Characterization of Ca-phosphate biological materials by scanning transmission X-ray microscopy (STXM) at the Ca L_{2,3}-, P L_{2,3}- and C K-edges

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Abstract

Several naturally occurring biological materials, including bones and teeth, pathological calcifications, microbial mineral deposits formed in marine phosphogenesis areas, as well as bio-inspired cements used for bone and tooth repair are composed of Ca-phosphates. These materials are usually identified and characterized using bulk-scale analytical tools such as X-ray diffraction, Fourier transform infrared spectroscopy or nuclear magnetic resonance. However, there is a need for imaging techniques that provide information on the spatial distribution and chemical composition of the Ca-phosphate phases at the micrometer- and nanometer scales. Such analyses provide insightful indications on how the materials may have formed, e.g. through transient precursor phases that eventually remain spatially separated from the mature phase. Here, we present scanning transmission X-ray microscopy (STXM) analyses of Ca-phosphate reference compounds, showing the feasibility of fingerprinting Ca-phosphate-based materials. We calibrate methods to determine important parameters of Ca-phosphate phases, such as their Ca/P ratio and carbonate content at the ~25 nm scale, using X-ray absorption near-edge spectra at the C K-, Ca L_{2,3}- and P L_{2,3}-edges. As an illustrative case study, we also perform STXM analyses on hydroxyapatite precipitates formed in a dense fibrillar collagen matrix. This study paves the way for future research on Ca-phosphate biomineralization processes down to the scale of a few tens of nanometers.

Keywords: X-ray microscopy; Ca-phosphates; polyphosphate; Nanoscale; XANES spectroscopy

1 Introduction

A diverse range of Ca-phosphate-based materials occur naturally, differing in their chemical composition and mineralogical structure (Table 1). The major mineral phase composing bones and teeth of vertebrates is carbonate hydroxyapatite (CHA) [1–3]. CHA can also be found in pathological calcification occurring in various parts of the body, including kidneys, cardiac valves and teeth, together with octacalcium phosphate, brushite or whitlockite [1,4]. Marine phosphorites, presumably formed by microbial activity, are mostly composed of carbonate fluoroapatite (CFA) [5,6]. Ca-polyphosphates, forming in eukaryotic and prokaryotic cells [7,8], are thought to play an important role in the formation of sedimentary CFA [9,10] and it has also been proposed that they could play a role in apatite biomineralization in bones [11]. Finally, brushite, monetite and tricalcium phosphate often compose biomimetic cements used for the repair of damaged bones and teeth [12,13].

The different Ca-phosphates and polyphosphates partly differ by their Ca/P ratios (Table 1). Ca/P ratios can therefore be used as a fingerprint of these phases. However, there can be significant variations of the Ca/P ratio for a given phase. For example, the apatitic mineral phase composing vertebrate bones is sometimes referred to as Ca-deficient hydroxyapatite (CDHA), with a Ca/P ratio of ~ 1.5 , compared to 1.67 for stoichiometric HA. Such a variation has great importance since the chemical and biological properties of CDHA are strongly dependent on their Ca/P ratios. For instance, the decrease in the Ca/P ratio is associated with increase in structural disorder (Ref. [14] and references therein), which could explain the higher solubility [15] and degradation rate in solution [16] of CDHA compared to stoichiometric HA.

The carbonate content is another important parameter influencing Ca-phosphate properties. CHA in bones and teeth or CFA in sediments contain carbonate ions (CO_3^{2-}),

which may be located in the *c*-axis anion channels of apatite (A-site) or substitute for tetrahedral PO₄³⁻ groups (B-site) [17]. Incorporation of CO₃²⁻ has important effects on the physical and chemical properties of apatite [3]. For example, the solubility product of CHA in enamel is several orders of magnitudes greater than that of stoichiometric HA [1,18]. The presence of CO₃²⁻ also increases the dissolution rate of apatite in acids [19,20]. CO₃²⁻ substitution in apatite is generally associated with a decreased crystallinity [21–23], which results in altered mechanical properties (modulus and hardness) [24].

Characterization of Ca-phosphate biological materials (e.g. phase identification, Ca/P ratio, CO₃²⁻ content) is usually achieved using bulk methods such as X-ray diffraction (XRD) (e.g. [25,26]), Fourier transform infrared (FTIR) spectroscopy (e.g. [27,28]), Raman spectroscopy (e.g. [29,30]) or nuclear magnetic resonance (NMR) (e.g. [31,32]). However, there is also a need for imaging techniques that provide information on the submicrometer-scale spatial distribution and chemical composition of the Ca-phosphate phases, as well as on the way they are associated with the organic matrix within which they formed. Such spatial information may provide insightful indications on how these materials formed. For instance, it has been suggested that the formation of bones proceeds through the replacement of transient non-apatitic mineral phases such as amorphous Ca-phosphate [33,34] or octacalcium phosphate [29,35]. However, the newly forming bone mineral might alternatively be composed of nanosized and poorly crystalline apatite, with non-apatitic minerals at its surface [36]. This question of the existence and nature of the precursor phase(s) of bone minerals is still debated [2]. Transient precursor phases of Ca-phosphate biominerals might sometimes be physically separated from mature phases, but they very often occur as fine mixtures with them (e.g. [25]). Discriminating and characterizing different Ca-phosphate phases at the few nanometers scale would be of great interest for gaining further insights into these issues.

