetically engineered or « isotopically-enriched » animals,[75] and *(iii)* the grinding provides an *average* information of the structure, which could potentially have been more-finely described by characterizing smaller fractions of the material, as recently demonstrated by Yon, Fayon and co-workers.[76] Hence, although the majority of initial solid state NMR investigations on bone and dental tissues concerned ground samples, several groups have proposed approaches to perform MAS experiments on intact ones.[73-79] The second issue regarding biological samples is that they need to remain hydrated to preserve their functional (and hence structural) properties. Indeed, it has been shown that bone dehydration can lead to changes in the NMR spectra,[73, 74, 80] meaning that strategies aiming at keeping the samples hydrated are to be privileged.[75, 80]

The question of sample preparation has recently been re-opened with the popularization of dynamic nuclear polarization, as DNP is now also starting to be used for studying bone tissue and other biominerals. In this case, the need to impregnate the sample by a radical solution raises the question

of the choice of the radical/solvent couple, the goal being to avoid (or minimize) any structural changes within the biological sample. When looking at the few studies reported so far, most bone sample preparations involved an AMUPol biradical solution in H_2O/D_2O,[81-83] leading to ^{13}C DNP-enhancement factors between ~ 20 and 60 (Figure 6). It is worth noting that these impregnation procedures differed slightly in terms of AMUPol concentration (between 12 and 20 mM), D_2O/H_2O ratio, duration of the impregnation, and size of the bone particles studied. Another preliminary DNP study of mice teeth was also reported, in which glycerol was added to the DNP-juice.[62] More importantly, it is worth noting that a broadening as well as shifts in some of the NMR resonances were observed on the DNP spectra (in comparison to conventional NMR analyses) ; this was ascribed to the lower temperatures used in the DNP analyses (but without verifying this point by performing a conventional $^{1}H \rightarrow {}^{13}C$ CPMAS NMR analysis at 100 K).[81, 82] Moreover, differences in DNP enhancements were also noticed for the mineral and organic components of bone (with stronger enhancements for the organic fraction, as shown in Figures 6b-c) ;[81] this implies that future optimizations of the impregnating conditions may also depend on the type of structural information which is sought.

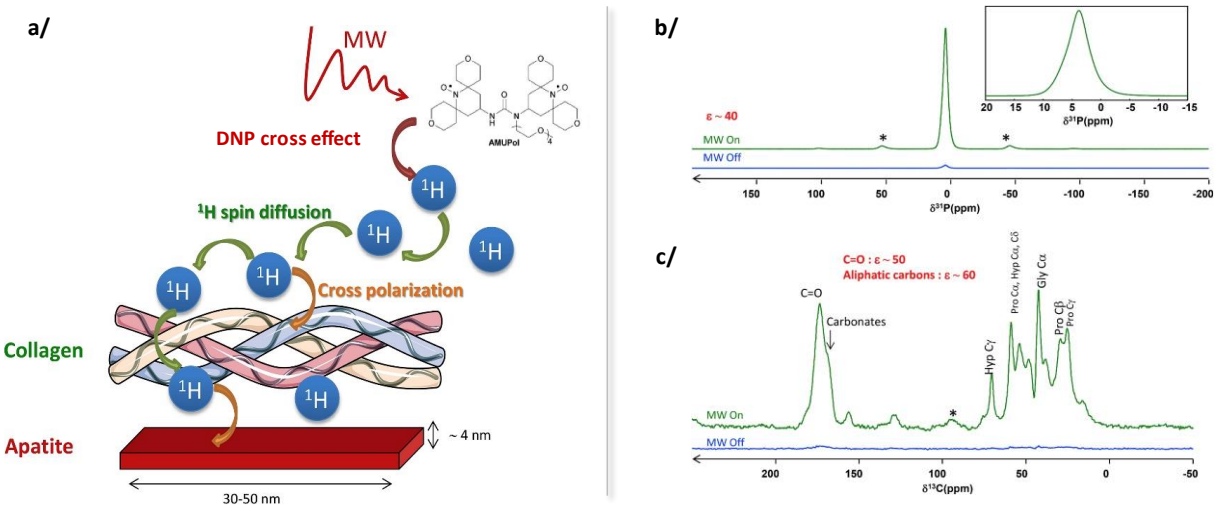


Figure 6. a/ Schematic representation of the DNP approach used in the study of bone. b/ $^{1}H–^{31}P$ and c/ $^{1}H–^{13}C$ DNP enhanced CPMAS NMR spectra of sheep bone. Adapted with permission from Ref. [81] (Copyright © 2019 Elsevier Inc.).

