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Cooling rate effects on thermal, structural, and microstructural properties of bio-hydroxyapatite obtained from bovine bone

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Abstract: This article is focused on the study of cooling rate effects on the thermal, structural, and microstructural properties of hydroxyapatite (HAp) obtained from bovine bone. A three-step process was used to obtain BIO-HAp: hydrothermal, calcinations, and cooling. Calcined samples in a furnace and cooling in air (HAp-CAir), water (HAp-CW), and liquid nitrogen (HAp-CN2), as well as an air cooled sample inside the furnace (HAp-CFAir), were studied. According to this study, the low cooling rate that was achieved for air cooled samples inside the furnace produce single crystal BIO-HAp with better crystalline quality; other samples exhibited polycrystalline structures forming micron and submicron grains. © 2015 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater, 104B: 339–344, 2016.

Key Words: hydroxyapatite, cooling rate, structural properties of bone, bio-hydroxyapatite

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INTRODUCTION

Hydroxyapatite (HAp) is a stoichiometric material formed of $Ca_{10}(PO_4)_6(OH)_2$, while a mineral component of the bone called Bio-hydroxyapatite (Bio-HAp) is a non-stoichiometric HAp that contains other amounts of ions such as Na, Zn, Mg, K, Si, Ba, F, CO_3.[1,2] Bio-HAp is growing in importance due to its potential applications in surgery, such as healing of segmental bone defects; but so far, it is a big problem due to the limited availability of bone processed material with special characteristics to be used in orthopedic, dental, and trauma surgery. The main application of Bio-HAp or Hap use is to fill the defects and promote bone growth.

From a clinical standpoint, the best way to repair a bone defect that can usually be done through the replacement of a bone part or by the filling of a hole in the bone is by using autologous bone or by using biomaterial or xenograft with the similar characteristics, as is the case of bovine bone. This can be done with the use of Bio-HAp which contains almost all the ions present in the human bone. Some important aspects related to the crystallinity of Bio-Hap, chemical composition, and HAP crystal grain size are still an open problem that need to be addressed in terms of micro and structural studies. Usually, the HAP contained in the human bone does not have a high crystalline quality[1]; therefore, it is necessary to study different annealing and cooling processes to obtain Bio-HAp with similar structural and microstructural properties of the human bone.

Bone from a biological source is composed of fat, proteins, water, and inorganic crystalline and amorphous compounds. In order to obtain a xenogenous bone, it is necessary to remove all organic components by using solvents or hydrothermal processes. The problem is that, in fact, organic and inorganic materials are forming a matrix in which these components are together. Several methods had been proposed in order to remove the organic phase. Giraldo-Betancur et al.[1] proposed a multistep process: first, the fat from the cortical bovine bone is removed; then, use an alkaline process to remove the protein in which some ions that are not part of the inorganic phase are removed; the final step is the calcination that removes any organic material for $T > 700^{\circ}C$. In this study, a physicochemical comparison between commercial and calcined samples was done, and the most important aspect found in their work was that commercial BIO-HAp contains organic materials. Another process to obtain BIO-HAp begins with the deproteination followed by calcination in air[3]; but some ions, present in fat and protein, could be present for the calcination process.

Calcium phosphate (CP)-based biomaterials are a group of compounds having Ca/P molar ratios in the range of

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0.5–2,[1,4] which are directly related to the source used to obtain the BIO-HAp (bovine, fish, etc). Bio-HAp is now used for the reconstruction of various bone defects especially in the field of dentistry, orthopedic, and trauma surgery.[5] However, the specific Bio-HAp properties for each one of these applications is still an open problem; and of course, these properties are directly related to the method used to obtain it.

Recently, Akram et al.,[6] highlights the importance of obtaining Bio-Hap from biological resources, with application in surgery. They explain in detail that fish and bovine sources are valuable candidates for the production of BIO-HAp, whereas use of biogenic and plant sources tend to furnish thermally unstable HAp, which could be due to the incomplete conversion of precursors to HAp or due to the presence of carbonate ions in natural materials.

