1 Title:

2 **Probing carbonate in bone forming minerals on the nanometre scale**

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18 Abstract:

19 To devise new strategies to treat bone disease in an ageing society, a more detailed characterisation of
20 the process by which bone mineralizes is needed. *In vitro* studies have suggested that carbonated
21 mineral might be a precursor for deposition of bone apatite. Increased carbonate content in bone may
22 also have significant implications in altering the mechanical properties, for example in diseased bone.
23 However, information about the chemistry and coordination environment of bone mineral, and their
24 spatial distribution within healthy and diseased tissues, is lacking. Spatially resolved analytical
25 transmission electron microscopy is the only method available to probe this information at the length
26 scale of the collagen fibrils in bone. In this study, scanning transmission electron microscopy
27 combined with electron energy-loss spectroscopy (STEM-EELS) was used to differentiate between
28 calcium-containing biominerals (hydroxyapatite, carbonated hydroxyapatite, beta-tricalcium
29 phosphate and calcite). A carbon K-edge peak at 290 eV is a direct marker of the presence of
30 carbonate. We found that the oxygen K-edge structure changed most significantly between minerals
31 allowing discrimination between calcium phosphates and calcium carbonates. The presence of
32 carbonate in carbonated HA (CHA) was confirmed by the formation of peak at 533 eV in the oxygen
33 K-edge. These observations were confirmed by simulations using density functional theory. Finally,
34 we show that this method can be utilized to map carbonate from the crystallites in bone. We propose
35 that our calibration library of EELS spectra could be extended to provide spatially resolved
36 information about the coordination environment within bioceramic implants to stimulate the
37 development of structural biomaterials.

- 1 Keywords:
- 2 bioceramics, bone mineral, carbonate, STEM-EELS
- 3
- 4

1. Introduction

During evolution, organisms have developed various external and internal skeletal systems. The skeleton performs a number of tasks: it provides a scaffold for the entire body; it is a vital component of the movement apparatus; and it protects internal organs. Any scaffolding material needs to possess particular properties, such as stiffness, strength and toughness. To achieve these characteristics soft, but elastic protein is reinforced with stiff mineral in the mineralisation process. In living systems this process typically occurs through a calcium based route. *Mollusca* and *Arthropoda* exoskeletons incorporate mainly calcium carbonates, while *Vertebrae* endoskeletons adapt calcium phosphates as building material [1,2].

For several decades hydroxyapatite $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ has been used as the closest approximation of the biomineral present in *Vertebrae* mineralised tissue [3–5]. Hydroxyapatites (HA) are the most common phase present in natural systems; however, there are other forms of apatite that contain ionic substitutions (e.g. carbonate, fluoride, sodium, potassium etc.). The composition of the mineral may vary between tissues (e.g. bone, dentin, enamel, calcified tendon) [6], with age [7], as a function of the mineralisation stage [8,9] and as a result of diseases such as osteogenesis imperfecta [10,11]. Modifications in apatite chemistry are present as substitutions into the lattice (e.g. carbonate or silicate ion substitutions), and as different calcium phosphate phases (e.g. beta tricalcium phosphate (bTCP) vs. HA) [8,12]. Compositional variations between, and within hard tissues, may control mechanical properties such as the hardness or fracture toughness of each biomineral [13]. For example, disruption to mineralisation processes may have significant implications in altering the mechanical properties of tissues. In aged bone, carbonate replaces phosphate in the mineral lattice contributing to bone brittleness [7].

Characterisation of minerals in tissues not only provides insight into disease states, but is also beneficial to synthetic bioceramics research, where biocompatibility, bioactivity (e.g. resorption or cardiovascular response), material properties and mineral nucleation are of paramount importance [14–17]. Apatites with various dopants (*i.e.* carbonate, silicon and fluoride), beta-tricalcium phosphate and various mixtures of them are among the most popular bone-like bioceramics made for medical applications [15–17]. These minerals are often used as a connective material between implant and bone or as a porous synthetic bone graft to reconstruct fractures; they are designed to encourage bioactive bone growth. Optimisation of the bioactivity of bioceramics requires precise control over their chemistry. Subtle changes in the chemical composition, e.g. as a result of the form of ionic substitutions, and phase purity leads to alteration in bioactivity [18–20]. Nano-scale modifications in the chemistry of these bioceramic implants have a direct impact on mechanical and chemical properties of the surrounding bone. For example, phase changes and changes in the local atomic order at grain boundaries of apatite crystals affects mineral dissolution and the ability of carbonate and silicate substituted HA to integrate with the surrounding collagen matrix [21,22]. Other surface changes may promote or demote creation of sacrificial layers, an important factor in mechanisms stopping fracture propagation [23]. In the future, the ability to analyse the coordination environment within these materials and probe substitution sites in the HA lattice will improve our understanding of mechanisms controlling their bioactivity which will open the door for synthesis of more bio-adaptive ceramics to replace diseased or fractured tissues.

One of the challenges in elucidating bioimineralisation processes is the ability to identify mineral compositions at the nanometre scale, during tissue formation and disease. Acquisition of this information is the first step in characterisation of different phases present in tissues and bioceramics.

1 Since mineralisation events frequently occur at the length scale of the collagen fibrils [24], it is critical
2 that compositional information is acquired with nanometre scale spatial resolution.