Transmission electron microscopy (TEM), which allows for high-resolution imaging, electron diffraction, energy-dispersive X-ray spectroscopy (EDXS) and electron energy loss spectroscopy (EELS) to be performed, has often been employed to study the initial stages of tissue mineralization (e.g. [37–42]). For example, single-crystal electron diffraction patterns have been used to characterize mixtures of several Ca-phosphates, including α -tricalcium phosphate (α -TCP), amorphous calcium phosphate (ACP) and hydroxyapatite (HA) [38,39,41], but, as pointed out by Leng et al. [38], electron diffraction ring patterns can be controversial and should be used for the identification of Ca-phosphates only very cautiously. Moreover, irradiation by an electron beam can induce damage in the precursor phases, as well as alter their spatial relations with the organic matrix [35]. For instance, Ca/P ratios of synthetic hydroxyapatites have been shown to be modified by beam-induced damage that occurs during TEM–EDXS and EELS analyses [43]. Finally, TEM usually provides only limited information on the chemical composition of the organic matrix.

Synchrotron-based scanning transmission (soft) X-ray microscopy (STXM) is a microscopy technique that provides chemical speciation-sensitive images at a spatial resolution down to ~25 nm, coupled with X-ray absorption near-edge spectra (XANES) over a relatively extended range of energies (between 100 and 2000 eV). It allows the characterization of the speciation (i.e. coordination and/or redox state) of various elements, including the major elements of Ca-phosphates (e.g. Ca and P) as well as associated carbonate ions and organic molecules. Moreover, STXM induces less beam damage compared to TEM-based techniques [44,45]. A number of studies on biomineral formation, including hydroxyapatite biomineralization (see Ref. [46] and references therein), have used STXM/XANES spectromicroscopy previously, but they did not use the information provided by this technique to its full extent.

Several studies characterizing reference Ca-phosphates by bulk-scale XANES spectroscopy at the Ca L_{2,3}-edges [33,47,48] and P L_{2,3}-edges [49,50] have been published. The present study includes XANES spectra at the P and Ca L_{2,3}-edges and the C K-edge acquired by STXM for a large number of reference Ca-phosphates and polyphosphates. It provides qualitative and quantitative approaches for the characterization of Ca-phosphate-based materials at the ~25 nm scale, including (i) fingerprinting of these phases, notably using Ca/P ratios, and (ii) estimation of the carbonate content of apatites.

2 Materials and Methods

2.1 Reference compounds

The reference compounds analyzed in this study are marked by an asterisk in Table 1, where abbreviations and generic formulae are also given.

Some of the Ca-phosphate and polyphosphate reference compounds were purchased from Sigma-Aldrich Co.: HA nanopowder, α -TCP, ACP and sodium polyphosphate (Na_(n+2)P_nO_(3n+1), $n = 45 \pm 5$; Na-PP). The composition and purity of these samples was checked by XRD using a Bruker D2 Phaser XRD desktop system with a Cu-K α radiation, except for the Na-PP, which is sold by Sigma-Aldrich as a glass (sodium phosphate glass) (Supplementary Information, Fig. S1). Other reference compounds were synthesized and provided by Frédérique Pourpoint: dicalcium phosphate dihydrate (mineral brushite, DCPD), monocalcium phosphate monohydrate (MCPM), β -tricalcium phosphate (β -TCP) and tetracalcium phosphate (TTCP), Ca-polyphosphate (β -Ca(PO₃)₂) (PP) and dicalcium phosphate anhydrous (mineral monetite, DCPA). These samples have previously been synthesized and characterized by bulk methods, and the results of these characterizations are published in Refs. [51–53]. Two synthetic biomimetic carbonated hydroxyapatites (CHA1, CHA 2), referred to as HA-1 and HA-2 in Ref. [54] and differing in their carbonate

concentrations, were also used. They were characterized by XRD, NMR and FTIR, as detailed in Ref. [54]. Their carbonate concentrations were measured by thermogravimetric analysis and are respectively ~ 2.2 and ~ 4.9 wt.% CO_3^{2-} (Stanislas Von Euw, personal communication).

Other reference compounds were natural samples from the field. The carbonate fluoroapatite (mineral francolite, CFA) sample was collected in the sedimentary phosphate deposit of Ouled Abdoun (Morocco) and characterized by bulk XRD measurements [55]. Its carbonate content was ~ 6.9 wt.% CO_3^{2-} , as shown by the empirical relation between the carbonate content of CFA and the $\Delta(2\theta)$ difference between the (004) and (410) XRD peaks derived from Schuffert et al. [56]. It contained 37.9 wt.% Ca, 14.5 wt.% P and 4.2 wt.% F, as assessed by electron microprobe analyses. A tooth from a camelid (T1 in Ref. [57]) and a bone from an unidentified animal (probably a donkey) [58] from the Tugen Hills (Great Rift Valley, Kenya) were also used and characterized by bulk FTIR [57,58]. Their elemental compositions, determined by atomic absorption spectroscopy, were 35 wt.% Ca, 17.8 wt.% P and 80 ppm F for the tooth sample, and 24.7 wt.% Ca, 13.21 wt.% P and 1622 ppm F for the bone sample (Haohao Yi, personal communication).

Finally, a bone-like matrix (Coll/CHA/SBF in Ref. [40]) obtained by co-precipitation of calcium phosphate salts and collagen molecules in simulated body fluid was studied.

2.2 Sample preparation for STXM

All reference samples except the Coll/CHA/SBF matrix were gently ground in pure ethanol in an agate mortar. About 2 μl of each sample was deposited on Si_3Ni_4 windows. The samples were then dried at ambient temperature.

The Coll/CHA/SBF matrix was fixed with glutaraldehyde, washed in a cacodylate/saccharose buffer solution, dehydrated and embedded in araldite. A thin section (80 nm) deposited on a TEM grid was used for STXM analyses.