Ooi et al.[7] studied the properties of porous HAp bioceramic produced by heat treatment (annealing) of bovine bone obtained for temperatures between 400°C and 1200°C. They found that "as-received" bovine bone contains organic compounds which, upon annealing at temperatures above 600°C, were completely removed from the matrices. Bovine bone annealed between 800 and 1000°C revealed the characteristics of a natural bone with the interconnecting pore network being retained in the structure. However, the cooling rate effects on the structural and microstructural properties of annealed samples were not studied in detail. Taking into account the aforementioned works, it is clear that the annealing process has been studied but the cooling process is still a problem which has to be studied in order to obtain both HAp and Bio-HAp.

The objective of this work is to study the effect of the cooling rate on structural and microstructural properties of Bio-HAp obtained by a three-step process: cleaning, calcination, and cooling. Thermogravimetric analysis (TGA) was used to study the thermal evolution of bare bone, and to determine the temperature for the calcination process while X-ray diffraction was used to study the structural changes that take place as a result of this multistep process, and scanning electron microscopy was used to study the changes in the morphology of the BIO-HAp.

MATERIALS AND METHODS

Bone is formed by two components: organic and inorganic. The organic phase is mainly composed by fat and protein, while the inorganic phase is composed by crystalline and amorphous components. The first step in order to obtain the inorganic phase of the bone (HAp) is the removing of the fat and proteins. Several methods had been used to remove these two components and, in this study, the hydrothermal method was used, which is a modification of the process reported by Giraldo-Betancur et al.[1]

Bone cleaning process

Three different processes were carried out in order to obtain pure Bio-HAp: defat/deproteinize, calcination, and cooling, respectively. Figure 1 shows the block diagram followed to carry out these three processes until Bio-HAp is obtained.

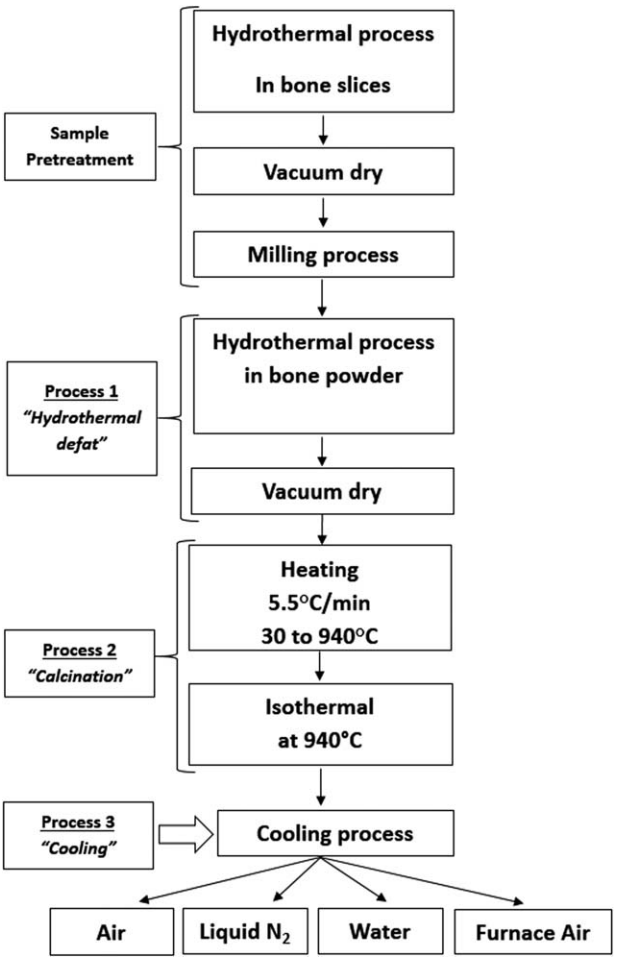

FIGURE 1. Block diagram of the three-step process to produce BIO-Hap; cooling process was carried out in air, liquid nitrogen, water, and furnace air.

