Crystallographic and Morphological Studies of Nanocrystalline Hydroxyapatite Synthesized 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 crystallographic features are similar to the inorganic segment of bone. In this paper, the comparative crystallographic and morphological analyses of nanocrystalline HAP extracted from bovine bone by calcination treatment were reported. The characterizations of the extracted HAP were carried out by X-ray diffraction (XRD) and Field emission scanning electron microscopy (FESEM). XRD analysis revealed that extracted HAP has a hexagonal crystal structure and crystallite size was in the range of 7.2-73.1 nm. Crystallinity degree and crystallite size gradually increased with the intensification of calcination temperature from 700-1100 °C. The lattice parameters and unit cell volume of extracted HAP were calculated using the standard least-squares equation and were analogous to reference ICCD (The International Centre for Diffraction Data) data. FESEM observation confirmed the hexagonal rod like structure. However, crystallographic and morphological properties of HAP extracted at different calcination temperatures (700°C, 900°C and 1100°C) are slightly different due to the presence of the important biological ions that are essential for bone growth. It is also revealed that the process of calcination prompts a change of the lattice parameter, resulting in lattice readjustment after the discharge of lattice carbonate and lattice water that cause an increase in crystallinity and crystal size.

Keywords: Bovine Bone, Hydroxyapatite, Nanocrystalline, Calcination, Crystallographic properties

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I. 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 (Fahami, Ebrahimi-Kahrizsangi, & Nasiri-Tabrizi, 2011). 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 (Cox, Jamshidi, Grover, & Mallick, 2014). Among this group of materials, HAP is the most distinguished because it is chemically and crystallographically similar to the mineralized component of hard tissues (Rogina, Ivankovic, & Ivankovic, 2013). 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 (Barakat, Khil, Omran, Sheikh, & Kim, 2009). This bioactive ceramic is broadly applied as bone fillers, bone tissue scaffolds, bioactive coatings and composites owing to its excellent biocompatibility, cytocompatibility and osteoconductivity. 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 (Miyaji, Kono, & Suyama, 2005). They are also promising candidates for the drug, protein, and gene delivery as well as fluorescence labeling, cell targeting, imaging materials (Lin, Wu, & Chang, 2014).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 (Champion, 2013). 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 (Akram, Ahmed, Shakir, Ibrahim, & Hussain, 2014), 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 (Bano, Jikan, Basri, Bakar, & Nuhu, 2017). 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(Ooi, Hamdi, & Ramesh, 2007). The aim of this research is the extraction of nanocrystalline HAP powders from bovine bonesince it is readily available in Malaysia and has been certified to be a good source of quality HAP.
II. Materials

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

Bone preparations

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 0.4 mPA 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.

Calcination

Pure HAP (inorganic phase of the bone) free from fat and protein was achieved by calcination. The powder samples of HAP were calcined at 700°C, 900°C and 1100°C temperatures for 3 hours holding time at a heating and cooling rate of 5 °C/minute in a furnace which were named as HAP-700, HAP-900 and HAP-1100 respectively. HAP was produced by the decomposition of the organic phase. These temperatures were designated after a Thermo gravimetric analysis (TGA).

Characterization techniques

The characterizations of HAP before and after calcination were performed using FESEM and XRD. FESEM was conducted to capture the surface morphology of the HAP samples. The presence of HAP phase, the crystallite quality, crystallinity percentage and crystallite size in powders samples were analyzed by using the XRD (Shimadzu XRD 6000 diffractometer) under ambient conditions using Cu-Ka (λ= 0.154 nm) as radiation source at current 40 mA with voltage 40 kV. The spectra of XRD are recorded at 2Ѳ= 20° to 60° 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 structural features of HAP powdered samples were repeated for one group; (002), (211) and (300) Miller's planes. The average of these measurements was chosen as a mean fraction of crystallite size and a crystalline phase. The Scherrer equation (1) is used to calculate the crystallite size (d)(Cengiz, Gokce, Yildiz, Aktas, & Calimli, 2008).

