



Fabrication and Characterization of Nanocrystalline Hydroxyapatite Extracted from Bovine Bone at Different Calcination Temperatures

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Abstract: Hydroxyapatite (HAP) is a calcium phosphate based bioceramics and a basic mineral component of teeth and bones of vertebrates. Its chemical and structural features are analogous to the inorganic components of bone. The present work focuses on the fabrication and characterizations of nanocrystalline HAP extracted from bovine bone at different calcination temperatures (600, 800, and 1000°C) without the use of chemicals/solvents. Therefore, it is a green technology. The characterizations of the extracted n-HAP were carried out by X-ray diffraction (XRD), Fourier transforms infrared (FTIR) and Field emission scanning electron microscopy (FESEM). The FTIR results confirmed the presence of phosphate PO_4^{3-} , hydroxyl OH^{-1} and carbonate CO_3^{2-} groups in the n-HAP powder. FESEM observation confirmed the hexagonal rod like structure. XRD analysis revealed that extracted n-HAP has a hexagonal crystal structure and crystallite size was in the range of 10.48-84.08 nm. Crystallinity degree and crystallite size gradually increased with the intensification of calcination temperature from 600°C -1000°C.

Keywords: Bovine Bone, Hydroxyapatite, Calcination, Characterizations, XRD, FTIR, FESEM,

1. Introduction

Nanocrystalline hydroxyapatite (HAP) based bioceramics are the new range of biomaterials in biomedical research. In spite of their suitable biological characteristics (superior biocompatibility and bioactivity), the poor mechanical properties of HAP severely hinder its clinical applications [1]. Nowadays, the new frontiers of nanotechnology open new ways to the synthesis of nanostructured materials which have the potential to revolutionize the field of biomedical science from bone regeneration to drug delivery. Calcium phosphates (CaP) occur in nature and have been widely used as biomaterials, specifically in the restoration or regeneration of hard tissues. Among this group of materials, HAP is the most distinguished because it is chemically and crystallographically similar to the mineralized component of hard tissues [2]. Its chemical formula is $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$. Beside this, there are other minor components of the inorganic phase, such as magnesium, sodium and fluoride ions that provide stiffness and strength to the bone [3]. This bioactive

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ceramic is broadly applied as bone fillers, bone tissue scaffolds, bioactive coatings and composites owing to its excellent biocompatibility, cytocompatibility and osteoconductivity [4,5]. However, it is important to annotate that native bone HAP differs from stoichiometric HAP in a number of ways because natural HAP is non-stoichiometric and have nanosized crystal dimensions [6]. They are also promising candidates for the drug, protein, and gene delivery as well as fluorescence labeling, cell targeting, imaging materials [7]. Due to the attractive properties of HAP, numerous techniques have been and are being developed to produce HAP. It can be synthesized from the inorganic components such as chemical precipitation, sol-gel, microemulsion and microwave irradiation [8]. However, very complex, biological unsafe, toxic byproducts and high cost production are the main difficulties associated with HAP synthesized from inorganic chemical methods. HAP can be obtained from natural resources such as bovine, chicken and fish bones [9]. Usually, the synthesis of HAP by most conventional chemical methods produced HAP without a trace of valuable elements. While extraction of HAP from some biogenic sources is biologically safe and economic [10]. Recently, calcination has fascinated the attention of scientists to extract biologically worthy HAP from bio wastes, to avoid the complicated procedures involved in the synthesis of HAP. From the previous research, there are only a few investigations that have been done on this extraction methodology as well as crystallographic studies at different calcination temperatures. In this study, the bovine bone was used to extract HAP by hydrothermal and followed by calcination at different temperatures. The bovine bone has been selected for this work because of its quantity (>60wt%), quality (purity>95%) and cost effectiveness [11]. The aim of this research is the extraction of nanocrystalline HAP powders from bovine bone since it is readily available in Malaysia and has been certified to be a good source of quality HAP [10]. Through this extraction method, organic components in bones can be thermally decomposed. Furthermore, morphological properties, crystallite size and crystallinity degree of HAP samples were determined. The high efficiency of this process offers an advantageous approach to produce nanocrystalline HAP powders commercially. The effect of calcination process on phase transformations and structural features of HAP samples were also examined. The prepared HAP powders were characterized using XRD, FTIR and FESEM.