Slices of the bovine bone from the central part of the femur (cortical bone) were cut in sections of 2 mm thickness and the adhering soft tissue was removed manually. After this, the fluids in bone, marrow, and any remaining soft tissue were eliminated by boiling the slices at high pressure (154°C, 4 atm) using an autoclave. The bone slices were then subjected to vacuum drying and a milling process using a stainless steel mill (Oster-USA) until the powder was fine enough to pass through a100 mesh sieve (147 μm). This bone powder was subjected to the following processes:

Process 1. Hydrothermal defat. This consists in the removal of fat/proteins from the bone powder without solvents; firstly, the bone powder US mesh 100 (147 μm) was heated at 154°C and 4 atm for 30 min (three times). In this way, the use of petroleum ether was avoided. Finally, the sample was washed twice with boiling water (92°C) and dried in a vacuum oven at 1.33 Pa and 70°C for 5 h. A sample obtained using petroleum ether (HS) was also obtained in order to compare the process.

Process 2. Calcination. Calcination of Bio-HAp was carried out as follows: a charge of 30 g of HAp powder was packed

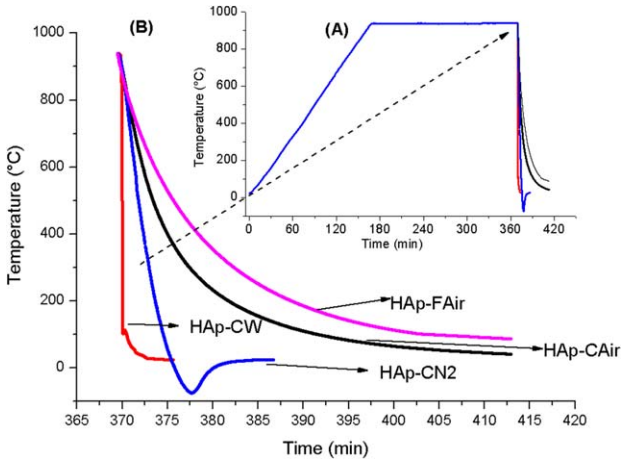

FIGURE 2. A: Shows the characteristic thermal profiles used to obtain the calcined samples at 5.5°C/min for the heating and isothermal temperature at 940°C, (B) shows the cooling rates for each one of the studied samples.

in a cylindrical stainless steel container: 2.54 cm internal diameter and 25 cm long; then it was heated up to 940°C in an electrical resistance furnace. Then, the temperature was maintained for a period of 3 h. Previously, two thermocouples were mounted at the center of the cylindrical container and at 2.5 cm from the top to the end [see Figure 2(A)]. The

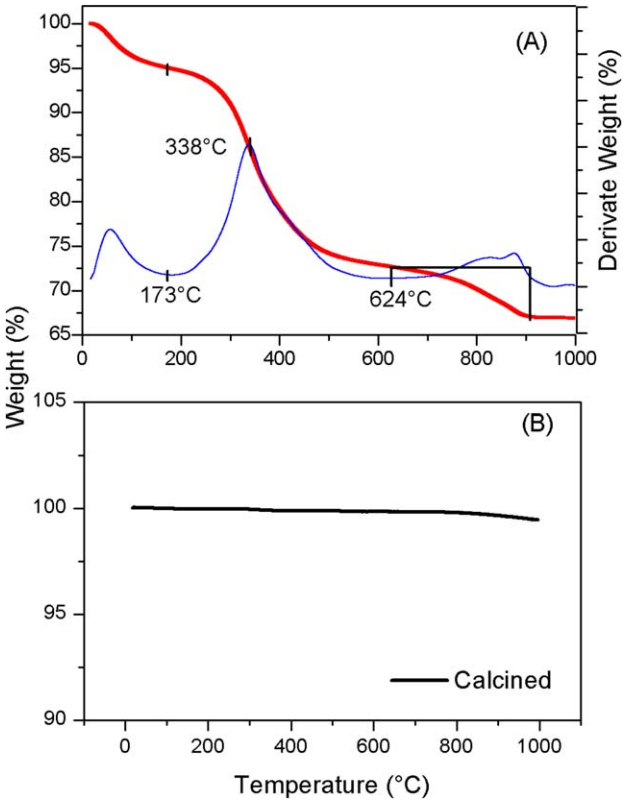

FIGURE 3. A: Characteristic TGA analysis of bone powder and its first derivative, (B) shows the TGA analysis of calcined sample HAp-FAir.

