Research paper

Revisiting the localisation of Zn$^{2+}$ cations sorbed on pathological apatite calcifications made through X-ray absorption spectroscopy

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A B S T R A C T

The role of oligo-elements such as Zn in the genesis of pathological calcifications is widely debated in the literature. An essential element of discussion is given by their localisation either at the surface or within the Ca apatite crystalline network. To determine the localisation, X-ray absorption experiments have been performed at SOLEIL. The Exafs results suggest that Zn atoms, present in the Zn$^{2+}$ form, are bound to about 4 O atoms at a distance of 2.00 Å, while the interatomic distance $R_{CaO}$ ranges between 2.35 Å and 2.71 Å. Taking into account the content of Zn (around 1000 ppm) and the difference in ionic radius between Zn$^{2+}$ (0.074 nm) and Ca$^{2+}$ (0.099 nm), a significant longer interatomic distance would be expected in the case of Zn replacing Ca within the apatite crystalline network. We thus conclude that Zn atoms are localised at the surface and not in the apatite nanocrystal structure. Such structural result has essential biological implications for at least two reasons. Some oligo-elements have a marked effect on the transformation of chemical phases, and may modify the morphology of crystals. These are both major issues because, in the case of kidney stones, the medical treatment depends strongly on the precise chemical phase and on the morphology of the biological entities at both macroscopic and mesoscopic scales.

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1. Introduction

From the medical point of view, pathological calcifications refer to either a concretion, e.g. a kidney stone [1], or an ectopic calcification [2] often associated with tissue alteration. Additionally, normal physiological calcifications such as bone may become pathological through the influence of diseases such as arthrosis or osteoporosis [3]. Different chemical phases constitute pathological calcifications, but calcium phosphate apatites are present in most of them. Biological apatites share various characteristics whatever their biological origin. These aspects include nanometer size, anisotropy along the c axis [4], the presence of carbonate groups [5], an OH$^{-}$ deficiency [6], and finally an amorphous region at the surface [7,8].

The presence of oligo-elements such as zinc, iron, copper or strontium is another common aspect. Some of these elements control the morphology of nanocrystals which constitute pathological calcifications. This morphology is a key parameter for medical diagnosis. Depending on the morphology, the associated pathology is either an intermittent hyperoxaluria of dietary origin [9], or a severe genetic problem such as primary hyperoxaluria type 1 [10,11]. Primary hyperoxaluria type 1 is a rare inherited disease leading to recurrent nephrolithiasis, nephrocalcinosis, systemic oxalosis, and renal failure, ultimately requiring combined kidney and liver transplantation [12]. Also, some elements have a marked effect on the transformation of chemical phases.
A recent investigation underlines the key role of different di- and trivalent metal ions on the transformation of brushite to more basic calcium phosphate at 37°C [13]. Again, such chemical transformation is of primary medical importance because the pathology linked to brushite is very different from those related to other calcium phosphates such as octacalcium phosphate or apatite [14].

In this paper, we determine the molecular environment of zinc atoms for biological apatites of different origins and linked to pathological calcifications. Such physical evidence on the chemical speciation of Zn in these samples, which is uniquely obtainable from X-ray absorption spectroscopy (XAS), yields valuable information for the understanding of the mechanisms responsible for pathological calcification formation. Different studies have already shown that XAS allows determination of the environment of a selected element minor through its absorption edge features [15–17]. In this study, information regarding the average coordination geometry as well as the average effective charge is obtained for Zn-bearing materials even at very low concentrations [18]. The notion of average is quite important, as this technique is insensitive to polydispersity [19]. The recent experiments were performed on the beamline DiffAbs, and included an analysis of the modulations of the X-ray absorption coefficient (EXAFS for Extended X-ray Absorption Fine Structure). This enabled comparison of the Zn–O inter-atomic distances in the phosphate calcifications with bulk Ca–O distances available from previous apatite structure refinements.

### 2. Materials and methods

#### 2.1. Material

The biological samples (Table 1) used in the present study came from different hospitals. The kidney stones comes from Necker hospital, cardiac valves from La Pitie Salpêtrière hospital and bones from CHR Orleans – Inserm U 658 laboratory. The samples were first characterized by infrared spectroscopy using a Fourier Transform infrared (F.T. I.R.) spectrometer Vector 22 (Bruker Spectrospin, Wissembourg, France) according to an analytical procedure previously defined [20,21].