2. Material and Methods

The fresh cortical bone of adult cow (~2-3 years old) was used as a raw material. It was purchased from the local slaughter market of Malaysia (Parit Raja).

2.1 Bone Powder Preparations

Extraction of n-HAP was carried out according to the method of Bano *et al.*[10]. Bovine bones were cleaned with water. Then it was cut into small pieces using the cutter. The bones were boiled with water for 2 to 3 hours. Subsequently, the fluids in bone marrow and residual soft material were removed with a knife and washed with distilled water several times. The cleaned bone pieces were sterilized in an autoclave using distilled water for 60 minutes at a high pressure of 0.4mPA and at a temperature of 129°C. Finally, the bone pieces were washed and dried in an oven at 80°C for 72 h (3 days) to denaturalize the protein. After that, dry pieces of bovine bone were abridged to small particles by means of the crusher. The crushed bone was subjected to ball milling process for 24 hours at 250 rpm until the powder was fine enough. Bovine bone powder was obtained by sieving. The achieved particle size of bone powder used in this study was 25µm.

2.2 Calcination

Pure n-HAP (inorganic phase of the bone) free from fat and protein was achieved by calcination. The powder samples of HAP were calcined at 600°C, 800°C and 1000°C temperatures for 3 hours holding time at a heating and cooling rate of 5°C/minute in a furnace. The samples were named as n-HAP-600, n-HAP-800 and n-HAP-1000 respectively. n-HAP was produced by the decomposition of the organic phase.

3. Characterization Techniques

Characterizations of raw and calcined n-HAP powder were done by using XRD, FTIR and FESEM, FESEM was conducted to capture the surface morphology of the HAP samples. Functional groups present in all HAP samples were detected by FTIR (FT-IR Perkin Elmer) using KBr technique. The FTIR spectra of the samples were obtained in the transmission mode in the mid-infrared range with wave numbers from 4000 to 450 cm⁻¹ at 4 cm⁻¹ resolution, 2 cm⁻¹ intervals and averaging 100 scans were selected. The presence of HAP phases was analyzed by XRD (Shimadzu XRD 6000 diffractometer) under ambient conditions using Cu-Kα (λ= 0.154 nm) as a radiation source at a current of 40 mA with a voltage of 40 kV. The spectra of XRD are recorded at 2θ= 20° to 70° at a scanning speed of 1°/min and step size at 0.02°. The XRD peaks were compared with standard reference ICDD file no. 00-009-0432. The Scherrer equation (1) was used to calculate the crystallite size (d) [12].

$$d = \frac{K\lambda}{\beta \cos\theta} \quad (1)$$

where d is the crystallite diameter in Å, k is the shape constant (~ 0.9), λ is the wavelength in Å, θ is the Bragg angle in degrees and β (FWHM) is the observed peak width at half-maximum peak height in radian. The fraction of the crystalline phase (X_c) of the HAP powders extracted at different temperatures was evaluated by the following equation (2) [13].

$$X_c = 1 - (I_{112/300} / I_{300}) \quad (2)$$

Where I_{300} is the intensity of (300) diffraction peak and $I_{112/300}$ is the intensity of the hollow between (112) and (300), which totally disappears in non-crystalline samples.

4. Result and Discussion

4.1 X-ray diffraction (XRD)

XRD patterns of n-HAP powdered samples obtained from bovine bone before and after calcination at 600°C to 1000°C for 3 hours, acquired for 2θ range of 20°–70° are shown in Fig. 1. It is clearly seen that before calcination, the HAP sample presents a typical poorly crystallized XRD pattern in a hexagonal symmetry. Phase analysis was done using (ICCD standard HA) PDF card no. 00-009-0432 for HAP, which provides information in the 2θ range of 10.820–78.229°.