3.2 Advances in NMR and DNP analyses of bone structure

Regarding bone mineral, although many ^{1}H and/or ^{31}P MAS NMR analyses have already been reported, recent investigations focussed more specifically on *(i)* demonstrating the « core-shell » structure of bone mineral (made of a crystalline HA core surrounded by a hydrated ACP layer, as mentioned previously in section 2.2),[58] and *(ii)* determining the location and concentration in HPO_4^{2-} anions. In the latter case, one of the key questions was to determine if HPO_4^{2-} anions were present inside the HA lattice or within the amorphous surface layer.[84] Using $^{1}H \rightarrow {}^{31}P \rightarrow {}^{1}H$ double CP MAS NMR experiments and studying the ^{1}H-detected CP dynamics (in comparison to model synthetic phases like monetite, octacalcium-phosphate and a biomimetic carbonated HA), Azaïs *et al* demonstrated that the protonated phosphates reside within the hydrated ACP layer. Moreover, thanks to additional quantitative ^{31}P NMR analyses, the thickness of the ACP layer could be estimated to ~0.8 nm.[84] In itself, the latter study constitutes one of the most thorough analyses of bone mineral reported so far.

Concerning the organic component of bone, the most significant advances over the past few years have been made possible thanks to the work of Duer and co-workers on the preparation of isotopically-enriched mice bone tissues (by feeding the animal with ^{13}C,^{15}N labeled Celtone powder),[85, 86] and the development of *in-vitro* protocols for producing enriched extracellular matrix collagen from osteoblasts.[86, 87] Thanks to the significant gain in sensitivity achieved by these isotopic labeling procedures,[85] it has become possible to use high resolution ^{13}C and ^{15}N NMR (e.g. 2D ^{13}C-^{13}C DQ-SQ, and DARR-assisted ^{15}N-^{13}C correlations) to highlight a number of major features concerning the organic component of bone and the development of the tissue. These include *(i)* the potential importance of poly-ADP-ribose in the extacellular matrix for developing bone, which is likely to play a role in calcification,[86, 88] *(ii)* the detection in native intact bone of nucleic acids and other low-abundant components (like cholines from phospholipid headgroups, as well as histidinyl and hydroxylysyl groups),[83] and *(iii)* the in-depth study of collagen structure and flexibility.[79]

Organic-mineral interfaces in bone have also been the focus of much attention. On one hand, the potential role of small molecular ions like citrate, acting as bridges between bone mineral platelets, was highlighted.[66, 89] On the other hand, using DNP, it was shown that a full set of $^{13}C\{^{31}P\}$ REDOR dephasing curves could be recorded on a sheep bone sample in just 11 hours (instead of several weeks in absence of DNP), and this allowed estimating the distance of citrate carbon atoms from the mineral surface.[81] In the latter case, based on these dephasing curves, it was suggested that only two out of three carboxylate functions attach to the mineral surface. This level of information is particularly important in relation to the different computational modeling studies of the organic/mineral interface.