Data were collected between 4000 and 400 cm⁻¹ with a resolution of 4 cm⁻¹. The F.T. I.R. spectra show characteristic absorption bands of the apatite phase (Fig. 1). The observed bands at 1092–1090 cm⁻¹ and 1049–1047 cm⁻¹ correspond to P₂ and P–O stretching vibration modes. The doublet at 602–573 cm⁻¹ can be assigned to the O–P–O r₁ bending mode. As is the case for biological apatites, the bands at 3570 and 633 cm⁻¹ corresponding to the stretching and vibrational modes of the OH⁻ groups are almost absent.

### Table 1

Composition given by Fourier Transform Infra Red Spectroscopy (F.T. I.R.) and the origin of the calcifications.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Origin of the calcification</th>
<th>Composition as given by F.T. I.R.</th>
</tr>
</thead>
<tbody>
<tr>
<td>N 13066</td>
<td>Kidney</td>
<td>79% CA, 15% ACCP, 4% Prot, 2% C₁</td>
</tr>
<tr>
<td>N 15048</td>
<td>Kidney</td>
<td>87% CA, 6% C₁, 4% C₂, 3% Prot.</td>
</tr>
<tr>
<td>N 20208</td>
<td>Kidney</td>
<td>79% CA, 10% C₂, 7% Prot, 4% C₁.</td>
</tr>
<tr>
<td>Sample 1</td>
<td>Cardiac Valves</td>
<td>56% CA, 20% ACCP, 20% Prot, 4% TGL</td>
</tr>
<tr>
<td>Sample 2</td>
<td>Cardiac Valves</td>
<td>47% CA, 20% ACCP, 10% Prot, 8% TGL</td>
</tr>
<tr>
<td>Sample 3</td>
<td>Bone</td>
<td>83% CA, 10% ACCP, 7% Prot.</td>
</tr>
<tr>
<td>Sample 4</td>
<td>Bone</td>
<td>88% CA, 5% ACCP, 7% Prot.</td>
</tr>
</tbody>
</table>

2.2. X-ray fluorescence spectroscopy

Initially, X-ray fluorescence spectra were collected to evaluate the Zn content of the samples. To attain this goal, a set of mixtures containing ZnO from Heraeus (with a purity of 99.999%) and synthetic Hydroxyapatite (H.A.P.) from Biorad were prepared. As previously discussed [22], an absolute quantification is not relevant for these biological samples.

A typical X-ray fluorescence spectrum of a biological sample is shown in Fig. 2 where we observe the contributions of different trace elements such as Zn (Kα = 8638 eV, Kβ = 9572 eV), Pb (Lα = 10549 eV, Lβ = 12613 eV) or Sr (Kα = 14165 eV) and obviously contributions coming from Ca (Kα = 3691 eV, Kβ = 4012 eV). In this set of measurements, the highest proportion of the heavy elements was observed for Zn (1000 ppm) a value in line with those found in previous work [22].

2.3. XANES/EXAFS measurements

The selected samples were investigated on the DiffAbs beamline situated at the D13-1 bending magnet at the SOLEIL synchrotron (St Aubin, France) in order to determine the electronic state as well as to describe accurately the first coordination sphere of Zn atoms. The SOLEIL synchrotron ring was running at 2.75 GeV with an average current of 250 mA. The X-ray beam was obtained with a two crystal Si(111) monochromator which offers an energy resolution of ΔE/E = 10−4 necessary to resolve the XANES structures. Calibration of the experiment was made with a reference ZnO foil.

Experiments were performed at room temperature and atmospheric pressure. Spectra were collected in an energy range between 9600 and 10200 eV, with energy steps of 0.3 eV (Xanes part) or 2 eV (Exafs part) and 3 s dwell time per point. XANES/EXAFS spectra were obtained in fluorescence mode using a SDD detector [23]. The size of the beam was determined by a set of slits (200 μm x 500 μm). More details regarding the experimental device can be found in a previous study dedicated to kidney stones [15].