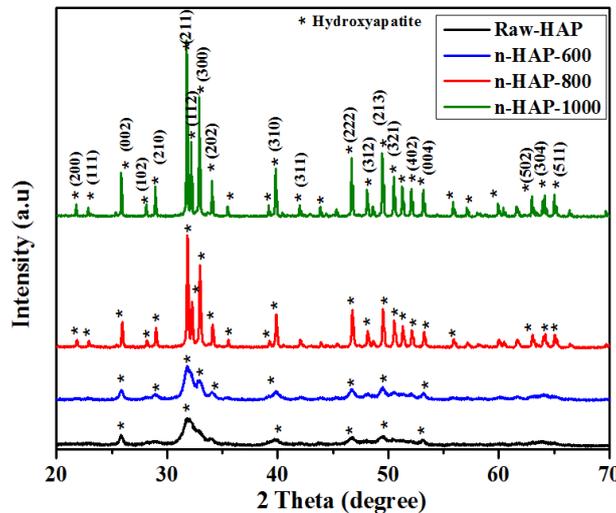


Fig.1. XRD patterns of n-HAP extracted from bovine bone before and after calcination temperatures at 600°C, 800°C and 1000°C

Phase analysis revealed that all major peaks of HAP were present in the samples extracted at different temperatures. These sharp peaks observed in Fig. 1 designate nanocrystalline nature of the extracted HAP samples. It has a hexagonal crystal structure and crystallite size is in the range of range of 10.48 nm to 84.08nm (Raw-HAP to n-HAP-1000). The prominent diffraction peaks at 2θ values of 25.8°, 28.9°, 31.8°, 32.8° and 34.0° corresponding to the (002), (210), (211), (300) and (202) Miller planes for HAP extracted at different temperatures exist in all samples. These sharp peaks deviated to some extent from the standard value because extracted n-HAP have some ions (Na^+ , K^+ , Zn^{2+} , Mg^{2+} , Sr^{2+}). It was found that the intensity of diffraction peaks increased and narrower which denoted to the increase in the crystallinity and crystal size with the increase in calcination temperature from 600°C and 1000°C as reported in the literature [14]. Obviously, calcination process enhanced the crystallinity of HAP phase with no secondary phase formation, development and readjustment of HAP crystals and comparatively due to the complete removal of collagen fibers and the liberation of lattice water and lattice carbonate groups [15]. Moreover, crystal growth has happened after the calcination process from 600 to 1000°C. It was revealed that the amorphous raw bovine bone was transformed into crystalline phase with more carbonates increasing, as the temperature increased from 600°C to 1000°C. Table 1 shows the crystallite size, degree of crystallinity % and degree of crystallinity for the peak (211) and (300).

Table 1. Crystallite size and Degree of Crystallinity

Samples	Crystal size (nm)		The degree of Crystallinity %	Degree of Crystallinity
	(211)	(300)		
Raw-HAP	10.48	13.9	57.45	low
n-HAP-600	9.615	17.62	69.87	low
n-HAP-800	49.41	64.81	99.19	high
n-HAP-1000	62.97	84.08	99.23	high

4.2 Fourier transforms infrared (FTIR)

FTIR spectra of raw and calcined n-HAP at different temperatures are presented in Fig. 2. In the spectra of n-HAP, many bands matched with the HAP reference band and are in agreement with the reported data on HAP [16].