2.3 Data acquisition and spectral processing

STXM/XANES analyses were performed on beamline 11.0.2.2 at the Advanced Light Source (Lawrence Berkeley National Laboratory, Berkeley, USA) [59] and the SM beamline at the Canadian Light Source (Saskatoon, Canada) [60]. Methods for STXM analyses and data processing can be found, for example, in Bourdelle et al. [61] and Cosmidis and Benzerara [46]. For each compound, stacks at the Ca $L_{2,3}$ -edges (340-370 eV) and sometimes the P $L_{2,3}$ -edges (125-190 eV) or the C K edge (260-320 eV) were acquired on an area containing one or several particles. When multiples stacks were acquired at the same area different edges, they had the same pixel number and size, so that they could be appended in a single stack. XANES spectra were then extracted from these stacks using aXis2000 [62]. Energy calibration at the C K-edge was performed using the 3*p* Rydberg peak at 294.96 eV of gaseous CO₂ flushed into the STXM chamber. The energy position of the major peak on the L_2 edge of Ca was set to 352.5 eV for all reference spectra. For spectra at the P $L_{2,3}$ -edges in Fig. 2, the energy of the first peak was set to 136.7 eV.

Absorption saturation effects, caused by sample thickness, are frequent at the Ca $L_{2,3}$ -edges, and cause distortions of the XANES spectra [63]. In order to avoid such spectral distortions, the spectra of the reference Ca-phosphate compounds were extracted from areas showing a maximum optical density (OD) value of <1 at 349.2 eV (energy of the major peak on the Ca L_3 edge) (see Supplementary Information, Fig. S2).

A linear background correction was applied to the spectra at the Ca $L_{2,3}$ -, P $L_{2,3}$ - and C K-edges by setting the baseline in the 340-345, 125-134 and 260-280 eV regions,

respectively, to an horizontal line at 0, in order to eliminate the contribution of lower energy absorption. The edge steps at the Ca L_{2,3}- and P L_{2,3}-edges resulting from transitions to the continuum were subtracted using double arctan functions, as described in Ref. [61]. These two operations (linear background correction and double arctan subtraction), applied to the Ca L_{2,3}-edge spectrum of HA, are illustrated in Fig. S3.

3 Results

3.1 XANES spectroscopy of Ca-phosphate reference compounds at the Ca and P L_{2,3}-edges

3.1.1 XANES analyses at the Ca L_{2,3}-edges of Ca-phosphates reference compounds

Ca L_{2,3}-edge XANES spectra were measured for nine Ca-phosphate reference compounds (Fig. 1). The energy positions of the peaks in these spectra are given in Table 2.

The positions of the main L₂ peak (~352.5 eV) and L₃ peak (~349.2 eV) are very similar for all analyzed compounds. However, variations exist in the positions and relative intensities of smaller pre-edge peaks (hereafter called a₃–d₃ and a₂–c₂) among the different Ca-phosphates. In most XANES spectra (TTCP, HA, α-TCP, DCPA, DCPD, MCPM), there are two small peaks (or a small peak and a shoulder), a₃ and b₃, of about the same intensity, with a third, more intense, peak, c₃. This pattern is characteristic of an octahedral crystal field [48]. Alpha- and β-TCP are two polymorphs of Ca₃(PO₄)₂, α-TCP being the high-temperature form and β-TCP the low temperature one. The structure of α-TCP is related to that of HA [64], which is reflected by very similar Ca L_{2,3}-edges XANES spectra for α-TCP and HA. In contrast, β-TCP has a very distinct spectrum, consistent with a different local electronic environment of Ca in this polymorph [65]. Ca-polyphosphate (also called Ca-metaphosphate)

β -Ca(PO₃)₂ (PP) exhibits long polymeric chains of PO₄³⁻ arranged in a zigzag configuration, where Ca²⁺ are intercalated as charge-balancing ions, sharing only partially covalent bonds with O atoms [52,66].

3.1.2 XANES analyses at the P L_{2,3}-edges of Ca-phosphate reference compounds

Most previous XANES studies of P minerals have focused on the P K-edge at ~2150 eV [67–71]. Soft X-ray STXM allows for the measurement of XANES spectra at the P L_{2,3}-edges which lie at around 130-150 eV. XANES spectroscopy at the P L_{2,3}-edges is technically more difficult to perform because of high absorption at these low energies, but it provides much information on P speciation [50] and therefore may allow a very good identification of crystalline and amorphous P-bearing minerals, which, owing to the use of STXM, can be conducted at the submicrometer-scale.

Fig. 2 shows XANES spectra at the P L_{2,3}-edges of several reference Ca-phosphate compounds. The two peaks a (at ~136.7 eV) and b (at ~137.6 eV) correspond to the L₃ and L₂ edges, respectively. The ~1 eV energy separation between these two peaks is insensitive to the chemical environment of P [50], and is therefore the same for all compounds. At higher energies, a broad peak (c) is observed for Na-PP and MCPM, whereas two peaks (c and d, at ~138.4 and ~139.0 eV, respectively) can be distinguished for the other compounds (DCPA, α -TCP and HA). The positions of peaks a, b, c and d are similar for all compounds (except for peak d in the spectrum of DCPA, which is shifted slightly towards higher energies, at ~139.3 eV). However, the relative intensities of these peaks vary among the different compounds spectra. The shoulder at ~142 eV (peak e) is a common characteristic of all Ca-phosphates [50], and has been interpreted as the result of transitions from P2p to empty Ca3d orbitals. It is, however, present (but much fainter) in the Na-PP spectrum (which does not

contain Ca). The broad peak at ~146.9 eV (peak f) is sometimes called the “shape resonance”, and its position is sensitive to the local chemical environment of P. It appears to be split into two broad peaks in the Na-PP spectrum. The spectrum of Na-PP presented here is similar to previously published spectra of calcium pyrophosphate (CPP) [49,50], indicating that the local chemical environment of P is similar in both compounds.