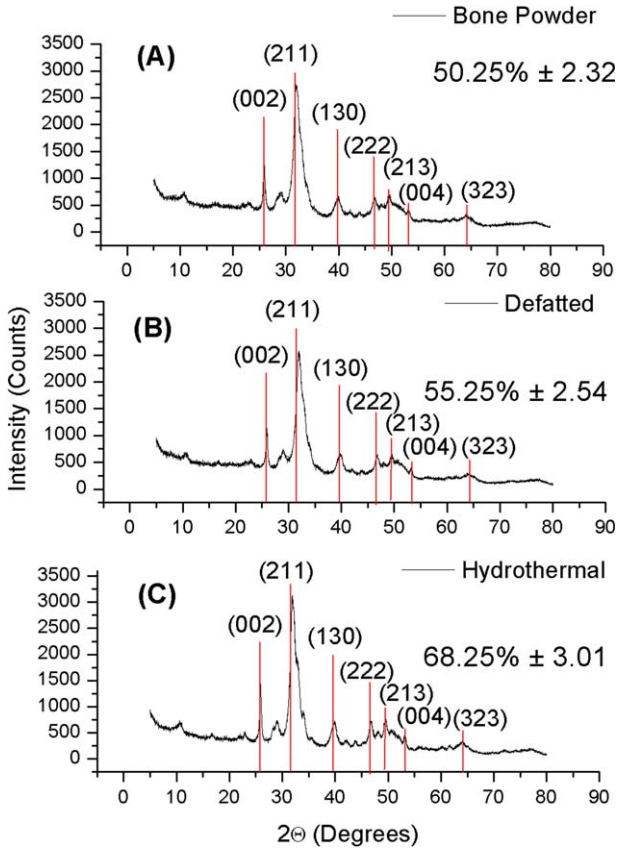

FIGURE 4. A: Shows the X-ray diffraction patterns of bone powder, (B) defatted sample using solvent process, and (C) sample defatted using hydrothermal process.

first ramp at 5.5°C/min was used to eliminate organic materials without promoting the generation of carbonate phases. The second isothermal part was used to reduce thermal stress due to fast heating in the first part of the ramp.

Process 3: Cooling. Four samples were calcined and cooled: HAp-CW, HAp-CN2, and HAp-CAir which correspond to defatted powders calcined in a furnace but cooled in water, liquid nitrogen, and air, respectively; and the fourth which corresponds to the sample calcined and cooled inside the furnace called HAp-FAir.The calcination process was carried out at Furnace Felisa, Mexico (see Figure 1). Each cooling process was carried out three times per sample.

Figure 2(A) shows characteristic thermal profiles for HAp-CW, HAp-CN2, and HAp-CAir, HApFAir; and Figure 2(B) shows the thermal profile corresponding to cooling process for each one of the aforementioned samples. For the cooling process, the stainless steel container with bone powder was immediately removed from the furnace and cooled in water, air, and liquid nitrogen and one sample was cooled in air inside the furnace. These three processes were carried out in order to study the influence of cooling process and specify the cooling rate on structural and microstructural properties of Bio-HAp.