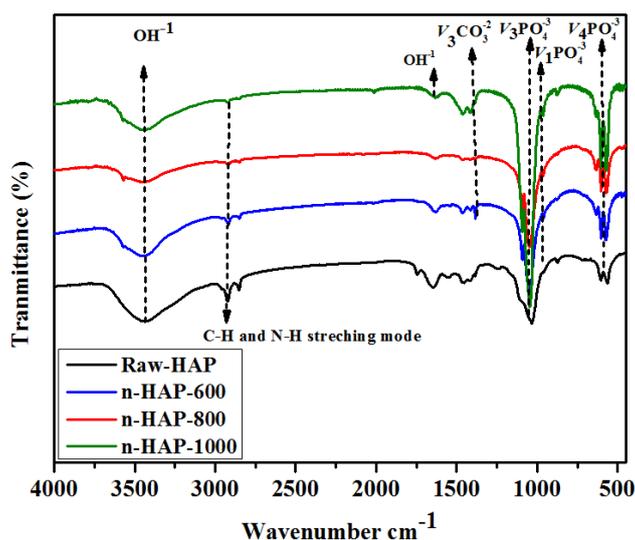


Fig.2. FTIR spectra of n-HAP extracted from bovine bone before and after calcination temperatures at 600°C, 800°C and 1000°C.

The FTIR spectra indicate the presence of phosphate (PO_4^{3-}), hydroxyl (OH^{-1}), and carbonate (CO_3^{2-}) groups. The bands of raw and calcined HAP are seemingly different due to the alterations in their chemical bonds during calcination at different temperature. These spectra are more clearly seen in calcined HAP samples because the calcination process has destroyed the cross linked structure in the raw HAP bovine bone powder. The color of raw HAP changes from yellowish white to white after calcination. This color change indicates the decomposition of organic substances which are collagen and proteins present in calcined HAP bone powder [11]. The FTIR band at 1637 cm^{-1} corresponds to Amide I of collagen [17] that exists in the raw HAP of bovine bone powder and is completely removed in calcined HAP samples. A strong and broad band due to symmetric stretching vibration that appears at 961.66 cm^{-1} , 962.99 cm^{-1} , and asymmetric stretching vibration bands at 1023 cm^{-1} and 1092 cm^{-1} in calcined HAP bone samples are associated with the phosphate group (PO_4^{3-}). The raw HAP bone powder exhibits a wide band near 3285.48 cm^{-1} which correspond to the absorbed water molecule. Low intensity peaks of carbonate groups are identified in the FTIR spectra at 1460 cm^{-1} and 1520 cm^{-1} due to asymmetric stretching ν_3 of (CO_3^{2-}) groups. The sharp narrow band at 630.92 cm^{-1} , 634.47 cm^{-1} and wide band at 3525.94 cm^{-1} are associated with the hydroxyl group that verifies the presence of HAP phase. In addition, after calcination, most of the bands have largely increased in intensity due to the phosphate vibrations of HAP as described by other authors [11].

4.3 Field Emission Scanning Electron Microscopy (FESEM)

The FESEM micrographs of the raw and calcined n-HAP samples are shown in Fig. 3 ((a)-(d)). It illustrates the morphology changes at the surface as calcination temperature increases from 600 to 1000°C. The morphology of HAP in the present study has been found to be rod-like as reported [18]. In spite of that extremely dense micro surface and particles agglomeration was seen in raw HAP particles.