3.1.3 Assessment of Ca/P ratios in Ca-phosphates using STXM at the Ca L_{2,3} and P L_{2,3}-edges

Three reference Ca-phosphate reference compounds were used to calibrate this method: MCPM (Ca/P = 0.5), DCPA (Ca/P = 1) and α -TCP (Ca/P = 1.5). For each reference compound, linear background-corrected spectra at the P and Ca L_{2,3}-edge spectra were used (Fig. 3A). According to the Beer–Lambert law, the height of the step measured at approximately 144 eV (h_P) in P L_{2,3}-edge spectra is proportional to the amount of P in the probed volume. Similarly, the height of the edge step of Ca at 360 eV (h_{Ca}) is proportional to the Ca amount in the same probed volume (Fig. 3C). Therefore, the parameter $R_{Ca/P}$, defined as: $R_{Ca/P} = h_{Ca}/h_P$, is proportional to the molar Ca/P ratio of a compound with a y-intercept of 0. In order to retrieve the factor of proportionality, $R_{Ca/P}$ was measured on ~1500 to ~9000 pixels for each of the four reference compounds. The mean $R_{Ca/P}$ ratios thus obtained for the four reference compounds were plotted vs. their molar Ca/P ratios (Fig. 4). A linear correlation was obtained between Ca/P molar ratios and $R_{Ca/P}$, with a correlation coefficient of 0.97, and following the equation: $Ca/P = 0.8895 \times R_{Ca/P}$. The residual standard error of the regression was ± 0.15 .

3.2 XANES spectroscopy of apatites at the Ca L_{2,3}- and C K-edges

A number of important naturally occurring Ca-phosphate-based materials belong to the apatite family. Specific XANES signatures at the Ca L_{2,3}-edges resulting from the CO₃²⁻

substitutions in apatite and associated alterations of crystallinity have been suggested by previous studies [33,47]. As biological materials are often intimately associated with an organic matrix, the determination of the C/Ca ratio does not provide a strict estimation of the carbonate contents of these materials. However, carbonate functional groups can be efficiently detected, distinguished from organic carbon functional groups and specifically quantified by STXM at the C K-edge, using the peak at ~290.2 eV, which corresponds to $1s \rightarrow \pi^*$ electronic transitions in carbonate groups [72–74]. We focused here on apatitic compounds with the aim of developing an approach to assess their carbonate content.

3.2.1 XANES spectra of reference compounds and test samples at the C K- and Ca L_{2,3}-edges

The C*/Ca weight ratios (C* referring to C atoms in carbonate functional groups) of the different reference compounds used in this study (CHA 1, CHA 2 and CFA) were calculated using the chemical data provided in Section 2.2, and are 0.012, 0.028 and 0.036 for CHA 1, CHA 2 and CFA, respectively.

Fig. 5 shows the XANES spectra at the C K-edge and Ca L_{2,3}-edges of the three reference compounds used in this study as well as two test samples with unknown carbonate concentrations (a tooth and a bone). They were normalized to the same height of the Ca edge step at 360 eV. The peak corresponding to the $1s \rightarrow \pi^*$ transitions in carbonate groups (at ~290.1-290.2 eV) is the main peak in the XANES spectra at the C K-edge for all reference compounds and test samples. Small peaks at 284.8 and 288.1 eV and a shoulder at 287.1 eV are also systematically present. They correspond to electronic transitions in organic C functional groups – $1s \rightarrow \pi^*$ transitions in aromatics, amides (e.g. peptide groups in proteins) and phenols and ketones, respectively [72–76]. Their presence in the spectra of CHA 1 and 2, which were synthesized inorganically, suggests that some contamination by organic

molecules occurred during sample preparation. XANES spectra of the bone sample presents a much stronger signal at the C K-edge than the reference compounds and other test samples, with a large peak at 288.2 eV and an additional peak at 289.4 eV. Bones typically have high organic contents (typically ~20 wt.%, which can be compared with the low organic content of mature enamel of ~0.1%), mostly as Type 1 collagen [1]. The peak at 289.4 eV is often attributed to $1s \rightarrow 3p/\sigma^*$ electronic transitions in alcohols [73,76] and may result from the presence of hydroxylated amino acids in collagen [77].

3.2.2 Rationale for CO₃/Ca quantification by STXM

Linear background-corrected spectra at the Ca L_{2,3}-edges and C K-edges were used here (Fig. 6). $R_{\text{CO}_3/\text{Ca}}$ is the ratio between the intensity of the peak corresponding to carbonate groups (at 290.2 eV) (i.e. the absorption at 290.2 minus the absorption just after the peak at 291 eV) and the height of the Ca edge step at 360 eV. It is calculated as: $R_{\text{CO}_3/\text{Ca}} = (\text{OD}_{290.2} - \text{OD}_{291})/\text{OD}_{360}$, where OD_{*x*} is the optical density value at *x* eV. $R_{\text{CO}_3/\text{Ca}}$ was measured for ~900-3100 pixels for each sample.

We plotted $R_{\text{CO}_3/\text{Ca}}$ vs. the C*/Ca weight ratio for the reference compounds (Fig. 7). The Beer–Lambert law predicts a linear relation, with a *y*-intercept of 0. The relationship between $R_{\text{CO}_3/\text{Ca}}$ and C*/Ca could thus be retrieved with the following equation: (C*/Ca)(wt.% ratio) = $0.030 \times R_{\text{CO}_3/\text{Ca}}$. The coefficients of correlation R^2 was ~0.97. The residual standard error of the regression was ±0.0018.