3 X-ray absorption spectroscopy (XAS) is one of the most common methods used to characterise
4 biomineral chemistry at the nanometre scale. X-ray absorption near edge structure (XANES) has
5 provided a new insight into chemical environment of biominerals and mineralised tissues [25–
6 30]. While XAS studies provide a very high energy resolution, the spatial resolution is not adequate to
7 resolve features below 15nm [31–33]. Although the average sized crystal platelets (100nm long, 50nm
8 wide, 5nm thick [34,35]) could be examined, investigation of smaller (5-10nm) features such as inter-
9 crystal spaces, grain boundaries and protein-mineral interfaces is below the spatial resolution limit of
10 XAS.

11
12 Scanning transmission electron microscopy (STEM) combined with electron energy-loss spectroscopy
13 (EELS) is the only technique capable of achieving nanometre scale resolved information about the
14 chemistry and coordination environment of minerals. Previous studies have attempted to identify
15 spectral fingerprints from bioceramics using STEM-EELS [36,37]. However, these studies did not
16 consider carefully the effects of irradiation of biominerals, which makes the results liable to
17 misinterpretation [38]. In addition, previous studies focused on selected edges, rather than comparing
18 edges of all characteristic elements present in the mineral (i.e. P, C, Ca, O). To our knowledge, no
19 previous studies have identified the presence of carbonate from bone mineral by studying fine
20 structure in the EELS spectra at these edges. Here we used EELS to discriminate between different
21 bioceramic standards. Phase pure hydroxyapatite (HA), carbonated HA (CHA) and beta tricalcium
22 phosphate (bTCP) were selected as these bioceramics are likely to be present in bone tissue at
23 different stages of mineralisation [8,9] or are relevant in clinically enhanced mineralisation. Calcium
24 carbonates were examined to determine if carbonate ion substitution in the HA lattice is detectable
25 with EELS. The near-edge core loss spectra of phosphate, carbon, calcium and oxygen were acquired
26 and analysed for various forms of synthetic biomineral and also for healthy mouse bone tissue. A
27 study of the effect of electron dose was conducted in order to observe changes in the spectra that
28 result from electron beam-induced damage.

2. Materials and methods

A range of standards was investigated to represent the calcium-containing minerals suggested to be present in calcified tissues [4,12] or bioceramics enhancing bone growth [21,22]. These minerals are pure hydroxyapatite (HA), carbonated hydroxyapatite (CHA) with carbonate substituted for hydroxyl and phosphate groups in various ratios (A vs. B type, respectively), beta-tricalcium phosphate (bTCP) and calcite (CAL) (Table 1).

2.1. Production of mineral standards

Hydroxyapatite (HA) with a Ca/P ratio of 1.67 was synthesised using a wet precipitation method described by Akao and Jarcho that involves a reaction between $\text{Ca}(\text{OH})_2$ and H_3PO_4 where the pH is kept above 10.5 using aqueous ammonia [39,40]. CaCO_3 (Sigma Aldrich ACS reagent grade 239216) was decarburised over night at 960 °C then cooled under vacuum. The resulting CaO was hydrated in deionised water to form $\text{Ca}(\text{OH})_2$, then H_3PO_4 aq (85 v/v % Fisher Scientific) was diluted in deionised water and was added at a rate of 5 ml.min⁻¹ to the $\text{Ca}(\text{OH})_2$. Upon completion the mixture was aged overnight then vacuum filtered. The resulting filter cake was dried then ground in an alumina crucible.

A mixed AB-type carbonated HA (CHA) was produced via a sodium free wet chemical precipitation reaction first described by Gibson & Bonfield [41]. Ca/P ratios of 1.76, 1.74 and 1.72 were considered. Similarly to HA, $\text{Ca}(\text{OH})_2$ was formed and CO_2 g was bubbled through deionised water until the pH dropped to around 4 then H_3PO_4 aq (85 v/v % Fisher Scientific) was added. This solution was added at a rate of 5 ml.min⁻¹ to the $\text{Ca}(\text{OH})_2$ solution. No pH control was necessary as the pH remained above 10.5.

A fraction of the apatite mineral standards were heat treated: at 1200 °C in air (HA) or 800-1000 °C in a wet CO_2 environment (CHA) for 2 hours. The other fraction was investigated without heat-treatment.

Beta-tricalcium phosphate (bTCP) precursors were formed through combination of $\text{Ca}(\text{OH})_2$ and H_3PO_4 in an aqueous environment with a Ca/P ratio of 1.5. This mixture was aged, dried and heated to 1100 °C for 4 hours to produce bTCP [42].

2.2. Preparation of bone samples

3 femurs of 8 week old wild type mice were prepared *via* high pressure freezing and freeze-substitution [43]. Bone samples were dissected, cut with a scalpel blade from the middle of each femur shaft, transferred into flat specimen carriers with 200 µm indents and fixed with 1-hexadecene. Rapid cryofixation was done using a Leica EMPACT2 (Leica Microsystems, Vienna, Austria) machine. Frozen samples were transferred into a Leica EM AFS2 freeze-substitution device, where the substitution using acetone solution containing 3% (v/v) glutaraldehyde was performed for 8h at -90 °C. The temperature was steadily increased (5°/h), until it reached 0 °C. Finally, samples were washed twice in acetone for 15 min, before they reached room temperature.

For the first three days, samples were immersed successively in 1:3, 1:1 and 3:1 resin:acetone solutions for 24 h. The resin was prepared from a mixture of 12.6 g of Quetol651, 15.5 g of nonenylsuccinic anhydride (NSA), 6.5 g of methyl nadic anhydride (MNA) and 0.6 g of benzyldimethylamine (BDMA), (Agar Scientific, Dorset, UK). Finally, samples were placed in pure embedding resin for 7 days allowing full infiltration under vacuum. The resin was changed daily. After eight days, the samples were moved into the curing oven and heated at 60 °C for 48 h. An

- 1 ultramicrotome PowerTome XL with an ultra 45° diamond blade (Diatome, Biel, Switzerland) was
- 2 used to prepare ultra-thin (70 nm) sections of embedded samples.