Regarding the XANES part of the absorption spectrum collected at the Zn K edge, we will use the edge position which indicates the effective charge of the Zn atoms (Fig. 3). According to a mono-electronic description of the absorption process, electronic transitions are allowed between 1s and 4p levels but prohibited towards 3d levels (the dipole selection rule gives Δl = ±1). In fact, some Zn compounds [24] display a similarity between the K and the L edge which points out an overlap between Zn (4s, 4p and 3d) and oxygen (2p) orbitals. Here, the analysis is based on previous discussion [25] on the form and the position of the Zn edge which emphasized the possibility of distinguishing using the XANES features between an octahedral first-neighbour anion shell and a tetrahedral one.

Regarding the analysis of the EXAFS modulations, data treatment was carried out using the EXAFS code [26,27]. As we have already discussed [28] inclusion of multiple scattering is essential only beyond the first shell. Thus, single scattering theory has been used here. Following Lengeler-Eisenberger normalization, EXAFS oscillations were Fourier Transformed (FT) using a Kaiser window.
(z = 2.5) between 2.5 and 8.5 Å⁻¹. Filtered EXAFS spectra were obtained by back FT between 0.8 and 2.14 Å. Errors were estimated after determination of the data mean standard deviation in χ(k) using the STATEXAFS code [20]. Example of simulation in k and R space are given in Fig. 4a and b.

The \( R^2 \) weighted EXAFS oscillations as well as the corresponding radial structure functions (RSF, not corrected for phase shifts) for the reference compounds selected for this study, namely Smithsonite (ZnCO₃), Willemite (Zn₃SiO₄) and Zincite (ZnO), are shown in Fig. 5. The significant difference in the Zn–O interatomic distance which exists between Zn in Smithsonite (N = 6, \( R = 2.11 \) Å), and in Willemite (N = 4 \( R = 1.94 \) Å, 1.95 Å, 1.97 Å, 1.98 Å) or Zincite (N = 4, \( R = 1.92 \) Å, 1.99 Å) is clearly visible in the analysis. Even if the extended environment of Zinc in Willemite and Zincite are quite different, the modulus and the imaginary part of the FT of the compounds have similar forms and positions.

### 3. Results and discussion

HAP can be described as a hexagonal stacking of \((PO₄)³⁻\) groups with two kinds of tunnel parallel to the c axis. The first one coincides with the ternary axis of the structure and is occupied by Ca\(^{2+}\) ions. The second one is lined by oxygen and other calcium ions, noted Ca(II) and is occupied by OH\(^⁻\) ions. Ca(I) and Ca(II) are present in a 2/3 ratio. At this point, it is interesting to underline that biological apatites consist of nanometer scale crystals. This small size is a crucial factor related to the solubility of biological apatites when compared with geological apatites.

Moreover, as discussed previously [29], the presence of carbonate groups which induces some vacancy regarding Ca(I) and/or Ca(II) ions leads to the fact that the stochiometric formulae of biological apatite is far from the classical one namely \(Ca_5(PO_4)_3OH\) corresponding to synthetic HAP. All these structural characteristics give biological apatites particular properties.

Numerous studies have been dedicated to the localisation of the foreign cations such as Mg [30], Si [31], Cd [32], Sr [33], Pb [34] in the HAP structure. According to several studies, the foreign cations occupy preferentially Ca(II) sites [see for example 35]. Occupation of Ca(II) site leads to a strict alignment in the columns and causes a stronger repulsion. Note that this occupancy depends on the concentration of the oligoelements [36].

Among these elements, Zn and Sr play a major role in medical research [37,38]. Regarding pathological calcification, we have proposed a classification of the different trace elements [39] in which we distinguish the case of zinc from that of strontium. Present in the human body in various metallo-proteins, Zn\(^{2+}\) ions can be remove by the kidney. This points to the element probably being adsorbed at the surface of the apatite. In contrast, Sr\(^{2+}\) ions follow the Ca\(^{2+}\) metabolic pathways. From a chemical point of view, Ca\(^{2+}\) and Sr\(^{2+}\) have similar chemistry (these two elements sharing the same column in the periodic table), and commonly substitute for one another in minerals [40–42]. From these observations, we can expect that Sr\(^{2+}\) ions are probably located within the apatite crystal structure [29]. In this study, we would like to discuss the localisation of Zn within apatite nanocrystals.