We used this relation to determine the C*/Ca weight ratios and the carbonate contents (by multiplying C*/Ca by Ca concentration in wt.%) of the two test samples. The teeth had a C*/Ca weight ratio of ~0.02 (±9%) and a carbonate content of ~0.72 wt.% C, and the bone had a C*/Ca weight ratio of ~0.03 (±6%) and a carbonate content of ~0.73 wt.% C. These

values are within the range of typical carbonate contents for bone and teeth minerals provided by Elliott [1].

3.3 Case study: STXM analyses of a bone-like collagen/apatite matrix at the C K- and the Ca L_{2,3}-edges

Fig. 9 illustrates the use of STXM/XANES to characterize and map Ca-phosphates in biological materials. The sample is composed of Ca-phosphate precipitated on a collagen matrix (Coll/CHA/SBF, [40]) and was mapped by STXM at the C K- and Ca L_{2,3}-edges with a spatial resolution of ~25 nm (Fig. 9A–D). Individual collagen fibrils, measuring ~80 nm in diameter, as well as Ca-phosphate precipitates (50–300 nm) are visible. XANES spectra at the C K- and Ca L_{2,3}-edges were obtained from these precipitates. The spectra at the Ca L_{2,3}-edges obtained on different grains were identical, showing that a single Ca-phosphate phase has precipitated in the collagen matrix. The average spectrum shown in Fig. 9F is similar to the spectrum of the reference HA (Fig. 1), confirming that the precipitates have an apatitic structure. The C K-edge spectrum (Fig. 9 E) is similar to the C K-edge spectra of collagen obtained by X-ray photoemission electron spectromicroscopy (X-PEEM/XANES) by Lam et al. [78], with two sharp peaks at 285.1 and 288.5 eV, assigned to $1s \rightarrow \pi^*$ electronic transitions in C=C bounds and carbonyl (C=O) groups, respectively, and a shoulder at 287.2 eV, assigned to $1s \rightarrow \sigma^*$ transitions in C–H bounds [78]. An additional peak at 290.2 eV ($1s \rightarrow \pi^*$ transitions in carbonate groups), indicated by an arrow in Fig. 9E, shows that the apatite precipitates are carbonated, confirming the bulk FTIR analyses performed by Wang et al. [40] on the same sample. The $R_{\text{CO}_3/\text{Ca}}$ ratio of this apatite as calculated using the method described in the previous section is ~0.19, corresponding to a C*/Ca ratio of 0.005 ($\pm 30\%$). No large spatial variations of this ratio were detected in the analyzed area. The calculated C*/Ca ratio is very low, with a higher error bar, but the peak at 290.2 eV, which is

characteristic of carbonates, is clearly significant (Fig. 9) and this overall demonstrates the low detection limit of STXM for carbonates.

4 Discussion

4.1 Fingerprinting of Ca-phosphate biological materials using STXM/XANES at the Ca $L_{2,3}$ - and P $L_{2,3}$ -edges

4.1.1 Fingerprinting of Ca-phosphate biological materials using XANES spectra at the Ca and/or P $L_{2,3}$ -edges

As described in Section 3.1.1, XANES spectra of Ca-phosphate-based materials (mainly differences in the positions and intensities of minor peaks) can be used as probes of the local geometry and electronic structure of Ca in these phases (Fig. 1 and Table 2). Besides its imaging capabilities at high spatial resolution (~ 25 nm), the main advantage of STXM/XANES over bulk methods such as XRD, FTIR or RMN is the possibility of obtaining structural information on very small quantities of sample. Indeed, the investigated area in our case study (Fig. 9) is only a few micrometers in width, and XANES spectra at the Ca $L_{2,3}$ -edges obtained on precipitates measuring only a few tens of nanometers could be used to demonstrate their apatitic structure.

Previous studies have shown the feasibility of using such specific fingerprints for micrometer- to nanometer-scale mapping of different Ca-phosphate phases in biological materials. For example, Benzerara et al. used STXM/XANES at the Ca $L_{2,3}$ -edges and the C K-edge to identify CHA, among other candidate phases, as the mineral formed by *Caulobacter crescentus* cells cultured in the presence of high calcium concentrations (8 mM) [72]. More recently, Beniash et al. used X-PEEM/XANES at the Ca $L_{2,3}$ -edges to compare, characterize and map newly formed enamel minerals at different stages of maturation, and

demonstrated that the phase formed at an early stage of enamel mineralization is ACP, which then transforms into CHA [33].

Ca-phosphates can be further characterized at the submicrometer scale by STXM using XANES spectroscopy at the P L_{2,3}-edges. Indeed, the P L_{2,3}-edge XANES spectra of Ca-phosphates and polyphosphates present distinctive features, which can be used for fingerprinting these different species (see Section 3.1.2 and Fig. 2). The spectra of MPCM, DCPA, DCPD, CPP, α -TCP, β -TCP and HA shown here and acquired by STXM were very similar to those obtained previously by Kruse et al. [50] and Demirkiran et al. [49] at the bulk scale, showing that it is possible to perform these analyses at the ~25 nm scale. XANES P L_{2,3}-edge spectroscopy has been used to study the nature of Ca-phosphates present in ceramics used for restoration of damaged bone tissues [49]. P K-edge X-ray fluorescence spectromicroscopy was used to identify apatite and polyphosphates in marine sediments and map their association with a sub-micrometer-scale spatial resolution, supporting the importance of polyphosphates in sedimentary formation of apatite [9,67]. Using P L_{2,3}-edges STXM/XANES spectromicroscopy for fingerprinting Ca-phosphates in such environmental samples at a higher spatial resolution is an exciting and yet unexplored area.