3.3 Solid state NMR and high field MAS MRI of teeth

Regarding dental tissues, the extensive investigations performed recently by Balan, Gervais and co-workers on a collection of teeth remains collected from Mio-Pliocene deposits in Kenya are worth mentioning. Indeed, by analyzing a series of fossil tooth enamel samples by different techniques, including ^{19}F MAS NMR, the distinct signature of a « defect » fluoride environment could be identified.[90] Using ^{19}F double quantum-single quantum (DQ-SQ) MAS NMR (using the SPIP sequence: SPin-locked symmetry-based double-quantum homonuclear dIPolar recoupling) and $^{13}C\{^{19}F\}$ frequency-selective REDOR experiments, as well as *ab initio* DFT calculations of NMR parameters, this environment could be unambiguously identified as fluoride ions in close vicinity to carbonate substituents within the apatite lattice.[53] It was suggested that such spectroscopic signatures can serve as tracers of the structure evolution of bioapatite during fossilization, and they were subsequently used to analyze fossilized bone samples from different geographic origin.[91]

As a final example, the research work recently reported by Yon, Fayon and co-workers is worth highlighting, as it brings the study on mineralized tissues a major step forward.[76] In this case, the key idea was to perform the *ex vivo* micro-imaging of an intact mouse tooth, under magic angle spinning conditions at 17.6 T, using ^{1}H and ^{31}P NMR analyses (Figure 7). By spinning the intact tooth at a moderate speed (10 kHz), it was possible to avoid potential undesirable effects due to centrifugal

forces, while keeping benefit of MAS. By performing the analyses at very high magnetic field (17.6 T) and using rotating pulsed field gradients, ^{31}P 2D slice-selected and 3D solid-state images could be obtained, with very good signal-to-noise and spatial resolution (approaching ~ 100 µm in some cases). Moreover, it was shown that several solid-state NMR sequences could be used in these conditions to achieve additional contrast in the images, such as CP MAS for ^{31}P imaging of the different mineral components (Figure 7b), or frequency-selective ^{1}H spin-echos for the chemically-selective imaging of either the organic component of the tooth by looking at aliphatic species, or the more mineralized zones by looking at the apatitic hydroxyl groups (Figures 7c-d). In the latter case, a spatial resolution close to 100 µm could be achieved. On a more general perspective, such high-field MAS MRI approaches are highly promising for the *ex vivo* imaging of mineralized tissues, as they can provide a more complete picture of both their structure and chemical composition. Indeed, they could also allow following the hard tissue modifications which can be caused by different diseases. Moreover, they could be used for studying the osteointegration of bone or dental implants from both spatial and chemical points of view, and thereby be highly complementary to other approaches described so far.[92]