At the atomic level [43], Ca(I) site is best described in terms of the different Ca–O bonds lengths as \(N_{CaO} = 6\) (\(R_{CaO} = 2.43\) Å) + \(N_{CaO} = 3\) (\(R_{CaO} = 2.79\) Å) while the Ca(II) site is best described as either \(N_{CaO} = 4\) (\(R_{CaO} = 2.36\) Å) + \(N_{CaO} = 2\) (\(R_{CaO} = 2.51\) Å) + \(N_{CaO} = 1\) (\(R_{CaO} = 2.71\) Å) or \(N_{CaO} = 5\) (\(R_{CaO} = 2.35\) Å) + \(N_{CaO} = 2\)

\[\text{Table 2}
\]

<table>
<thead>
<tr>
<th>Sample</th>
<th>CN</th>
<th>(R(\text{Å}))</th>
<th>(\Delta r^2(\text{Å}^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>N 13066</td>
<td>3.7</td>
<td>1.98</td>
<td>0.00</td>
</tr>
<tr>
<td>N 15048</td>
<td>4.3</td>
<td>2.00</td>
<td>0.02</td>
</tr>
<tr>
<td>N 20308</td>
<td>4.7</td>
<td>1.98</td>
<td>0.03</td>
</tr>
<tr>
<td>Sample 1 Cardiac Valves</td>
<td>4.5</td>
<td>1.98</td>
<td>0.03</td>
</tr>
<tr>
<td>Sample 3 Bone</td>
<td>4.5</td>
<td>1.99</td>
<td>0.01</td>
</tr>
<tr>
<td>Sample 4 Bone</td>
<td>5.0</td>
<td>2.01</td>
<td>0.01</td>
</tr>
</tbody>
</table>

CN – Coordination Number, \(R\) – Zn–O interatomic distance, \(\Delta r^2(\text{Å}^2)\) – Variation of the Debye–Waller factor versus its value in zincite.
at low Zn concentrations the first coordination sphere is constituted by four oxygen at 1.96 Å [24]. Such a structural environment suggests that Zn species are located at the surface of the apatite, as sorbed species or a in different Zn phase.

For the samples analysed here (Fig. 6a and b, Table 2), we emphasize that we are in the low concentration regime. The amount of Zn in biological apatite is around 1000 ppm (on the basis of X-ray fluorescence measurements) consistent with the low content of Zn in the human body (around 2–3 g for a 72 kg body). In the case of synthetic HAP, we have previously underlined the effect of the preparation procedure. This is an important consideration since with biological samples, significant variations of physiological parameters may also occur. For example, in kidneys, cyclic day- and night variations of pH between 5 and 8 exist. The concentration of calcium and phosphate ions greatly varies in patients, with daytime and spatially within the nephron (for example Ca in urine may vary from 0.1 to 25 mmol/l and P from 0.1 to 100 mmol/l). More precisely, in the proximal tube, the Henle’s loop or the distal tube, which are three distinct parts of the nephron, the content of water varies significantly. Finally, the carbonate content of apatite crystals constitutes another important parameter. In the case of kidney stone, a large variation of this parameter is observed through F.T.I.R. measurements [58]. In the case of calcifications developed in other tissues, such as cardiac valves, variations of pH are excluded, as the pH of blood is strictly constant around 7.4. Finally, in the case of bone, an intermediate situation is observed, where controlled variations of pH are involved in both calcification and release of apatite from the bone tissue.

Regarding the Xanes part of the absorption spectra (Fig. 3), it seems that a significant distorsion of the Zn environment exists for biological apatite. For Zn insertion in apatite, due to the low content of Zn and if we neglect relaxation of the site, $R_{ZnO}$ would be expected to close to the $R_{CaO}$ interatomic distance. This experimental fact suggests the localisation of zinc atoms at the surface but not inside the HAP nanocrystals whatever their biological origin. Work is in progress to complete these data by another set of experiments aimed at determining the second neighbour of Zn. At this point of the discussion, we would like to recall that biological apatites consist of nanometer scale particles with an average length of 50 nm, 25 nm in width and thicknesses of just 2–5 nm. On the basis of these structural characteristics, a significant number of calcium atoms can be considered as “surface atoms”, the maximum distance between calcium atoms and one of the crystal surface

### Table 1
First coordination sphere around Zn atoms determined through Xas in the case of biological apatites. Selected details regarding the preparation are given.