4.1.2 Fingerprinting of Ca-phosphate biological materials using Ca/P ratios

We have shown that Ca/P ratios of Ca-phosphate biological materials can be measured using STXM/XANES at the Ca and P L_{2,3}-edges, with an error of ± 0.15 . This can be performed down to the ~25 nm scale. As stated in the introduction, Ca/P ratios can be used for fingerprinting Ca-phosphates. Mapping Ca-phosphates based on their whole Ca or P L_{2,3}-edges spectra requires the use of stacks, which can be time-consuming. The method described in Section 3.1.3 requires the acquisition of only six images: two images in the P L_{2,3}-pre-edge

region, two images in the Ca L_{2,3}-pre-edge region for linear background removal, one image at 144 eV and one image at 360 eV, allowing relatively fast mapping of Ca/P ratios while maintaining good spatial resolution. Although Ca/P ratio can be determined more accurately with bulk chemical methods, STXM offers the possibility to map variations of this ratio in a sample at the 25 nm scale.

4.2 Assessing carbonate content of apatitic biological materials using STXM/XANES at the C K- and Ca L_{2,3}-edges

The incorporation of carbonates in apatites greatly influences their physical and chemical properties (e.g. [3]). Incorporation of CO₃²⁻ ions in apatite and their distribution in A- and B-type sites are usually assessed by bulk techniques such as XRD, FTIR and NMR (e.g. [1,27,28,54]). High-resolution TEM and electron diffraction do not allow for the detection of CO₃²⁻ substitution in apatites (the electron diffraction patterns of CHA are similar to those of pure HA [42,79,80]). Moreover, CO₃²⁻ groups in apatites are difficult to map by TEM–EDXS when they are mixed with organic carbon, as is often the case in biological materials. Fig. 9 shows that STXM, on the other hand, allows the detection of CO₃²⁻ groups in apatites even within an organic matrix. STXM moreover provides information on the composition of organic matrix associated with the precipitates. In Section 2.2.3, a STXM/XANES-based method is proposed that allows the specific quantification and mapping of carbonates in apatites at the 25 nm scale, even when organic carbon is present.

Increased carbonate contents in apatites are generally associated with decreased crystallinity [22–24]. The replacement of PO₄³⁻ groups by CO₃²⁻ in nanosized apatites moreover leads to (i) the development of an ACP-like hydrated disorder surface layer at the expense of the crystalline apatitic core and (ii) a decrease in the crystallite size [81]. Beniash et al. [33] compared XANES spectra at the Ca L_{2,3}-edges of HA, low-crystallinity HA and

ACP, and found that the position of peak d_3 (at ~ 348.5 eV) was sensitive to crystallinity, appearing at lower energy in crystalline compared to amorphous phases. This finding is not confirmed by our analyses. Indeed, the position of this peak for CHA 2 is shifted slightly towards lower energies compared to CHA 1 (Fig. 8), while CHA 2 contains more carbonate than CHA 1 (~ 2.2 wt.% CO_3^{2-} for CHA 1 vs. ~ 4.9 wt.% CO_3^{2-} for CHA 2). The peak at ~ 348.5 eV also occurs at lower energies in the tooth and bone sample spectra compared to the CHA 1 spectra, which, according to Beniash et al. [33], would indicate a relatively higher crystallinity, in disagreement with higher carbonate contents. This suggests that either (i) the crystallinity of these compounds is not controlled by carbonate content only (but also for instance by crystallite size) or (ii) the position of peak d_3 is influenced by parameters other than crystallinity.

CO_3^{2-} ions in carbonate apatites can be located in the c -axis channels of apatite (A-site) or they can substitute for tetrahedral PO_4^{3-} groups (B-site) [17]. Fleet and Liu [47] compared the Ca $L_{2,3}$ -edges XANES spectra of synthetic A-type carbonate hydroxyapatites. They found that the only feature characteristic of the presence of CO_3^{2-} was the weak intensity of peak b_3 (at ~ 347.6 eV). This was interpreted as a change in Ca coordination due to the presence of CO_3^{2-} in the apatite channels. Here, we observe that the intensity of peak b_3 is quite similar in the spectra of the different reference compounds and test samples. CO_3^{2-} ions are mainly present in B-sites in CHA 1 and 2 according to Nassif et al. [54]. It has been shown by FTIR and magic-angle spinning NMR studies that most CO_3^{2-} groups ($\sim 75\%$) in sedimentary CFA occupy the B-sites (e.g. [28,82]), and it is also known that carbonate anions mainly substitute for PO_4^{3-} (B-sites) in the mineral phase of bones [1]. However, Yi [58] showed that a significant portion of CO_3^{2-} ions are present in A-sites in the tooth samples studied here. The relationship between the presence of A-type carbonates in apatites and b_3 peak intensity proposed by Fleet and Liu [47] therefore does not apply here.

The influence of carbonate substitution on the XANES spectra at the Ca L_{2,3}-edges of apatites requires further investigation. Simulation of experimental Ca L_{2,3}-edges XANES spectra of different types of Ca-carbonates using multiplet calculations is an effective method to provide information about the local coordination of Ca in these biological materials [83]. This approach therefore appears to be a promising way to gain further understanding of the interpretation of apatite spectra at the XANES L_{2,3}-edges.

5 Conclusion

This study shows the feasibility of identifying and mapping Ca-phosphate minerals as well as important parameters relevant to biomineralization studies, such as the Ca/P ratio and carbonate content of these phases at the sub-micrometer scale. Unlike bulk methods, such as XRD, FTIR or NRM, STXM requires minimal amounts of samples, and offers imaging capabilities. Moreover, STXM is unique in allowing the detection, mapping (at the ~25 nm scale) and quantification of carbonate groups in apatite minerals embedded in an organic matrix.