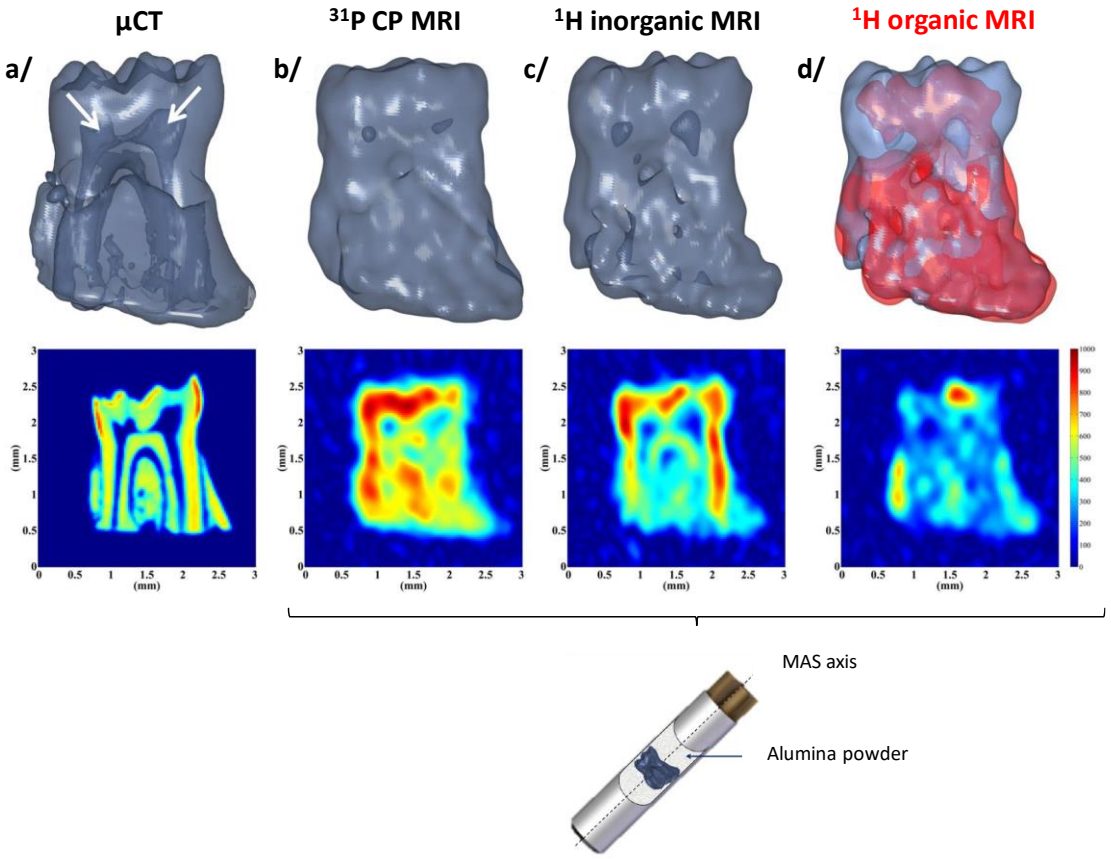


Figure 7. a/ 3D micro-computed tomography (µ-CT) image of a mouse tooth (the white arrows indicate the location of the pulp channels). b/ 3D $^{1}H \rightarrow {}^{31}P$ CP MAS MR image of tooth mineral (experimental duration of 69 h), c-d/ 3D ^{1}H MAS MR chemically selective images of the (c) OH^- hydroxyl groups of apatite and (d) aliphatic species in the organic part of the mouse tooth sample (experimental duration of 18 h). All micro-imaging NMR analyses were performed under magic angle spinning (10 kHz spinning frequency) at very high magnetic field (17.6 T), with the tooth sample introduced in the rotor as shown below the MRI images. Adapted with permission from Ref. [76] (Copyright © 2017 Springer Nature).

4. Outlook

This overview of recent investigations of calcium phosphate based biomaterials demonstrates how the major instrumental and methodological advances made in recent years by the NMR community (ultra-high magnetic fields, DNP, MAS MR imaging…) have enabled to deepen our knowledge of the structure of calcium-phosphate based biomaterials. In the case of synthetic materials, the most complete studies have consisted in combining high resolution NMR / DNP to other analytical studies (X-ray diffraction, PDF, IR…), and computational modeling (including GIPAW-DFT calculations of NMR parameters). For native bone and dental tissues, the most advanced NMR / DNP studies have enabled measuring the size of the ACP layer at the surface of apatite bone crystallites,

identifying the presence of yet-undetected minor organic species (e.g. nucleic acids), and determining the distance and binding geometry of small molecular ions like citrate at the surface of mineral particles. Moreover, by comparing NMR signatures of the natural materials to those of synthetically derived models, including *in vitro* cultured tissues, it has been possible to obtain important information on the inorganic and organic components, and to rule out previously proposed models concerning bone structure and formation.

While most investigations still concern ^{1}H, ^{31}P and ^{13}C NMR analyses, due to their more favorable NMR properties, ^{43}Ca NMR is now increasingly being used. Indeed, recent NMR and DNP studies show how calcium-43 is a good target to help refine the description of the surface-structure of synthetic apatites, and of the bulk-structure of (amorphous) calcium phosphates. Oxygen-17, however, has more rarely been studied, despite the high level of information that ^{17}O NMR can provide.[20, 93-95] Indeed, only a few investigations have looked into the ^{17}O NMR study of enriched model calcium-phosphates and related bioglasses,[21, 66, 69, 96, 97] and only one analysis of a bone sample has been reported, though the sensitivity was quite low.[66] With the current development of novel ^{17}O-labeling schemes based on mechanochemistry[98, 99] and of ^{17}O DNP,[100, 101] it can be expected that a much larger number of analyses on this nucleus will be performed in the future for these materials, including to help better understand the role played by one of the key components of bone, namely water. This is a point we are currently looking into.

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