2.3. Bulk characterisation

The phase purity of heat-treated powders was investigated with X-ray diffraction (XRD). Powder XRD scans were performed using a Phillips PW1050 diffractometer with monochromatic Cu K- α x-rays operating at 40 kV and 40 mA. 0.5° divergence and anti-scatter slits, a 10 mm mask and a 0.2 mm-receiving slit were used. Scans used a 0.05° step size and a sweep rate of 1° 2 θ /min. Phillips HighScore plus software was used to identify phases in the heat-treated CaP powders. ICDD (International Centre for Diffraction Data) powder diffraction files of HA (09-0432), alpha-tricalcium phosphate (29-0359), beta-tricalcium phosphate (bTCP, 70-2065), calcium oxide (37-1497), tetra calcium phosphate (TTCP, 25-1137), calcite (CAL, 85-1108) and aragonite (41-1475) were considered during phase analysis.

Carbonate groups in hydroxyapatites can occupy two possible positions; hydroxyl (A-type) or phosphate (B-type) group substitutions [44,45]. Several variants of CHA were produced, in order to obtain a high and low A/B type carbonate ratio, to reflect clinical findings that A/B ratio varies in bone between species [45].

To determine the substitution sites of carbonate ion in the HA lattice, apatites were examined using Fourier transform infrared spectroscopy (FTIR, Perkin Elmer Spectrum 100 spectrometer), and the A/B ratio was estimated as the ratio of areas of peaks corresponding to A and B substitutions [46].

To obtain a spectrum, 32 scans were performed with a resolution of 1 cm⁻¹. The FTIR spectra were cropped to the region of interest showing carbonate peaks (840-900 cm⁻¹). Spectra were deconvoluted using the Peak Analyzer tool in Origin (OriginLab, Northampton, MA, USA). After subtraction of a linear, integrated background, three Gaussian peaks were fitted corresponding to A (~880 cm⁻¹), B (~873 cm⁻¹) and C (~867 cm⁻¹) type substitutions (Figure SI 1). Integrated intensities of respective, deconvoluted peaks were used to establish the A/B ratio.

2.4. STEM-EELS experimental data and simulation

For STEM-EELS investigations, the mineral powders were dispersed in 100% ethanol and then transferred onto copper 300-mesh grids coated with a lacy carbon film as a support (Agar Scientific Ltd., Dorset, UK).

The specimens were examined on the FEI Titan 80-300 field emission, Cs corrected electron microscope fitted with a Gatan Tridiem electron energy-loss spectrometer. The instrument was operated at an accelerating voltage of 300 kV and an emission voltage of 4500 V, conditions frequently used to study mineralised tissues to minimise radiolysis damage [12,47]. For EELS analysis, the microscope was aligned in STEM mode with a 50 μm condenser aperture, spot size 9, a camera length of 60 mm and a spectrometer entrance aperture of 2.5 mm, corresponding to convergence and collection semi-angles of 8 and 14 mrad, respectively. The core loss signal was acquired in 10 second acquisitions with sub-pixel scanning. Beam parameters were optimised to ensure the total electron dose for the specimen would not exceed 10^4 electrons/ nm^2 , which is below threshold dose for damage of these minerals [38]. Each spectrum was collected with energy resolution of 0.6-0.7 eV using an energy dispersion of 0.05 eV/channel.

For the damage study, doses higher than 10^4 electrons/ nm^2 were also tested. Samples were exposed to dose rates of 10^3 electrons/ nm^2 per second. The signal was acquired in 1 second increments for 120 seconds.

A background subtraction was performed on all acquired edges. The background signal was removed by subtraction of a power-law fit to a 25 eV-wide window (10 eV for the phosphorus edge) preceding the edge of interest. The window position was selected to ensure that background-subtracted spectrum does not become unphysical i.e. intersects the energy-loss axis. Examples of background subtraction are shown in Figure SI 3 & Figure SI 4. The background subtraction was performed using Digital Micrograph software (Gatan, Pleasanton, CA, USA). All EELS figures show spectra after background subtraction. Resulting spectra were normalised and calibrated to the characteristic peaks (for details of calibration see the results section).

For the examination of possible impurities in the calcite sample, where overlapping structures of carbon film and mineral were observed in the carbon K-edge, a principal components analysis (PCA) approach was applied to separate superpositioned spectra [48]. In PCA, the original spectra are decomposed into a set of orthogonal spectra. These spectra are weighted according to their contribution to the original spectrum, while spectra not showing any meaningful features can be discarded as noise. Finally the data set may be reconstructed from spectra carrying valuable information. For this processing Hyperspy open source software was used [49]. The spectra collected from a cluster of unidentified mineral on a carbon film (Figure SI 4) were decomposed using Hyperspy software. The decomposition procedure used the singular value decomposition algorithm and assumed a Gaussian distribution of noise. The resulting scree plot, scores and loading of first nine components are given in Figure SI 5. The decomposition was followed by the independent component analysis (ICA), which aims to identify the real (or at least more physically meaningful) components of the multiple original signals mixture. Finding the real components depends on the selection of “contrast functions”. Contrast functions indicate the degree of componential independence, and the most independent components are considered as the original signals. The ICA procedure (via the FastICA algorithm [50]) was used in the attempt to reconstruct independent components of the overlapping materials.

