A novel biphasic calcium phosphate derived from fish otoliths

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Abstract. Calcium phosphates are bioceramics that have been widely used as bone substitutes because they encourage the formation of bone on their surface and can improve the healing of the bone. Hydroxyapatite HA (calcium/phosphorus ratio of 1.67) and tricalcium phosphate TCP (calcium/phosphorus ratio of 1.50) are the most common calcium phosphates. Natural materials have begun to be tested to make HA or TCP such as shells of cardiidae (family of mollusks) and eggshells. The calcium phosphate obtained has a high ability to precipitate apatite. In this work, the mixed phase ceramic of beta-Tri-calcium phosphate / hydroxyapatite (β-TCP/HA) was synthesized by aqueous precipitation from fish otoliths, which are monomineralic species composed of aragonite. Otoliths of the specie Plagioscion squamosissimus, commonly called the river croaker, were used. Techniques such as DRX, Raman spectroscopy and SEM-EDS were used to characterize the raw material and the obtained material. X-ray diffraction analysis revealed the presence of two crystalline phases of calcium phosphates with 86.2% crystallinity. SEM micrographs showed agglomeration of particles with porous structure and submicron particle sizes.

1. Introduction
Calcium phosphates (CaPs) that were used as coatings or bulk implants improved bonding between bone and implant. The most known CaPs are Beta–Tri–calcium phosphate (Ca₃(PO₄)₂, β-TCP) and calcium hydroxyapatite (Ca₁₀(PO₄)₆(OH)₂, HA), which have been used as corrective therapies for bone defects and diseases [1-3]. HA has excellent biocompatibility and its components are similar to those of natural bone and teeth. However, it has been found that dense hydroxyapatite hinders bone growth due to its low biodegradability, so in these cases β-TCP works better, the latter has been proposed for orthopedic applications because its bioresorption rate is higher compared to pure hydroxyapatite. A mixture of HA and β-TCP called "biphasic calcium phosphate (BCP)" was first introduced by Moore and Anuta in 1985. The BCP consists of a mixture of CaPs phases with similar Ca/P molar radius [4,5]. However, there are controversies in the appropriate amount of each of the present phases and the physico-chemical properties in the BCP. The higher relative biological solubility of tricalcium phosphate in biological medium and the partial dissolution of HA, increases the local concentration of phosphate (HPO₄²⁻, PO₄³⁻) and calcium (Ca²⁺) ions, followed by a precipitation of calcium carbonate phosphate, these precipitates induce the growth of bone to the implant by attraction of osteoblasts, followed by osteoclasts that dissolve the carbonaceous precipitates in native bone in the defects related to low resorption of HA. A high concentration of TCP results in the rapid formation of bone at the expense of long-term osseointegration. In addition, a high amount of HA can occlude the porosity of...
the metal surface so that adequate bone apposition is not achieved. Resorption kinetics governs the fixation of the implant and therefore the formation of bone [1,5,6].

Calcium phosphates are synthesized by chemical techniques such as aqueous precipitation, sol-gel, hydrothermal method and thermal deposition, however raw material costs are high and yield is small. Other naturally occurring materials have begun to be tested to make HA or β-TCP such as shells of cardiidae (family of mollusks) and egg shells. The HA obtained has a high ability to precipitate apatite [4].

Natural materials not widely studied in this type of applications are the otoliths. Otoliths are a material found in the inner ear cavity of fish, located in the saccule, utricle and canals, they have a few grams or milligrams in weight and its morphology depends on the specie. An X-ray diffraction analysis shows that otoliths are monomineralic species composed of aragonite. The otolith grows from a central core of organic material called primordium. The organic matrix provides a support that favors the nucleation of aragonite crystals which grow on its c axis, so apparently the aragonite is accompanied by proteins called otoline [7-9]. In a biocompatibility assay in mice, polyethylene tubes containing otoliths were implanted subcutaneously into the back of Wistar mice. The results showed that after 30 days the tissue surrounding the implants was composed of fibroblasts and new corrugated collagen fibers [10]. In this research fish otoliths of the Sciaenidae family of the Plagioscion squamosissimus specie, commonly called Curvinata, river croaker or stone head were used. From otoliths a BCP was synthesized with physicochemical characteristics suitable to be used in the application of orthopedic implants.