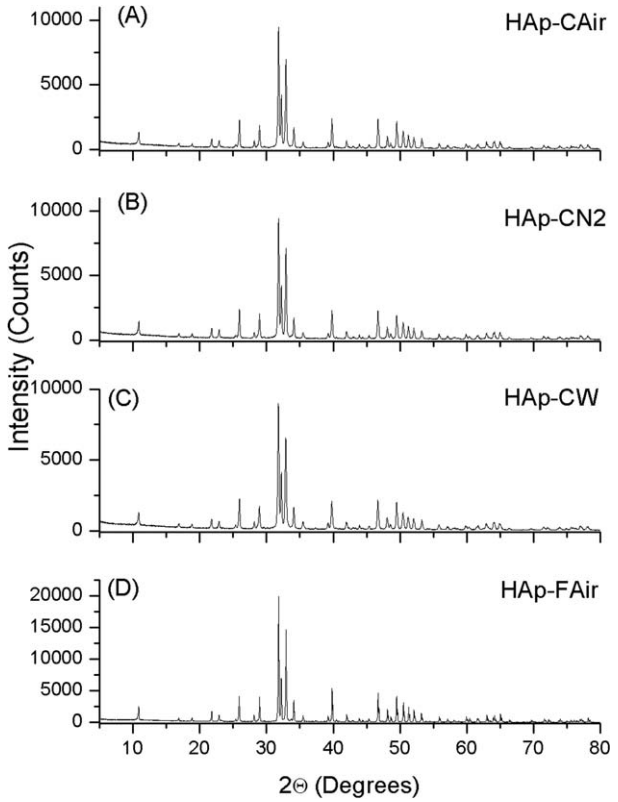

FIGURE 5. Shows the X-ray diffraction patterns of (A) HAp-CAir, (B) HAp-CN2, (C) HAp-CW, and (D) sample cooled in furnace HAp-FAir.

Thermal behavior: TG analysis

The thermogravimetric curves and their derivative, in relation to temperature, were obtained for bone and calcined samples by using TG Q500 equipment (TA Instruments). The sample mass was 12.0 ± 1.0 mg of each sample and these were placed in the platinum thermo balance crucible (TA Instruments, USA). The samples were heated from room temperature to 800°C, at a heating rate of 10°C/min; the measures were carried out in a constant N_2 flow. The TG data was processed using the Universal Analysis 2000 TA software.

Structural properties: X-ray diffraction (XRD)

X-ray diffraction patterns of samples HAp-CWater, HAp-CAir, HAp-CN2, and HAp-FAir were used to determine the presence of crystalline phases, as well as changes in crystalline quality, and crystalline percentage for samples obtained at different cooling rates. Powder samples (mesh 100) were densely packed in an Al holder. X-ray diffraction patterns of the samples were carried out on a Rigaku Ultima IV diffraction instrument operating at 40 kV, 30 mA with Cu Kα radiation wavelength of $\lambda = 1.5406$ Å. Diffractograms were obtained from 5° to 80° on a 2θ scale with a step size of 0.02°. Full width at half maximum (FWHM), crystalline quality, and crystalline percentages were obtained by the analysis of the patterns using MDI Jade 5.0 (free version). Lanthanum hexaboride powder from National Institute Standards and Technology (NIST), (Standard Reference Material 660a) was used as an internal standard.

Surface microstructure: Scanning electron microscopy (SEM)

Morphologic analysis of all samples was carried out in a Jeol JSM 6060 LV Scanning Electron Microscope. The analysis was performed using 20 kV electron acceleration voltages. Prior to the analysis, the samples were fixed on a copper specimen holder with carbon tape and covered with gold thin film in order to make them conductive before testing.

RESULTS AND DISCUSSION

Thermal degradation analysis

Figure 3(A) shows a characteristic TGA analysis of bone powder without removing water, fat, and protein as a function of the temperature as well as its first derivative, and Figure 3(B) shows the TGA analysis of the calcined sample HAp-FAir obtained by three-step process. TGA gives information about the parentage of components, its interaction, and also its structural transformation, which takes place as a result of the heating process commonly referred to as degradation of different phases (organic and inorganic).