1 To aid the interpretation of the oxygen K-edge data, the energy-loss near-edge structure (ELNES) was
2 simulated using density functional theory. The site projected unoccupied density of states (DOS) on
3 the oxygen atomic sites was calculated using VASP 5.3.5 with PAW-PBE pseudo-potentials and a
4 2x2x3 supercell [51–58] To evaluate whether core-hole effects are reflected in spectra, a Z+1
5 approximation was utilized to simulate an excited-state system[59]. The resulting density of states
6 was convoluted with a Gaussian function to simulate the product of experimental, final state, and
7 initial state broadening. The energy dependence of final state broadening was not considered as this
8 would have a minor effect on the simulation of the ELNES in the energy range of interest here.

3. Results and discussion

3.1. Bulk characterisation

CHA standards with the highest and lowest A/B ratio were selected for subsequent characterisation to represent the extremes of carbonate substitutions (Figure 1, Table 1, Table 2).

XRD revealed that all mineral standards were phase pure (Figure 1). The only phase present in heat treated HA and CHA was hexagonal HA (ICDD 09-432). bTCP (ICDD 70-2065) did not contain any α -TCP or HA impurities. The as-received CaCO_3 from Sigma Aldrich was a pure calcite phase (ICDD 85-1108). One should keep in mind that the estimate of the purity at the macroscale might give an error of 2-6% [47]. This inaccuracy in the estimation may lead to observations of various phases at the nanometre scale.

3.2. EELS characterisation

No **meaningful** differences in EELS spectral features were observed between non-sintered hydroxyapatite and hydroxyapatite sintered in 1200°C (data not shown). No **meaningful** differences in EELS spectral features were observed between various carbonated hydroxyapatites (type A or B substitution) (data not shown). Representative hydroxyapatite spectra were labelled as HA, and representative carbonated apatite spectra were labelled as CHA.

3.2.1. Phosphorus $L_{2,3}$ -edge

EELS data from the phosphorus-containing minerals are shown in Figure 2. The phosphorus $L_{2,3}$ -edge displays three main peaks in the energy loss near-edge structure (ELNES): peaks A, B and C at ~138, ~141 and 146-149 eV, respectively. These features precede a broad peak D at ~160 eV. Phosphorus $L_{2,3}$ -edge spectra were normalised and aligned to the first peak A set to 138 eV based on the literature [60]. Background subtraction of the phosphorus $L_{2,3}$ -edge was performed using a pre-edge, 10 eV window.

Peak A can be assigned to transitions from the phosphorus 2p core states to unoccupied states of p-like symmetry [60]. Monopole transitions (p-to-p state) are not allowed by the dipole selection rule. However, if a hybrid sp^3 (combination of 3s and 3p state) state is formed, the dipole selection rule can be satisfied by transitions to the s-like component of this state [61]. Additional mixing with hybrid sd^3 states may further increase the probability of this transition occurring [61,62]. By analogy, peak B has been attributed to transitions from 2p state to p-like final states. Kruse *et al.* suggested that peak B arises due to mixing of phosphorus p-states and calcium d-states [60]. Peak C was formed by electrons excited to d-like states [60,63]. Peak D with a maximum at ~160 eV is attributed to a cross section maximum of 2p state excitations [63] and multiple scattering. There was no **observable** difference in the phosphorus $L_{2,3}$ -edge from phosphorus-containing minerals shown in Figure 2.

3.2.2. Carbon K-edge

The carbon K-edge spectra of carbonated minerals were normalised and aligned to the carbonate peak D (Figure 3) set to standard 290 eV, based on the literature [27]. The amorphous carbon spectrum (AC) of the carbon film was collected during the same acquisition as the calcium carbonate spectra. The AC spectrum was normalised to peak A of CHA.

The spectrum from calcite, CaCO_3 (CAL, Figure 3) was consistent with the literature [64] and exhibits a distinct, sharp peak D at ~290 eV, less intense peaks E and G at ~296 and ~298 eV, respectively, and a broader peak H at ~302 eV.

The CAL standard was nominally a 99% pure phase calcite. Impurities detected during in the EELS study exhibited spectra with characteristics similar to aragonite. In addition to aragonite-like features, these spectra showed additional peaks typical for organic compounds [65,66]. Impurity particles were found exclusively on the support film. Therefore spectra acquired from impurities represent a superposition of amorphous carbon film and unknown phase spectra, and were analysed using ICA.

In Figure 3, IMP is the spectrum obtained via the ICA blind source separation method [48], which extracts a full spectral signal of each individual phase present in the examined region. Original spectra and extracted spectra are presented on Figure 4. We repeated the ICA to reconstruct up to 6 independent components. However, the combination of just first two components gave a good approximation of the initial spectrum (Figure SI 6III); hence we assume that the original signal is a mixture of two signals. After application of ICA, the first spectrum obtained exhibited features consistent with an amorphous carbon film, while the second spectrum represented the unknown mineral impurity.

The impurity spectrum (IMP), in comparison to calcite (CAL), displayed additional double peaks B and C at ~287 and ~288 eV, respectively. Peaks E and G, seen in calcite, are barely visible in the impurity spectrum and peak H is shifted towards lower energies.

Peaks B and C observed in the calcite impurity (IMP) were assigned to $1s \rightarrow \pi^*$ states transitions of carboxyl and carbonyl groups, which are often connected with organic material in aragonite in XANES studies [67–69]. Previous EELS studies did not record peaks B and C in aragonite [64], while XANES studies show the presence of peaks B and C, and attributed their origin to the surface contamination or surface states [70].