2. Experimental

To synthesize the biphasic calcium phosphate, the otoliths were washed with ethanol and deionized water by sonication for 1 hour for each solution and could dry in a horizontal laminar flow biosecurity chamber for 30 minutes. The natural material was calcined at 1000 Celsius degrees for 2 hours at a heating rate of 2°C/min to produce calcium oxide (CaO). Since CaO is highly hygroscopic, calcium hydroxide (Ca(OH)₂) was formed according to equation (1).

A solution of phosphoric acid 0.6M (H₃PO₄, 85%, Merck) is mixed with a solution of calcium hydroxide 1M to pH 4.5, at a rate of 5ml/min with continuous stirring at 90°C for one hour and another hour more without temperature. The mixture is left without stirring for 48 hours at room temperature. The precipitated calcium phosphates are washed with deionized water and dried for 24 hours at 105°C. The reaction carried out is shown in equation (2), after that, the calcium phosphates were calcined at 1050°C for 3 hours.

\[
\text{CaCO}_3 \xrightarrow{1000^\circ\text{C}} \text{CaO} \xrightarrow{\text{H}_2\text{O}} \text{Ca(OH)}_2 \tag{1}
\]

\[
10\text{Ca(OH)}_2 + 6\text{H}_3\text{PO}_4 \xrightarrow{1050^\circ\text{C}} \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 + 18\text{H}_2\text{O} \tag{2}
\]

The phase formation in the ceramic was carried out by X-ray diffraction (XRD, Bruker D8 advance, Germany) in 2θ=20-70°. The qualitative analysis of the phases present in the calcium phosphates was performed by comparing the profile observed with the diffraction profiles reported in the PDF-2 database of the International Center for Diffraction Data ICDD (PDF-2 010-80 -3956 for HA, and for β-TCP 010-70-2065). The quantitative analysis of the phases was performed by refinement by the Rietveld method of the observed profile, adding a known internal standard quantity (Aluminum oxide, -100 mesh, 99%, Corundum, α-phase. Aldrich No. 23.474-5) corresponding to 20.28%.

The morphology of the otolith and the sample obtained was analyzed by scanning electron microscopy (SEM Evo Ma 10 Carl Zeiss Detector X-act oxford instruments). To observe the otolith sample, it was covered with gold (equipment Quorum Q1SORES) in the vacuum for 100 seconds,
producing a layer of thickness of approximately 6nm. The functional groups of CaPs were verified by Raman spectroscopy (Labram HR Evolution HORIBA Scientific) with a 532nm laser.

3. Results and discussion

The X-ray diffraction pattern of otolith powder and Raman spectrum of fish otolith are shown in Figure 1. It is evident that among the calcium carbonate polymorphs, the aragonite peaks (PDF number 000-75-2230 (ICDD)) coincide with the diffraction pattern shown in Figure 1 and 2. According to Pattanaik, the growth of the calcic matrix is mediated by kinetic factors such as the degree of super-saturation of calcium carbonate, which favored the formation of aragonite [11]. Besides that, others environmental conditions like temperature, pollution and biological activity affect the formation of the biomineral structure [12].

The quantitative analysis of the XRD resulted in 82.5% aragonite and 17.5% amorphous. This biomineral structure cannot be formed in a biological environment and is accompanied by acidic glycoproteins, part of the percentage of amorphous is due to the content of acidic groups in the proteins, which are involved in biomineralization processes resulting in a higher capacity of attract Ca\textsuperscript{2+} ions. Additionally, this structure can construct water-insoluble gel structures in the biomineral matrices. Because the proteins are not easily separable, the identification is very complicated [9,13,14].

![Figure 1. XRD pattern of fish otolith.](image1)

![Figure 2. Raman spectrum of fish otolith.](image2)

Figure 2 shows the Raman spectrum of fish otolith. The crystallization of calcium carbonate in its aragonite phase is shown marked by the principal peaks 152, 207, 701-705 y 1085cm\textsuperscript{-1}. The bands between 115 and 273cm\textsuperscript{-1} were assigned to the vibrational modes of the cell, two bands of 701 and 705cm\textsuperscript{-1} were attributed to CO\textsubscript{3} in plane bending, 855cm\textsuperscript{-1} to δ(CCH) ring and C-C stretching, 1085cm\textsuperscript{-1} to symmetrical stretching of C-O and the band 1464cm\textsuperscript{-1} to CO\textsubscript{3} asymmetrical stretching. Both the 855 and the 1464cm\textsuperscript{-1} bands are also attributed to the vibrational modes of organic fractions in the otolith as proline, glutamic acid and serine for the first case [15].