According to Lozano et al.,[8] the changes in loss of mass located between 220 and 570°C correspond with the degradation and combustion processes of collagen. In the case of defat samples, these processes occurred between 180 and 624°C. One peak located between 270 and 338°C is related to the collagen degradation and protein denaturalization or

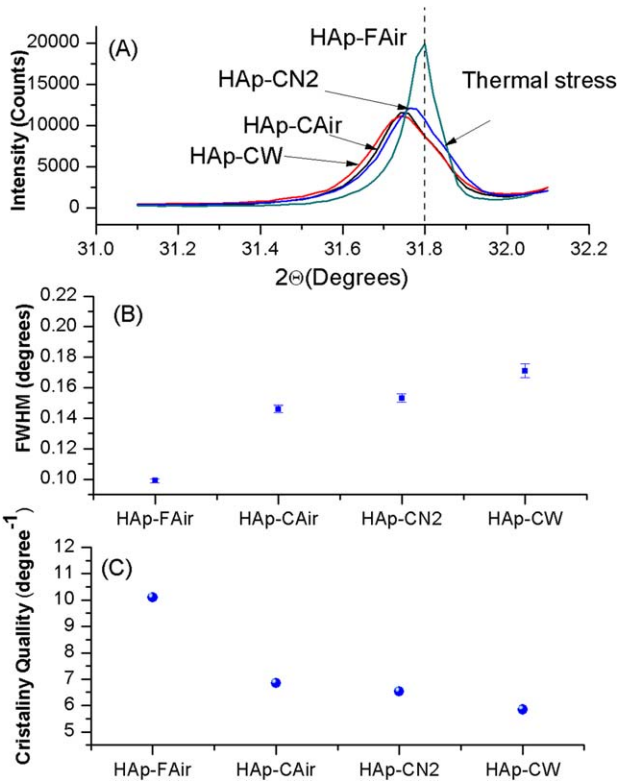

FIGURE 6. A: Shows the (211) peak for all studied samples, the vertical dash line represents the position of the (211) peak for pure HAp, (B) shows the FWHM values for this peak, the bars indicate the standard deviation and (C) shows the inverse of FWHM (crystallinity quality).