The most distinct peak in calcium carbonates (D at ~290 eV) originates from main core $1s$ transitions to the vacant π^* -A states of CO_3 groups [27]. Peaks E (CAL, IMP) and G (CAL) were assigned to carbonate $1s \rightarrow \sigma^*$ -C and π^* -C transitions [28,70]. Peak H results from the main $1s \rightarrow \sigma^*$ -A transitions [70,28].

Carbonated HA (CHA) exhibited four peaks: A at ~285 eV, D at ~290 eV, F at ~297 eV and H at ~302 eV. Low signal-to-noise ratio of CHA carbon K-edge is the effect of relatively low carbonate content and the low dose exposure. Pure calcium phosphates (HA and bTCP) exhibit no carbon signal or a weak signal corresponding to amorphous carbon, which could be attributed to impurities introduced during preparation of standards or accumulation of mobile hydrocarbons originating from carbon support film (data not shown). The amorphous carbon edge consists of two peaks a sharper one A at 285 eV characteristic for core electron transitions from $1s$ to vacant π^* state and a broad peak F at ~297 eV characteristic for $1s \rightarrow \sigma^*$ transitions in the carbon-carbon bonding environment [71].

1 Peak D is characteristic of the presence of carbonate ions (Figure 3) [66]. Analysis of the carbon K-
2 edge alone is not sufficient to distinguish between CaCO_3 and CHA [25], as the CHA spectra hold
3 strong resemblance to edges collected from calcite and aragonite and may be approximated as a
4 superposition of features visible for amorphous carbon and calcite.

3.2.3. Calcium $L_{2,3}$ -edge

Calcium $L_{2,3}$ -edge spectra were aligned and normalised to the peak B, set to 348 eV based on literature [30]. The calcium $L_{2,3}$ ELNES (Figure 5) consists of two principal sharp peaks: B at ~348 eV and D at ~350 eV, corresponding the spin-orbit split L_3 and L_2 edges, respectively [72,73].

In the calcite spectrum each of the principal peaks (B, D) was split, creating additional, smaller peaks (A, C) at ~346 and ~350 eV, as a result of the crystal field splitting [28,70–72]. We did not observe these features in other minerals, possibly due to insufficient energy resolution. Nevertheless, some carbonated hydroxyapatite species showed a slight broadening of the FWHM (around 0.3 eV), which may indicate the multiplet splitting [29,74].

The features characteristic of multipet splitting were observed in the calcium $L_{2,3}$ -edge of calcite spectrum due to the highly symmetric calcium coordination environment. In calcite, the calcium atom is in octahedral configuration with six oxygens; in aragonite, calcium is positioned in irregular environment of nine oxygens; in apatites, calcium atoms occupy two positions: one in coordination with nine oxygen atoms (Ca1), other with seven (Ca2) [29]. In the impurity found in calcite and HA and CHA, the d orbitals undergo a complex crystal field splitting resulting in multiple peaks that are unresolved. In comparison, XANES experiments showed calcite-like crystal field splitting for carbonated hydroxyapatites, aragonite [29] and even hydroxyapatite [75].

Most examined minerals (HA, CHA, bTCP, IMP) did not exhibit meaningful modifications in the calcium $L_{2,3}$ -edge. Only calcite displayed distinctive features (A, C), which are characteristic of multiplet splitting.

3.2.4. Damage observed in oxygen K-edge

Changes were observed in the oxygen K-edge structure, which are connected with two different damage processes. In the first process, minerals exposed to electron doses above 10^4 electrons per nm^2 exhibited a characteristic peak X at ~ 530 eV. In the second process, minerals exposed to doses above 10^5 electrons per nm^2 showed the transformation of the oxygen K-edge structure into spectra consistent with calcium oxide features. These observations are presented first as the damage peak X was used to align oxygen spectra.

We also observed electron radiation induced damage in the HA and CHA oxygen K-edge for samples exposed to higher doses (above 10^5 electrons per nm^2). During the exposition, formation of peaks B* and E* (HA into CaO, Figure 6) and a decrease in the intensity of peak C were observed (HA damaged, Figure 6). The final structure of oxygen K-edge transformation (HA into CaO, Figure 6) is consistent with the pattern attributed to calcium oxide found in the literature [76].

For doses exceeding 10^4 electrons per nm^2 [38], many minerals exhibit a strong peak X at 530 eV (HA damaged, CAL damaged, Figure 6). Peak X is a sign of damage induced by formation of O_2 oxygen molecules [77] or other forms of oxygen radicals, like OH or CO, and is a good marker of early damage modifications of the sample's chemistry. Some studies interpreted the X peak damage signal as the hydroxyl group or a water presence marker [36,37]. Garvie questioned this approach and showed that a direct relation between hydroxyl or water presence and a damage signal cannot be derived [38]. After prolonged exposure to the beam radiation, HA transforms into calcium oxide [78]. It was previously reported, that this transformation occurs in apatites exposed to an electron dose exceeding 10^8 electrons per nm^2 [79]. However, we observed HA-to-CaO transformation at a much lower dose.

As biominerals are prone to degrade under specific beam conditions, precautions need to be taken to preserve their structural and chemical integrity. However, these changes are frequently not considered in the literature, which makes data liable to misinterpretation [38]. Prolonged beam exposure leads to modifications, such as breakdown of elemental bonds, formation and reconstruction of voids and transformations into other compounds [80].

The oxygen coordination environment is sensitive to beam-induced modifications, which makes it an excellent marker for the low dose damage. A total dose of 10^4 electrons per nm^2 is sufficiently low to avoid the radiation damage observed in biominerals. Nevertheless, we recommend monitoring the oxygen edge to minimise the possibility of damage.

