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Probing carbonate in bone forming minerals on the nanometre scale

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ABSTRACT

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

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 $Ca_{10}(PO_4)_6(OH)_2$ 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

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

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

Scanning transmission electron microscopy (STEM) combined with electron energy-loss spectroscopy (EELS) is the only technique capable of achieving nanometre scale resolved information about the chemistry and coordination environment of minerals. Previous studies have attempted to identify spectral fingerprints from bioceramics using STEM-EELS [36,37]. However, these studies did not consider carefully the effects of irradiation of biominerals, which makes the results liable to misinterpretation [38]. In addition, previous studies focused on selected edges, rather than comparing edges of all characteristic elements present in the mineral (i.e. P, C, Ca, O). To our knowledge, no previous studies have identified the presence of carbonate from bone mineral by studying fine structure in the EELS spectra at these edges. Here we used EELS to discriminate between different bioceramic standards. Phase pure hydroxyapatite (HA), carbonated HA (CHA) and beta tricalcium phosphate (bTCP) were selected as these bioceramics are likely to be present in bone tissue at different stages of mineralisation [8,9] or are relevant in clinically enhanced mineralisation. Calcium carbonates were examined to determine if carbonate ion substitution in the HA lattice is detectable with EELS. The near-edge core loss spectra of phosphate, carbon, calcium and oxygen were acquired and analysed for various forms of synthetic biomineral and also for healthy mouse bone tissue. A study of the effect of electron dose was conducted in order to observe changes in the spectra that 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 $Ca(OH)_2$ and H_3PO_4 where the pH is kept above 10.5 using aqueous ammonia [39,40]. $CaCO_3$ (Sigma Aldrich ACS reagent grade 239,216) was decarburised over night at 960 °C then cooled under vacuum. The resulting CaO was hydrated in deionised water to form $Ca(OH)_2$, 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 $Ca(OH)_2$. 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 and Bonfield [41]. Ca/P ratios of 1.76, 1.74 and 1.72 were considered. Similarly to HA, $Ca(OH)_2$ 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 $Ca(OH)_2$ 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 h. The other fraction was investigated without heat-treatment.

Beta-tricalcium phosphate (bTCP) precursors were formed through a combination of $Ca(OH)_2$ 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 h to produce bTCP [42].

Table 1
List of standards used in the EELS study.

Mineral name and formula	Abbreviation	Notes
Hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$	HA	As precipitated and heated to 1200 °C
Carbonated hydroxyapatite $Ca_{10}(PO_4)_{6-x}(CO_3)_x(OH)_{2-y}(CO_3)_y$	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 $Ca_3(PO_4)_2$	bTCP	Heated to 1100 °C
Calcium carbonate - calcite $CaCO_3$	CAL	Sigma Aldrich 239216, ACS Reagent >=99.0%,

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