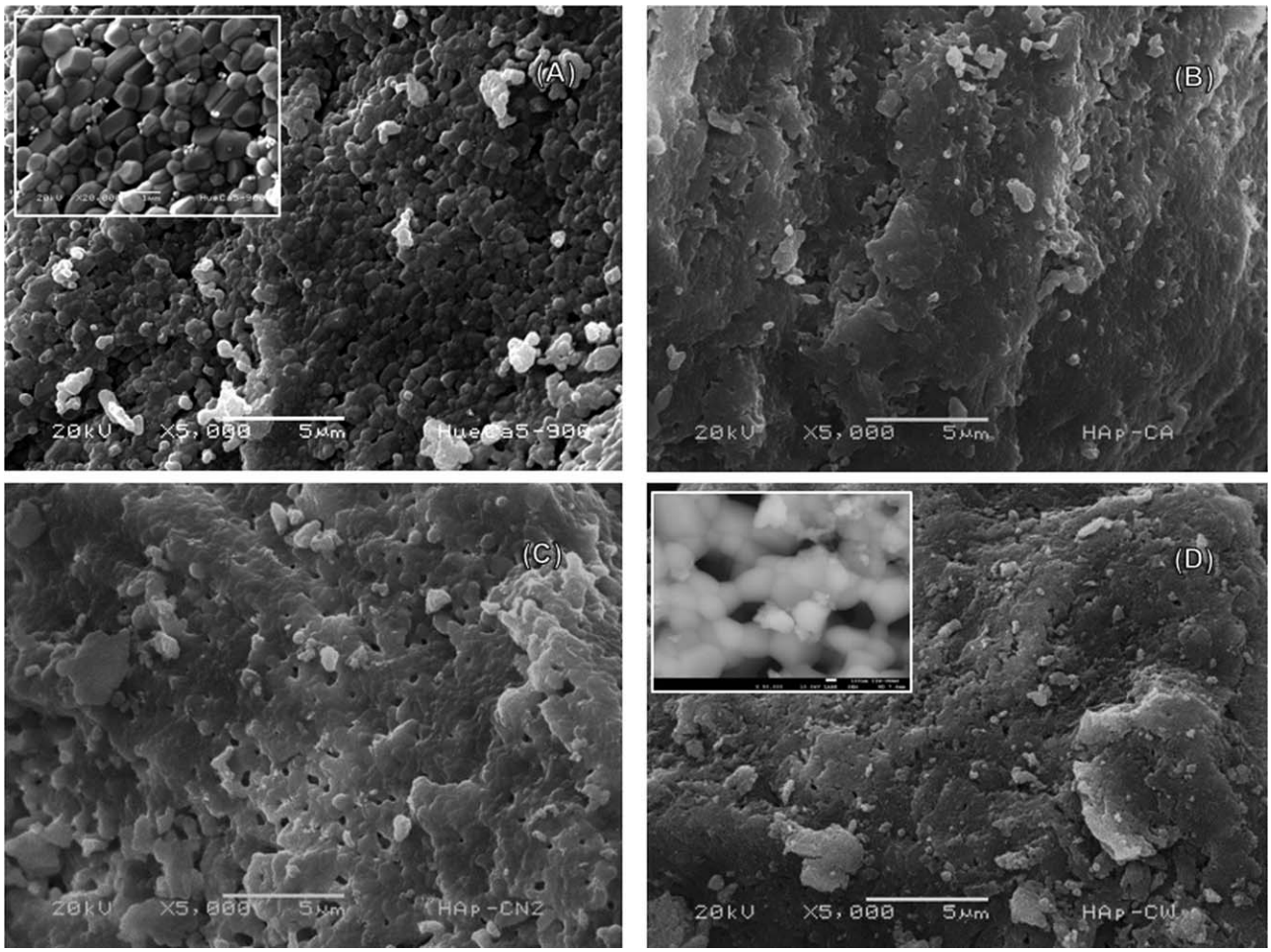

FIGURE 7. A: SEM images of HAp-FAir taken at 5000×, (the insert was taken at 20,000×), (B) HAP-CAir, (C) HAp-CN2, and (D) HAp-CW, (the insert was taken at 50,000×).

de-branched. After 900°C no drastic changes in the TGA curve are present and this fact was used to select 940°C as the calcination temperature.

On calcinating HAp-FAir sample, no thermal changes are present indicating that no organic materials are present and no thermo-structural changes take place in this range of temperature. According to this figure, the high to the low cooling rate was found as follows: Cooled in water, liquid nitrogen, and air; and the lower value correspond to the furnace cooled sample. The cooling rate depends on the thermal properties of the fluid which are also a function of the temperature. For samples cooled in water and air, the fluid was at room temperature, while for liquid nitrogen it was −77°C. For samples cooled inside the furnace, the temperature of the air chamber changed in the same way the cooling profile changed.

Structural characterization

X-ray is an excellent tool to study the structural changes that take place in a sample as result of a process. This technique has been used to study structural changes in bovine HAp as a function of the sintering time and temperature.[9] In order to study the structural transformation in the samples as a result of the defatted process, Figure 4(A) shows the X-ray diffraction pattern of bone powder, Figure 4(B) the X-ray diffraction pattern of defatted samples using a solvent process, and Figure 4(C) shows the pattern for defatted samples using the hydrothermal process. As can be seen, after the use of a solvent, a better definition of some HAp peaks is revealed; but by using a hydrothermal process, the intensity of these peaks increases indicating an improvement in the crystalline percentage due to the removal of fat and some protein. The following crystalline directions detected: (002), (211), (130), (222), (213), (004), and (323) characteristics of pure HAp according to JCPD file No. 09-0432. This result is important because by using a hydrothermal process at high temperature and pressure, it is possible to avoid the use of solvent and improve the crystalline percentage. The crystalline percentage was: 50.25 ± 2.32, 55.25 ± 2.54, and $68.25 \pm 3.01\%$, for bone powder without treatment, defatted samples using solvent, and defatted samples using the hydrothermal process, respectively.