<table>
<thead>
<tr>
<th>Biological apatites</th>
<th>$N_{ZnO}$</th>
<th>$R_{ZnO}$ (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human dentine treated with ZnCl$_2$ [55]</td>
<td>4.1</td>
<td>1.96</td>
</tr>
<tr>
<td>Remineralized enamel [56]</td>
<td>/</td>
<td>2.37–2.40</td>
</tr>
<tr>
<td>Human dental calculi [57]</td>
<td>4.2</td>
<td>2.08</td>
</tr>
<tr>
<td>Human dental calculi [57]</td>
<td>5.1</td>
<td>2.06</td>
</tr>
</tbody>
</table>

($R_{CaO} = 2.51 \text{ Å} \times N_{CaO} = 1 = R_{CaO} = 2.71 \text{ Å}$). Due to the difference in ionic radius between Zn$^{2+}$ (0.074 nm) and Ca$^{2+}$ (0.099 nm), a significant decrease of the lattice parameters $a$ and $c$ is measured at low Zn concentration in the case of Zn-substituted HAP [44].

Several structural investigations on the incorporation of Zn in synthetic HAP through X-ray absorption spectroscopy have been performed [45–48] and some results are shown in Table 3. For example, using theoretical models of Zn substituted for calcium, only two models agree with experimental data, zinc substituted for calcium in site I and zinc in the interstice between the two OH$^-$ [49]. Based on X-Ray diffraction and spectrophotometric data, one study has indicated that the greatest amount of zinc must be on the crystal surface and/or in an amorphous phase. These workers also noticed a reduction of the crystallite size and a decrease in thermal stability in Zn-bearing HAP [50]. More recently, experimental data as well as DFT calculations seem to show a Zn substitution process in the Ca(II) site [51].

Such discrepancy can be explained by the fact that some parameters in the preparation protocol have a major influence. For example, several workers have pointed out the significant role of the pH [52] in the mechanisms of Zn interaction with apatite. While the formation of Hopeite [Zn$_3$(PO$_4$)$_4$]$_2$H$_2$O was observed at low pH, Zincite was formed at high pH [53]. More recently, the influence of dissolved CO$_2$ as well as the Zn concentration on the mechanism of Zn uptake by apatite has been considered. At low Zn concentrations, retention of Zn by HAP occurs through adsorption [54]. At neutral and higher pH, where dissolved carbonate is more abundant, the adsorbed Zn from solution increases with pH.

Regarding biological apatite [55,56], EXAFS results (Table 4) have shown that, under in vivo conditions, the zinc atoms are fully incorporated into the crystalline structure of the calcium phosphates [57]. In contrast, another EXAFS investigation points out that at low Zn concentrations the first coordination sphere is

![Fig. 6. Modulus (6a) and imaginary (6b) parts of the Fourier Transform uncorrected for phase shifts of the two reference compounds zincite (solid line) and smithsonite (dotted line) compared with the different samples. We can see clearly that the position of the first peak indicates short zinc-oxygen bonds, a signature of a tetrahedral geometry.](image-url)
being equal to 2.5 nm. This nanometer scale implies also that the definition of Ca(I) and Ca(II) occupation site which comes from an “infinite” repetition of the crystallographic units is probably not relevant. The presence of the nature and the content of oligoelements has up to now been neglected. [61].

4. Conclusion

In summary, X-ray absorption experiments performed on biologicalapatites of different origin lead to the conclusion that Zn atoms are not present in the apatite nanocrystals structure but rather present as a surface element in line with the medical classification. Although relationships between pathology and the surface state, the morphology and the color of kidney stones have previously been proposed, the influence of the nature and the content of oligoelements has up to now been neglected [61].

5. Competing interests statement

There are no potential conflict of interest for the authors.

Acknowledgments

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References