3.2.5. Oxygen

Oxygen spectra were aligned using the damage peak X set to 530 eV based on the literature [38]. First, the undamaged signal was acquired, and then acquisition was continued until a peak at 530 eV started to form. Spectra were normalised to the most intense peak of each individual spectrum.

All phosphorus-containing minerals examined (HA, CHA, bTCP, Figure 7) exhibited a characteristic double peak C-D at ~ 537 and ~ 539 eV, respectively. On the high energy shoulder of peak D, a small peak F is positioned at ~ 545 eV. Carbonated HA exhibited an additional, low-energy shoulder B at ~ 533 eV.

Calcium carbonates (CAL & IMP) exhibited a pronounced peak B at ~533 eV followed by smaller peaks C, E and F at ~537, ~540 and ~545 eV. Although the calcite and calcite impurity show a similar distribution of peaks, the intensity of particular peaks fluctuates. Namely, the intensity of peak B is relatively lower in IMP, while E is the highest in IMP. The impurity spectrum also displays shoulder A at ~531 eV. The oxygen K-edge of IMP is consistent with aragonite spectra in the literature [64].

Literature on the oxygen K-edge configuration in calcium-containing minerals is limited. Transitions of core 1s electrons to molecular vacant π^* states of carbonate are responsible for the formation of peak B, while 1s transitions to vacant σ^* states result in formation of E peak at approximately 540 eV [81].

EELS and XANES studies on metal oxides suggests peaks C and D to originate from transitions to hybrid 3d calcium/2p oxygen orbitals (π^* and σ^* , respectively) [82,83]. Similarly, peak E and F would represent transitions to higher energy hybrid states 4s- and 4p-like [82,83]. In oxides, separation of peaks C, D, E and F should be consistent with peak separation seen in metal $L_{2,3}$ -edges (calcium peaks A-D). However, such consistency was not observed in the minerals examined here, which suggests a more complex hybridisation of oxygen orbitals. For example, formation of peak D may be also attributed to hybrid phosphorus-oxygen orbital transitions, as this peak is observed only in phosphorus-containing minerals.

Previously, Gregori *et al.* [84] proposed that the presence of peak C is characteristic of HA, while a decrease in the intensity of peak C indicate presence of bTCP. We detected presence of both peaks in HA, CHA and bTCP. No meaningful variation in the C/D intensity ratio was observed.

In CHA, the C-D double peak is preceded by a broad shoulder B at ~533 eV (Figure 7). This shoulder corresponds to calcium carbonates (CAL, IMP) initial shoulder and peak. Shoulder B observed in CHA has a low intensity, which might be attributed to relatively low amount of carbonate present in the crystal lattice.

The oxygen K-edge structure changes most significantly between minerals allowing discrimination of examined ceramics. The oxygen signal can distinguish between calcium phosphates and calcium carbonates. The presence of carbonate in carbonated HA (CHA) is confirmed by formation of peak B at 533eV (Figure 7).

The ability to identify the presence of carbonate ions with good spatial resolution would be an important analytical tool for the study of wide range of problems in biomineralisation research. In order to confirm the interpretation of the experimental data, we attempted to simulate the oxygen K-edge ELNES for both HA and CHA.

Oxygen K-ELNES for HA was simulated using experimental structure parameters obtained from x-ray diffraction studies [85]. Since partial occupancy is not possible in explicitly atomistic simulations, a random structure program was utilized to populate two of the four possible hydroxide positions to remove investigator bias [86]. Besides the ground-state site-projected electronic DOS, we have utilized the Z+1 approximation to simulate an excited-state that includes a core-hole [59,87]. Comparison of the site projected DOS of the ground state and excited state Z+1, calculations with the experimental oxygen K-ELNES (Figure 8) shows that the effect of the core-hole is to move intensity towards the edge onset. A linear combination of the ground state and excited state models gives an excellent agreement with the experimental data, suggesting partial screening of the core hole.

1 For carbonated hydroxyapatite a relaxed structure was used in which both, an A site and a B site,
2 carbonate substitution is present in the primitive cell [88]. The objective was to establish if the
3 carbonate bonded oxygen atoms contribute additional low energy intensity to the oxygen K-edge seen
4 in the experimental spectra (Figure 7). In the CHA, only the projected DOS was calculated, as there
5 are twenty-seven unique oxygen atoms that would each need to be treated independently to construct
6 a Z+1 model. The contributions from all oxygen atoms to the unoccupied p-like states were compared
7 with the contributions from those of non-carbonated oxygen atoms (Figure 9), which showed that
8 carbonate oxygen atoms increase the relative intensity of the lowest-energy peak. Finally a model in
9 which the carbonate groups in CHA were replaced with phosphate and hydroxyl groups with AB
10 substitution was also considered. The results in Figure 9 show clearly that introduction of carbonate
11 ions results in a small pre-peak on the oxygen K-ELNES which is consistent with the experimental
12 data.

3.3. EELS examination of bone mineral

Changes in fine features of elemental edges observed in bioceramic standards will help to interpret fine structure seen in the EELS spectra taken from bone mineral (Figure 10) and other mineralising tissues.

To demonstrate that STEM-EELS has the spatial resolution to map the distribution of compositional variations within ceramics, we have acquired spatially resolved chemical maps and shown, that there are small (>20 nm) clusters within the bulk of the CHA with a higher concentration of carbonate than is present in the surrounding material (Figure SI 7).

