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Hydroxyapatite-based materials of marine origin: A bioactivity and sintering study

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ABSTRACT

Single phase hydroxyapatite (HAp) and biphasic material hydroxyapatite/β-tricalcium phosphate (HAp/β-TCP) were obtained from a marine source (Atlantic cod fish bones).
Here we report a study on the biological properties of these materials, including cytotoxicity, bioactivity and haemocompatibility. Results showed that the materials are not cytotoxic, neither in their powder nor in pellet form; indeed growth of Saos-2 cells was comparable to that of commercial. The haemolysis rate was lower than 2%; hence the materials can be classified as non-haemolytic. Moreover, when immersed in Simulated Body Fluid (SBF), crystal formation was observed on the surface of both materials.
The sintering behaviour of the samples was also studied; both powders showed very high sinterability (density higher than 95% of the theoretical value).
Overall, these results confirm the suitability of these materials for biomedical applications.

1. Introduction

Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$ — HAp) is a material belonging to the calcium phosphate family, frequently used for bone and dental implant fabrication due to its high biocompatibility [28]. Other calcium phosphates are also employed in this field, often combined with HAp; tricalcium phosphate ($Ca_3(PO_4)_2$, TCP), for instance, shows good biocompatibility and resorbability in both its α and β forms [9]. HAp and other phosphate-based materials are also bioactive, promoting bone growth and formation [30].

Biocompatibility and/or bioactivity can be affected by several parameters; the presence of some minor elements, for instance, can play a crucial role. According to literature data, chlorine ions can increase the bioactivity [7], while fluorine can have a positive effect on osteoblast growth [10]. Positive ions such as sodium or magnesium can also improve the material's performance [8,27]. Moreover, microstructural features such as porosity and surface roughness can also be important [21,29].

For HAp to be used for bone implant fabrication, its mechanical properties are also crucial; its sintering behaviour, in particular, is important, as this can affect other mechanical properties [23]. Sintering can depend on different parameters, which include particle size and shape. The presence of other ions in minor amounts can also have an effect.

HAp in powder form can be prepared by different methods, which generally are based on a chemical reaction between calcium and phosphorus salts. For single phase HAp to be obtained, the Ca/P ratio has to be equal to the stoichiometric HAp ratio (1.67). If Ca/P = 1.5, on the other hand, single phase TCP is prepared [11]. If the Ca/P ratio is within these two values, bi-phasic materials of HAp-TCP can be obtained. Additional reagents can also be added to the synthesis, if minor elements like those mentioned above are to be introduced in the HAp lattice [24].

HAp can also be obtained from biological sources [1]; corals and sea shells, for instance, contain calcium carbonate, which can be converted into HAp with a reaction with an appropriate phosphorus source. Animal and fish bones, however, are a particularly good HAp source, as they already contain it naturally. Therefore, only an extraction process in necessary to obtain HAp, without any chemical reaction [18]. Pig bones, for instance, were successfully converted into HAp [14]; several fish have also been used, including swordfish, different species of tuna and sardines [5,19,31]. In the majority of cases, the extraction was achieved simply with a treatment at high temperatures (from 700 °C onwards); in this way, organic matter was removed, leaving only the inorganic phosphate. In other cases, however, different treatments were employed to eliminate the organic matter, such as alkaline hydrolysis or subcritical water process [2,31]. One of the advantages of HAp derived from these sources is that it already contains the minor elements described above, which can have beneficial effects on HAp properties [1].

Calcium phosphate-based materials derived from cod fish bones were previously investigated by the authors of this paper. Results

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showed that a thermal calcination of the bones led to a HAp/β-TCP biphasic material, the proportion of each phase being different depending on the calcination temperature [18]. The formation of β-TCP was due to the Ca/P being lower than 1.67. It was also shown that treating the bones in solution before the calcination could change the composition of the bones and, consequently, of the calcined material. This was because, using suitable solutions, selected ions could be introduced into the lattice structure. A calcium-containing solution, for instance, increased the Ca content in the bones and led to a single phase HAp material; other ions such as sodium or fluorine could also be included in the material. However, biocompatibility of these materials has not yet been demonstrated.

Therefore, in the present work, we report on the biological properties of the HAp-based materials produced from cod fish bones as described above. Both biphasic HAp/β-TCP and single phase HAp samples were investigated; properties such as bioactivity, cytotoxicity and haemolysis were tested. Moreover, sintering experiments were also performed.

2. Materials and methods

2.1. Sample preparation

HAp extraction from cod fish bones was previously described in literature [18]. Briefly: fish bones were washed and crushed manually into large pieces up to 1 cm wide/long. They were then calcined at 700 °C using a Nabertherm furnace, with a heating ramp of 5 °C/min and a calcination time of 1 h. Samples prepared like this will be indicated as **CB** (calcined bones). To increase the calcium content, bones were pre-treated in a $CaCl_2$ solution for 16 h at 65–70 °C, before calcination. The $CaCl_2$ concentration was 5 times higher than the HAp content in the bone, which was estimated as 70% weight. Samples prepared in this way will be indicated as **CBCa** (calcined bones $CaCl_2$).

The composition of the **CB** samples calcined at different temperatures was determined by XRD as reported in literature [18] and it is shown in Table 1 and Fig. 1. For sample **CBCa**, the composition was always single phase HAp, regardless of the calcination temperature.