The Ca-phosphate fingerprinting methods presented here can be applied to a range of studies dealing with the formation of biological materials. An interesting potential application is the characterization of Ca-phosphate mixtures in such materials. For instance, apatite composing the mineral fraction of bones and teeth is thought to precipitate via the formation of other non-apatitic Ca-phosphate precursor phases [33,34,29,35]. As a consequence, mixtures of mature apatite and precursor phases resulting from the incomplete transformation of the transient phases to apatite, or the co-precipitation of apatite with other Ca-phosphates, may occur in such materials. Cements used for bone and tooth implants are also very often composed of mixtures of Ca-phosphates (e.g. [84–86]), and HA sometimes coexists with other Ca-phosphate phases in pathologic calcifications [30,87,88]. Few studies have dealt

with the characterization of Ca-phosphates mixtures despite the abundance of such mixtures in natural systems (see Ref. [25] and references therein). STXM/XANES is a promising method for the sub-micrometer-scale characterization of Ca-phosphates mixtures, with the potential to improve our understanding of the formation mechanisms of these materials.

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Captions

Table 1

List of several calcium phosphate compounds (adapted from Elliott (2002) [1] and Dorozhkin (2009) [13])

{TABLE FOOTNOTE} The compounds indicated by an asterisk were analyzed in this study.

Table 2

Energy positions of the peaks in the Ca L_{2,3}-edge spectra of reference Ca-phosphate compounds

713 {TABLE FOOTNOTE} The numbers in brackets refer to energy positions of shoulders in the
714 spectra.

715 Fig. 1. XANES spectra of the Ca L_{2,3}-edges of several Ca-phosphate reference compounds.
716 Vertical dotted lines correspond to the peak energies of HA (see Table 2).

717 Fig. 2. XANES spectra of the P L_{2,3}-edges of reference Ca-phosphate compounds. Vertical
718 dotted lines correspond to energy positions of the peaks in HA spectrum (136.7, 137.6, 138.4,
719 139.0, 142.0 and 146.9 eV).

720 Fig. 3. (A) XANES spectrum of MCPM at the P and Ca L_{2,3}-edges before (black line) and
721 after (gray line) performing linear background correction. (B) Linear background-corrected
722 spectrum at the P L_{2,3}-edges. The red arrow shows the positions of the edge step used for
723 measuring $R_{Ca/P}$. (C) Linear background-corrected spectrum at the Ca L_{2,3}-edges. The black
724 arrow shows the position of the edge step used for measuring $R_{Ca/P}$.

725 Fig.4. Ca/P molar ratios vs. $R_{Ca/P}$ measured for MCPM, DCPA and α -TCP. the regression
726 line and correlation coefficient (R^2) are shown. Error bars correspond to standard deviations
727 for $R_{Ca/P}$ measurements on ~1500-9000 pixels.

728 Fig. 5. Linear background-corrected XANES spectra at the C K-edge (left) and Ca L_{2,3}-edges
729 (right) of reference compounds and test samples. The position of the Ca L₂-edge has been
730 adjusted to 352.5 eV (vertical dotted line). The spectra have been divided by the height of the
731 edge step at 360 eV.

732 Fig. 6. XANES spectrum of CFA at the C K-edge and Ca L_{2,3}-edges. Linear background
733 correction has been performed at both C K and Ca L_{2,3} edges. $R_{CO_3/Ca}$ is calculated by
734 measuring the height of the peak at 290.2 eV and the height of the Ca edge step at 360 eV
735 (blue arrows).

736 Fig. 7. C*/Ca weight ratio (C* are C atoms present as carbonates) vs. $R_{\text{CO}_3/\text{Ca}}$ measured for
737 reference compounds CHA1, CHA2 and CFA. The regression line obtained and correlation
738 coefficients (R^2) are shown. Error bars correspond to standard deviations for $R_{\text{CO}_3/\text{Ca}}$
739 measurements on ~900-3100 pixels.

740 Fig. 8. Magnification of the Ca $L_{2,3}$ -pre-edge region of the XANES spectra of reference
741 compounds and test samples.

742 Fig. 9. STXM/XANES maps and spectra of apatite nanoparticles precipitated on a bone-like
743 collagen matrix (Coll/CHA/SBF in Ref. [40]). (A) Image at 288.2eV. (B) Map of organic
744 carbon obtained by subtracting an image at 280 eV (below the C K-edge) from an image at
745 288.2 eV (corresponding to $1s \rightarrow \pi^*$ electronic transition in amide groups). (C) Corresponding
746 calcium map obtained by subtracting an image at 340 eV (below the Ca $L_{2,3}$ -edge) from an
747 image at 349.2 eV (corresponding the Ca L_3 edge). (B) and (C) were acquired from the same
748 sub-area, designated by a square in image (A). The pixel size on maps (B) and (C) is 20 nm.
749 (D) Composite map obtained using (B) and (C), showing collagen fibers in red and calcium
750 (apatite precipitates) in blue. (E) XANES spectrum at the C K edge obtained on apatite
751 precipitates. Three main peaks are present, at 284.8 eV ($1s \rightarrow \pi^*$ transitions in aromatics),
752 288.2 eV ($1s \rightarrow \pi^*$ transitions in amides) and 290.2 eV ($1s \rightarrow \pi^*$ transitions in carbonates)
753 (arrow). (F) XANES spectrum at the Ca $L_{2,3}$ -edge obtained on apatite precipitates.

754 Fig. S1. XRD patterns of commercial Ca-phosphate compounds ACP, α -TCP and HA.
755 Squares and circles correspond to reference ICDD patterns of HA (ICDD #09-0432) and α -
756 TCP (ICDD #29-0359), respectively. All observed diffraction peaks relate to reference
757 spectra and no impurity phases were found.