Figure 5 shows the X-ray diffraction patterns of samples obtained from (A) HAp-CAir, (B) HAp-CN2, (C) HAp-CW, and (D) HAp-FAir as a function of the 2θ scale. All peaks corresponding to the JCPD file No. 09–0432 for HAp were found.

A very important aspect related to these patterns is that the intensity of the HAp-FAir exhibits the most intense peaks; by direct inspection of Figure 2, it is clear that this sample was obtained using the lowest cooling rate. The high intensity of the peaks for this sample could indicate the existence of a crystalline phase for this cooling condition, but this has to be confirmed with the study of FWHM and scanning electron microscopy images.

The crystalline quality of the sample can be determined by using FWHM value of the main XRD peak which, in the case of HAp, is located at 31.773° and corresponds to the (211) direction. This parameter is inversely proportional to the average crystalline size according to the Scherrer equation[10]; indicating that for FWHM small values, the crystallinity quality[11] increases or maybe that the polycrystalline samples undergo a re-crystallization process. In order to have more detailed information about changes in the structural properties of these samples as a function of the cooling rate, Figure 6(A) shows the (211) peak for all studied samples, and the vertical dash line represents the position of the (211) peak according to the JCPD file No. 09-432 of pure HAp. In this figure, the presence of a shoulder for Hap-CAir, Hap-CW, and Hap-CN2 on the right side of the peak is indicative of the existence of a mechanical stress. It means that due to the faster cooling process, the lattice does not release stress and maybe some ions change their position from substitutional to interstitial positions, and these extrinsic and intrinsic behaviors affect the crystalline quality. Figure 6(B) shows the average value of FWHM values for the (211) peak. The sample cooled in furnace (low cooling rate) exhibits the lower value for this parameter, while samples cooled faster showed a high value for this parameter.

As it is well known, the inverse of the FWHM reflects the crystalline quality as can be seen in Figure 6(C). The decreases of the FWHM in this case can be related to a re-crystallization process or the transformation of poly-crystals to a single crystal; this fact can be studied by SEM images.

Morphological characterization

In order to determine the changes in the morphology or microstructure of the samples due to the different thermal cooling processes, SEM images were carried out. Figure 7(A) shows the SEM image taken at ×5000 for HAp-FAir, (B) HAp-CAir, (C) HAp-CN2, and (D) HAp-CW. For the HAp-FAir with the lowest cooling rate, the formation of micron and submicron HAp single crystals was found (see inset in Figure 7(A) taken at ×20,000); these crystal have a hexagonal shape and their length is around 1 μm. It is interesting to see that in Figure 5(D), the (002) direction exhibits the highest intensity value and, by direct comparison with Figure 6(B), this sample showed the lowest value for FWHM and a high crystalline value. In the case of HAp-CN2, HAp-CW, and HAp-FAir SEM, the images showed the formation of micro grains which, according to Figure 5(A–C), correspond to polycrystalline structures of HAp. A detailed analysis of these structures is shown in the insert of Figure 7(D) taken at ×50,000; where it is evident that the micro grains are formed by crystals of HAP and we can also see that the origin of thermal stress is in part due to inter crystal forces.

CONCLUSIONS

The three-step process proposed in this work, to obtain BIO-HAp with different structural and micro structural properties, indicated that process 1 eliminated the use of solvents and produced samples with better crystalline percentage, as was shown in Figure 4. The removing of fat and protein of bone powder is important because the extracted products do not interfere with HAp components, in order to create new crystalline or amorphous compounds during the calcination process. TGA experiments showed that the calcination temperature in which no organic compounds are present is 900°C, which was used as the final temperature for the heating ramp and no decomposition in calcined samples were found. Changes in the cooling rate (Process 3) produce changes in the structural and microstructural properties of BIO-HAp giving the possibility of obtaining different products for different application. Fast cooling rates produce thermal stress which, according to SEM images, is originated by forces between the HAP crystals. Low cooling rate (furnace air) produces crystalline BIO-HAp with better crystalline quality and percentage. Further studies in this direction are needed to elucidate this problem.

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