To validate this approach for biomaterials, we performed EELS of bone taken from a murine model. Bone mineral possesses the major characteristic features of carbonated apatite. In the phosphorus L-edges (Figure 10 I), peaks A and C, assigned to transitions from the 2p core states to unoccupied states of p-like and d-like symmetry, were observed. In the carbon K-edges (Figure 10 II), peaks A and D, assigned to 1s- transitions in C=C bonds and in carbonate, respectively, were detected. In the calcium L-edges (Figure 10 III), peaks B and D assigned to spin-orbit splitting, were measured. In oxygen K-edges (Figure 10 IV), peaks C and D were observed, which were attributed to various transitions to p-like states of all oxygen atoms. Spatially resolved maps at the carbon K-edge showed variation in the fine structure between intra- and extra-fibrillar regions of bone (Figure SI 8 & SI 9). The intrafibrillar regions usually exhibited a peak at 290eV corresponding to the presence of carbonate.

Examination of carbon K-edge successfully revealed the presence of carbonate in bone (peak D), confirming that it is possible to detect carbonate in bone with the nanometre scale spatial resolution using EELS.

4. Conclusions

The chemical composition of biologically formed minerals at the nanometre scale is complex and employment of standards is essential to unravel the exact composition of biological systems. In particular, to establish whether the carbonate signal originates from calcium carbonate, carbonated HA or other minerals. We have successfully shown that EELS can be used to detect the presence of carbonate in bone mineral and can be utilised to differentiate between calcium carbonates, carbonated HA and pure HA in hard tissues and synthetic bioceramics, by comparing the carbon and oxygen K-edges. We also discovered a nanometre scale impurities and inhomogeneities in prepared mineral standards. Observation of EELS spectra did not reveal any features that might indicate carbonate substitution site in carbonated apatite (CHA), but carbonate substitution site location and orientation has been previously shown using other techniques such as FTIR [41] and neutron powder diffraction [88].

Discrimination between calcium phosphates (HA, CHA, bTCP) and calcium carbonates (CAL, IMP) can be performed directly via observation of the oxygen K-edge (Figure 5). The oxygen edge also provides sufficient information to discriminate between carbonate polymorphs: calcite and aragonite-like impurities. EELS was also capable of detecting small amounts of carbonate present in HA by observation of fine features in the carbon and oxygen K-edges. However, we were not able to distinguish between HA and bTCP.

At the oxygen K-edge, calcium phosphates exhibited the presence of a characteristic double peak at ~537 and ~539 eV and the absence of a strong peak at ~533 eV, which is characteristic of calcium carbonates. Calcium carbonate polymorphs (calcite and aragonite) might be discriminated between by the presence (CAL) or absence (IMP) of the peak at ~537 eV. The presence of carbonated HA can be confirmed by mutual observation of the peak at ~290 eV in the carbon K-edge and the double peak at ~537 and ~539 eV in the oxygen K-edge. CHA also displayed a shoulder at ~533 eV in the oxygen edge. This interpretation was supported by calculations of the oxygen K-ELNES.

In contrast, the phosphorus and calcium $L_{2,3}$ -edges displayed only subtle changes between bioceramics, such as peak shifts. Changes in the separation of peaks might originate from alterations in the bonding environment caused by the differences in crystal structures and/or the presence of dopants (Figure 1, Figure 4). Employment of monochromated EELS, which provides a better energy resolution (<0.5eV), would be beneficial for further examination of elemental edges. Especially observation of small magnitude shifts, which may be easily obscured by experimental noise, in phosphorus and calcium edges would provide additional information, which would be helpful in discrimination of bioceramics.

The main limiting factor in analytical studies of biominerals is their relative susceptibility to beam damage, like oxygen ionisation. Some studies neglected the importance of damage [38]; studies, which do consider damage, do not report the acquisition conditions in a reproducible manner [89]. Here, we described the most characteristic damage markers observed in the oxygen K-edges and their threshold conditions. Selection of appropriate experimental conditions is crucial to reduction of possible damage, while maintaining a high signal-to-noise ratio at the same time to distinguish between different species. Data collected without appreciation of damage mechanisms will be liable to misinterpretation about the native mineral composition and structure.

Studies of bone and other mineralizing tissues may provide additional challenges. One of the concerns is the effect of sample preparation for electron microscopy. Previous studies using both

1 ultramicrotomy and focus ion beam milling for the preparation of tissues shown that there is no
2 change in chemical signatures, which might arise from the fixative and resin [12,90]. Another
3 challenge arises due to the intrinsic heterogeneity of tissues. The emphasis needs to be put on the
4 examination of characteristic nanofeatures, such as collagen gap and overlap regions.

5 In summary, STEM-EELS was successfully used to differentiate between calcium-containing
6 biominerals (hydroxyapatite, carbonated hydroxyapatite, beta-tricalcium phosphate and calcite) and
7 we show that this method can be utilized to map carbonate from the crystallites in bone. We also
8 show that EEL spectrum images can be used to map small variations in carbonate distribution within
9 CHA with a spatial resolution of 5 nm.

10 In mineralising tissues, the nanometre scale resolution is necessary to distinguish between
11 extrafibrillar and intrafibrillar mineral distributed in gap and overlap regions of a collagen fibril and to
12 observe initial nucleation clusters. This technique would be also beneficial for studying bioceramics
13 chemistry across grains, grain boundaries and other nanometre scale features. It is important that these
14 materials are phase pure to ensure that we have full control over their bioactivity.