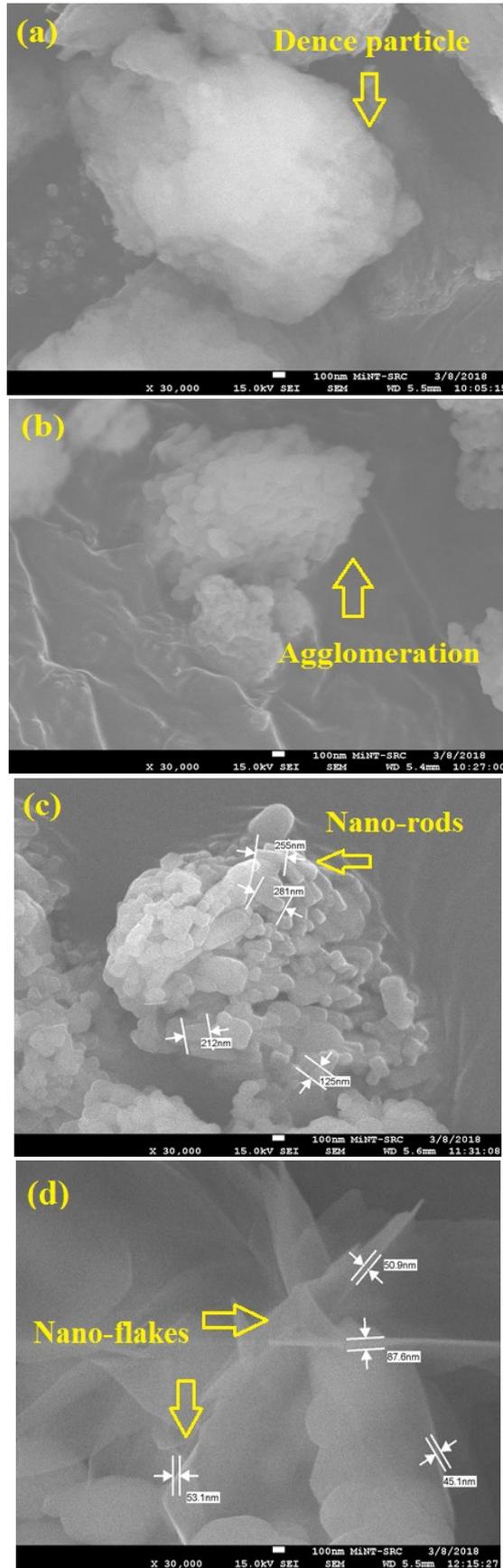


Fig.3. FESEM micrographs of HAP particles (a) raw-HAP and calcined n-HAP (b), at 600°C, (c) 800°C and (d) 1000°C of calcination temperature.

It is revealed that calcined n-HAP samples contain multiple pores produced by disintegration of organic constituents as reported in the literature [19]. The hexagonal HAP particles, including single crystals, with a wide range of dimensions having length and breadth of the HAP crystal were found to increase with the increase in the calcination temperature. The average size of nano-rod crystals was in the range of 125–281 nm. The average size of nano-flakes was in the range 45.1–87.6 nm. Therefore, this result confirms that the major phase transformation occurred in between 800 to 1000°C. This observation was owing to lattice diffusion and morphology conversion in the range of 800 to 1000°C. It is interesting to note that the calcined samples showed reasonable interconnectivity such as those present in natural bone HAP. Furthermore, calcination temperature brings direct impact to changes in crystal growth where higher calcination temperature yields an increase in crystallite size and change in morphology from nano-rods to nano-flakes. This crystal growth and morphological changes may associate with the absorption of heat energy by the particles. Moreover, the particles showed a high tendency to agglomerate as can be seen from the FESEM micrographs. The high surface energy stored in HAP nano rods induced the morphology change from the rod shape to flakes like and provides an increased driving force for densification during calcination [20].

5. Conclusion

The present research confirms the possibility of producing pure nanocrystalline HAP powder from bovine bone deprived of any impurities as shown by XRD and FTIR analysis. XRD data showed that the extracted n-HAP is pure, high crystalline and have a hexagonal crystal structure with crystallite size in the range of 10.48–84.08 nm. This process involved hydrothermal followed by heat treatment of bovine bone powder at different temperatures to eliminate its organic components. The FESEM micrographs showed the nano-rod like morphology that was changed to nano-flakes at higher calcination temperature. Hence this study paved the way to make value-added healthcare biomaterial like HAP from the bovine bone waste at economical limits. The HAP extraction methodology is simple, low cost, reduced pollution effect of the waste and subsequently conversion of the waste into an extremely valuable product. It is a green technology. Finally, the extraction processes have produced nanocrystalline HAP with considerably good yield and the average yields were found to be 65–70%. This outcome is obliging for future biomaterial design, preparation and applications.

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