![Figure 3. SEM micrograph of fish otolith.](image3)

![Figure 4. SEM micrograph of translucent zone.](image4)

![Figure 5. SEM micrograph of white zone.](image5)
SEM micrograph of fish otolith shows a different morphology on its outer surface. Two areas are clearly distinguished, which we will call “white zone” and “translucent zone” (Figure 3). The translucent area shows an aggregate structure in the form of flakes formed from small crystals of aragonite (Figure 4). These crystals are similar in appearance to otoliths of the Hoplostethus Atlanticus fish, which were described as aragonite [16]. The white zone has aggregates in the same direction as those described above, however, the morphology is not the same, and the aragonite crystals are not so evident (Figure 5). Despite this, there was no significant difference in the percentage of calcium in the EDS analysis of the two zones, these percentages oscillated between 18 and 22% (atomic %) of calcium.

The X-ray diffraction patterns of BCP powder synthetized from otoliths of River Croaker are shown in Figure 6. The presence of two crystalline phases of hydroxyapatite and tricalcium phosphate in proportions 18.3% and 67.9% respectively is evident. A total of 13.8% of amorphous was found. Figure 6 shows the main characteristic peaks of HA at 2θ diffraction angles relevant to each peak: 25.9°, 31.7°, 32.2°, 32.8°, 34.1°, 46.9° and 49.4°. For β-TCP, the main peaks are; 25.9°, 26.5°, 29.6°, 31.0°, 32.8° and 34.3°, corresponding to the results shown by Ebrahimi [5].

The Raman spectrum of BCP powder synthetized from otoliths is shown in Figure 7. According to the literature, two peaks at 948 and 970cm⁻¹ were assigned to the symmetric vibration P–O stretching mode (v₁PO₄³⁻) of β-TCP. Between 500 and 650cm⁻¹, the peaks 548, 611 and 630cm⁻¹ were assigned to the asymmetric vibration bending mode (v₃PO₄³⁻) of β-TCP and the peak 595cm⁻¹ were assigned to the asymmetric vibration P–O of HA. Typical HA vibrations (v₂PO₄³⁻) bending mode (O–P–O) at about 430-450cm⁻¹ were presented, as well as, asymmetric stretching (v₃PO₄³⁻) mode at 1015-1089cm⁻¹. The vibrations (v₂PO₄³⁻) bending mode to 406cm⁻¹ was identified for β-TCP [17-19]. The peak 276cm⁻¹ could not be identified.

Figure 6. (a) XRD pattern of BCP derived from fish otolith (■ HA peaks, ● β-TCP peaks).

Figure 7. Raman spectrum of BCP.

SEM micrograph and chemical mapping for oxygen, phosphorus and calcium are shown in Figure 8 and Figure 9. The BCP particles were larger spherical, in porous structure, with a particle size up to 2μm. This morphology is similar to that reported by Gao et al [20] for 100% β-TCP with a particle size of 1μm. The percentage of HA and amorphous causes the particle size to increase in the BCP. The distribution of oxygen, phosphorus and calcium elements is homogeneous throughout the sample and no precipitates are found (Figure 9).

Bioceramics with a high dissolution rate encourage important pH changes that can result in inflammatory processes [21], while bioceramics with very slow dissolution rates can hamper bone growth. Therefore, manufacturing and evaluating BCP with different proportions of HA and β-TCP is of scientific interest, since the results regarding kinetics of degradation can be varied. According to the results found the obtained proportion of HA and TCP can provide a surface suitable for cell proliferation, especially if they come from natural materials.
4. Conclusions

Otoliths of the Plagiomicron squamosissimus specie are monomineral species from the aragonite type accompanied by organic fractions. The XRD and Raman analysis of BCP synthesized from fish otoliths confirmed the presence of two crystalline phases HA and β-TCP and the different modes of vibration of the $PO_4^{3-}$ ion. On the other hand, the morphology shows that there was more crystallization of the TCP phase with respect to the HA phase and the percentage of amorphous that modifies the particle size. The material synthesized 18% HA/67.9% β-TCP is a novel BCP derived from a natural material, with physicochemical characteristics similar to synthetic BCP, since they are materials that can encourage both bone replacement and bone regeneration.

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