758 Fig. S2. Assessment of spectral distortions in XANES spectra at the Ca- $L_{2,3}$ edge due to
759 absorption saturation effects. (A) Map at 349.2 eV of TTCP particles showing different

760 ranges of absorption (in OD units). The different colors represent areas from which the
761 spectra shown in (B) were extracted, corresponding to different OD ranges (i.e. different
762 thicknesses of the particles). (B) XANES spectra obtained on the different areas represented
763 in (A), extracted using the Mask tool of aXis2000. It can be seen that some distortions of the
764 spectra occur when the sample becomes too thick. (C) Plots of peak a_3 height (ha_3) vs. peak
765 L_3 height (hL_3) for each pixel of the sample in (A). The dashed line corresponds to the
766 ha_3/hL_3 ratio obtained for pixels with a maximum OD (hL_3) of <1 . For thin areas of the
767 sample, the ratio between ha_3 and hL_3 is roughly a constant. However, for thicker areas (e.g.
768 areas with $OD > 1.5$), this ratio increases with the thickness of the sample, indicating
769 variations in the relative heights of the peaks (pixels deviate from the dashed line at high
770 maximum OD values, showing that the ha_3/hL_3 ratio increases with increasing sample
771 thickness). In order to avoid such spectral distortions, the spectra of the reference Ca-
772 phosphate compounds were extracted from areas showing a maximum OD value of <1 at
773 349.2 eV (the energy position of the peak L_3). For example, only the purple and blue regions
774 in (A) would be selected for the TTCP XANES spectra at the Ca $L_{2,3}$ -edges.

775 Fig. S3. Ca $L_{2,3}$ -edges XANES spectra processing. The black line is the measured spectrum
776 of HA. First, a linear background background correction was applied to obtain the gray line,
777 with 0 OD and a zero slope in the pre-edge region (340–345 eV). Second, a double arctan
778 function (blue line) was subtracted to obtain the final spectrum (in red).

Graphical Anstract

Chemical mapping
(~25 nm spatial resolution)

Ca
Corg

XANES spectroscopy

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

Table 1

Name	Abbreviation	Formula	Ca/P
Polyphosphates*	PP	$M^I_m H_{(n+2)} P_n O_{(3n+1)}$ $M^I = Ca^{2+}, Na^+, Fe^{3+}, Fe^{2+}, \dots$	ε
Monocalcium phosphate monohydrate*	MCPM	$Ca(H_2PO_4)_2 \cdot H_2O$	0.5
Monocalcium phosphate anhydrous	MCPA	$Ca(H_2PO_4)_2$	0.5
Brushite, Dicalcium phosphate dihydrate*	DCPD	$CaHPO_4 \cdot 2H_2O$	1.0
Monetite, Dicalcium phosphate anhydrous*	DCPA	$CaHPO_4$	1.0
Calcium pyrophosphate (α , β or γ)	α -, β - or γ -CPP	$Ca_2P_2O_7$	1.0
Calcium pyrophosphate dihydrate	CPPD	$Ca_2P_2O_7 \cdot 2H_2O$	1.0
Amorphous calcium phosphate *	ACP	$Ca_x H_y (PO_4)_z \cdot n(H_2O)$ ($n=3-4.5$)	1.2-2.2
Whitlockite		$Ca_{18}(Mg, Fe^{2+})_2 H_2 (PO_4)_{14}$	1.29
Octacalcium phosphate	OCP	$Ca_8 H_2 (PO_4)_6 \cdot 5H_2O$	1.33
Tricalcium phosphate (α or β)*	α - or β -TCP	$Ca_3(PO_4)_2$	1.5
Calcium-deficient hydroxyapatite	CDHA	$Ca_{10-x}(HPO_4)_x(PO_4)_{6-x}(OH)_{2-x}$ ($0 < x < 1$)	1.4-1.67
Hydroxyapatite	HA	$Ca_{10}(PO_4)_6(OH)_2$	1.67
Amorphous hydroxyapatite	HAa	$Ca_{10}(PO_4)_6(OH)_2 \cdot nH_2O$	1.67
Oxyapatite	OXA	$Ca_{10}(PO_4)_6O$	1.67
Fluoroapatite	FA	$Ca_{10}(PO_4)_6F_2$	1.67
Carbonate hydroxyapatite*	CHA	$Ca_{10-p}(PO_4)_{6-p}(OH)_{2-p}(CO_3)_p$ ($0 < p < 1$)	>1.67
Francolite, Carbonate-fluoroapatite*	CFA	$(Ca, Mg, Sr, Na)_{10}(PO_4, SO_4, CO_3)_6 F_{2-3}$	>1.67
Hilgenstockite, Tetracalcium phosphate*	TTCP	$Ca_4(PO_4)_2O$	2.0

Table 2

Compound	Energy positions (eV)									
PP	347.9	349.3	351.2	352.5						
MCPM	347.0	347.6	348.3	348.6	349.3	351.4	352.5			
DCPD	347.1	(347.6)	348.2	348.6	349.2	351.4	352.5			
DCPA	347.1	347.6	348.2	348.6	349.3	351.4	(351.8)	352.5		
α -TCP	347.0	(347.6)	348.1	348.5	349.2	351.4	352.5			
β -TCP	347.2	347.8	348.3	348.6	349.2	351.1	351.5	351.7	352.5	
HA	347.0	(347.6)	348.1	348.5	349.2	(351.0)	351.4	351.8	352.5	
TTCP	347.1	(347.9)	348.2	348.6	349.3	351.4	351.8	352.5		