15 EELS is currently the only technique capable of providing spatially resolved maps of the chemistry
16 and coordination environment of bioceramics and mineralizing tissues with nanometre (potentially
17 sub-nanometre) resolution. Synchrotron XRD [91,92] and nanoFTIR [93] can be used to map
18 minerals with the micron and sub-100 nm resolution, respectively, and provide important
19 complementary information to STEM-EELS about the chemical composition within bioceramics and
20 bone. Applied in combination with EELS these methods will open the door for possibilities to develop
21 more detailed mineralisation models and will enable nano-scale characterisation of the distribution of
22 substituted ions in bioceramics and of tissue/implant interfaces *ex vivo*. The combination of bulk
23 techniques (in which average composition is assessed) and spatially resolved techniques, such as
24 EELS, allows for composition to be assessed and compared at multiple length scales. High resolution
25 techniques are particularly critical for understanding biological processes that occur on the nanometre
26 length scale, such as mineralization or the mineral-collagen interface.

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Mineral name and formula	Abbreviation	Notes
Hydroxyapatite $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$	HA	As precipitated and heated to 1200°C
Carbonated hydroxyapatite $\text{Ca}_{10}(\text{PO}_4)_{6-x}(\text{CO}_3)_x(\text{OH})_{2-y}(\text{CO}_3)_y$	CHA	Ca/P=1.76, heated to 800°C, A/B = 0.099 Ca/P=1.74, heated to 900°C, A/B = 0.168
β-tricalcium phosphate $\text{Ca}_3(\text{PO}_4)_2$	βTCP	Heated to 1100°C
Calcium carbonate - calcite CaCO_3	CAL	Sigma Aldrich 239216, ACS Reagent ≥ 99.0%,

3 **Table 1. List of standards used in the EELS study**

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Ca/P ratio	Temperature °C	A/B ratio
1.76	800	0.99
1.76	900	1.02
1.74	900	1.27
1.76	1000	1.46
1.72	900	1.54
1.72	800	1.63
1.74	800	1.68

5 **Table 2. A/B ratios in ascending order for CHA produced using various Ca/P and heat treatment temperatures. The two extreme A/B**
6 **ratios were used in the TEM-EELS study.**

Figure 1. Representative XRD patterns of selected mineral standards used in STEM-EELS studies. The lower crystallinity shown in carbonated HA (lowest A/B ratio) is due to the lower heat treatment temperature and lower Ca/P ratio.

Figure 2. Phosphorus $L_{2,3}$ -edge structures of hydroxyapatite (HA), carbonated hydroxyapatite (CHA) and beta-tricalcium phosphate (bTCP). All phosphate-containing minerals displayed a characteristic pattern of two main peaks: peak A and peak C followed by a broad peak D.

Figure 3. Carbon K-edge structures of carbonated hydroxyapatite (CHA), calcite (CAL), an impurity (IMP) in the sample, and amorphous carbon (AC). The carbonate-containing minerals (CHA, CAL, IMP) display a sharp peak D at 290 eV and a less intense peak H at 302 eV. The IMP edge was obtained via independent component analysis (ICA; Figure 4).

Figure 4. The original carbon K-edge spectrum acquired from an impurity crystal grain found within the calcite sample. The amorphous carbon film (AC) and impurity (IMP) spectra were reconstructed by the ICA method.

Figure 5. Calcium $L_{2,3}$ edge structures of hydroxyapatite (HA), carbonated hydroxyapatite (CHA), beta-tricalcium phosphate (bTCP), calcite (CAL) and the impurity (IMP) in the calcite sample. All specimens displayed two peaks B and D characteristic of the calcium L-edge. All minerals containing carbonate groups showed a small shift of peak D towards higher energies, in comparison to the pure calcium phosphates (HA, bTCP). Only CAL displayed additional peaks A and C characteristic of multiplet splitting.

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Figure 6. Damage markers seen in the oxygen K-edge. CAL (calcite) and HA (hydroxyapatite) display an additional peak at 530 eV when exposed to an electron dose above 10^4 electrons/nm². The spectrum obtained from HA exposed to an electron dose above 10^5 electrons/nm² (HA into CaO) is consistent with calcium oxide spectra presented in the literature. CaO exhibits additional peaks marked as B* and E*.

Figure 7. Oxygen K-edge structures of hydroxyapatite (HA), carbonated hydroxyapatite (CHA), beta-tricalcium phosphate (bTCP), calcite (CAL) and the aragonite-like impurity (IMP). Calcium carbonates (CAL, IMP) display a characteristic peak B. Calcium phosphates (HA, CHA, bTCP) display a characteristic double peak C-D.

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Figure 8. EELS spectra plotted along with the unoccupied site projected density of $\langle p \rangle$ states of all oxygen atoms present in hydroxyapatite structure. The blue curve is a ground state calculation while the green curve represents an excited state approximation obtained through the use of a Z+1 approximation. The light blue curve is a linear combination of both the ground-state and Z+1 calculation.

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Figure 9. The ground-state unoccupied density of oxygen «p» states for type AB carbonated hydroxyapatite. The green curve includes contributions from all oxygen atoms while the blue curve shows the contributions from all oxygen atoms except those involved in a carbonate group. The red curve represents contributions from all oxygen atoms except carbonated bonded oxygen atoms, and in lieu of these carbonate bonded oxygen atoms contributions from the ions that were substituted for carbonates are included. The red curve effectively models non-substituted hydroxyapatite. All plots are normalized.

Figure 10. Representative EELS spectra of phosphorus (I), carbon (II), calcium (III) and oxygen (IV) edges collected from wild type mouse bone. The major peaks in each of the edges were labelled as in Figure 2, Figure 3, Figure 5 and Figure 7.