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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.

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

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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 PO_4^{3-} groups (B-site) [17]. Incorporation of CO_3^{2-} 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 CO_3^{2-} also increases the dissolution rate of apatite in acids [19,20]. CO_3^{2-} 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, CO_3^{2-} 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 tricalcium phosphate (TCP), octacalcium phosphate (OCP) 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-induce 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.

Table 1
List of several calcium phosphate compounds and their Ca/P molar ratios (adapted from Elliott (2002) [1] and Dorozhkin (2009) [13]).

Name	Abbreviation	Formula	Ca/P
Polyphosphates*	PP	$M^I_mH_{(n+2)}P_nO_{(3n+1)}$ $M^I = Ca^{2+}$, Na^+, Fe^{3+}, Fe^{2+},...	ε
Monocalcium phosphate monohydrate*	MCPM	$Ca(H_2PO_4)_2,H_2O$	0.5
Monocalcium phosphate anhydrous	MCPA	$Ca(H_2PO_4)_2$	0.5
Brushite, Dicalcium phosphate dihydrate*	DCPD	$CaHPO_4,2H_20$	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	$Ca_2P_2O_7,2H_2O$	1.0
Amorphous calcium phosphate*	ACP	$Ca_xH_y(PO4)_z,n(H_2O)$ (n = 3-4.5)	1.2–2.2
Whitlockite		$Ca_{18}(Mg,Fe^{2+})_2H_2(PO_4)_{14}$	1.29
Octacalcium phosphate	OCP	$Ca_8H_2(PO_4)_6,5H_20$	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	$Ca_{10}(PO_4)_6(OH)_2,nH_2O$	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	$(Ca,Mg,Sr,Na)_{10}(PO_4,SO_4,CO_3)_6F_{2-3}$	>1.67
Hilgenstockite, tetracalcium phosphate*	TTCP	$Ca_4(PO_4)_2O$	2.0

* The compounds indicated by an asterisk were analyzed in this study.

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