Samples prepared in this way were in powder form; in some cases the powder was used without any further treatment, while in others it was compressed in the form of pellet.

2.2. Sintering studies

To investigate the sintering process, **CB** and **CBCa** powders were prepared through calcination at 700 °C, as described above. The powder obtained was milled in a Fritsch high-energy planetary ball mill at 200 rpm for 24 h, in Teflon pots with zirconia balls and iso-propyl alcohol (IPA) as a solvent. Previous work [16] showed that after calcination at 600 °C and milling, the powder had a BET surface area (measured by N_2 adsorption) of ~19 $m^2\ g^{-1}$, decreasing slightly to ~15 $m^2\ g^{-1}$ after heating to 700 °C. The average spherical particle size can be estimated from surface area, using the equation $d = (6000 / SA * \delta)$, where d = average particle diameter in nm, SA = BET surface area and δ = density of HAp (3.15 g cm^{-3}). This yielded an average particle size of 127 nm for the powders calcined at 700 °C and then milled.

Table 1
Phase composition for the samples **CB** calcined at different temperatures (wt.%).

Calcination temperature (°C)	HAp:β-TCP
700	73.2:27.8
1100	69.7:30.3[a]
1200	67.1:33.9[a]

[a] Data previously published [17].

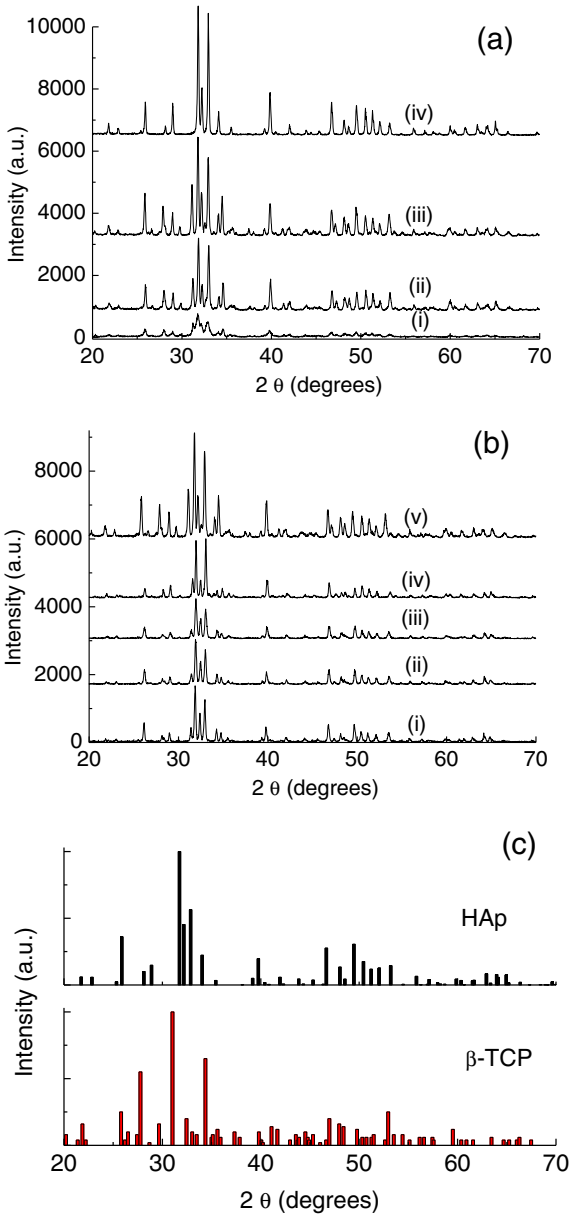

Fig. 1. (a) XRD pattern for the samples (i) **CB** 700 °C, (ii) **CB** 1100 °C, (iii) **CB** 1200 °C, (iv) **CBCa**; (b) **CB** pellets calcined at (i) 900 °C, (ii) 1000 °C, (iii) 1100 °C, (iv) 1200 °C, (v) 1250 °C; (c): standard for HAp and β-TCP.

The milled powders were dried, and pressed into disc shaped pellets with a diameter of 13 mm in a uniaxial press at a pressure of 1 ton. The pellets were then sintered over a range of temperatures between 900 and 1250 °C, with heating/cooling rates of 5 °C/min and dwell time of 2 h. The density of each sintered pellet was obtained by the geometrical method, calculated from the measured weight (±0.0001 g) and diameter and height (±0.01 mm). The maximum (X-ray) densities used for comparison were obtained from the XRD standard XRPDFs 01-010-6315 (HAp = 3.15 g cm^{-3}), 01-072-7587 (β-TCP = 3.067 g cm^{-3}) and 01-070-1454 (ClAp = 3.185 g cm^{-3}). The density of a 75% HAp/25% TCP mixture for sample **CB** was estimated to be 3.13 g cm^{-3}.

X-ray diffraction (XRD) measurements of the pellets were also performed to see whether any significant change in composition took place during the sintering studies. The diffraction patterns were acquired on a PANalytical X'Pert Pro diffractometer, equipped with a fast RTMS detector (PANalytical PIXcel 1D) and with graphite monochromated Cu Kα radiation, measured at 40 kV and 40 mA, over a 2θ range of 20–75°, with a virtual scan step of 0.0167° and integration time of 100 s. The