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Hatijah Binti Basri et al.
where $d$ is the crystallite diameter in Å, $k$ is the shape constant ($\sim 0.9$), $\lambda$ is the wavelength in Å, $\theta$ is the Bragg angle in degrees and $\beta$(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 Eq. (2)(Landi, Landi, Tampieri, Celotti, & Sprio, 2000).

$$x_c=1-\left(\frac{I_{112}}{I_{300}}\right)$$

Where $I_{300}$ is the intensity of (300) diffraction peak and $v_{112/300}$ is the intensity of the hollow between (112) and (300), which totally disappears in non-crystalline samples. The lattice parameters $a$ and $c$ for the hexagonal HAP structure was calculated from peaks (211) and (002) respectively, Miller's planes using the method of least squares Eq. (3):

$$\frac{1}{a^2} = \frac{4}{3} \frac{h^2 + hk + k^2}{a^2} + \frac{l^2}{c^2}$$

Where $h$, $k$, and $l$ are the Miller indices, $d$ is the distance (nm) between adjacent planes in the set of Miller indices. The unit cell volume (Eq. (4)) of HAP samples was determined using the following equation. The volume $V$ of the hexagonal unit cell was determined for each HAP samples from the following equation (4) (Fahami & Nasiri-Tabrizi, 2014).

$$V = a^2 \cdot c \sin 60^0$$

Where $a$ and $c$ is the lattice parameters

### III. Results and Discussion

**Morphology**

The FESEM micrographs of the uncalcined and calcined HAP samples are shown in Fig. 1(A) to (D). It illustrates the morphology changes at the surface as calcination temperature increases from 700 to 1100 °C. The morphology of HAP in the present study has been found to be rode-like as reported by (J. Liu, Li, Wang, Zhu, & Yan, 2004). In spite of that extremely dense micro surface and particles agglomeration was seen in raw HAP particles. It is revealed that calcined HAP samples contain multiple pores produced by disintegration of organic constituents as reported in the literature (Murugan, Rao, & Sampath Kumar, 2003).
Fig. 1. FESEM micrographs of HAP particles (A) uncalcined and calcined HAP (B), at 700°C, (C) 900°C and (D) 1100 °C of calcination temperature.

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 length of the crystals was in the range of 101–470 nm and the average breadth was in the range 50.1–250 nm. Therefore, this result confirms that the major phase transformation occurred in between 900 to 1100 °C. This observation was owing to lattice diffusion and morphology conversion in the range of 900 to 1100 °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 (Pramanik, et al., 2012).

XRD Analysis

The XRD technique is employed to evaluate the phase purity and crystallographic changes of the HAP bioceramics extracted from various sources. Fig. 2 shows the XRD patterns of HAP powdered samples obtained from bovine bone before and after calcination at 700 to 1100 °C for 3 hours, acquired for 2θ range of 20°–60°. 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°. Phase analysis revealed that all major peaks of HAP were present in the samples extracted at different temperatures. These sharp peaks observed in Fig. 2 designate nanocrystalline nature of the extracted HAP samples.
The prominent diffraction peaks at $2\theta$ 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 shape peaks deviated to some extent from the standard value because extracted HAP have some ions ($\text{Na}^+$, $\text{K}^+$, $\text{Zn}^{2+}$, $\text{Mg}^{2+}$, $\text{Sr}^{2+}$). Existence of these sharp peaks suggests that nanocrystalline HAP were formed. 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 700 °C and 1100 °C as reported in the literature (Khoo, et al., 2015; Londono-Restrepo, et al., 2016). 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 (Wang, et al., 2010). Moreover, crystal growth has happened after the calcination process from 700 to 1100 °C. It was revealed that the amorphous raw bovine bone was transformed into crystalline phase with more carbonates increasing, as the temperature increased from 700 °C to 1100 °C.

Crystalllographic features

Fig. 3 shows the lattice parameter (a- and c-axis), unit cell volume (Å³), average crystallite size ($D$), and crystallinity degree (%) of HAP powdered samples before and after calcination. According to Fig. 3, after calcination, the breadth of the fundamental diffraction peaks decreases as compared to uncalcined sample (Raw-HAP) which can be accredited to an increase in crystallinity and crystallite size. Fig. 3(a) presents the lattice parameter calculated from XRD data by least squares fit method for uncalcined and calcined HAP samples.

According to the crystalllographic evidence, the a and c-axis lattice parameters of the standard HAP are 9.418 Å and 6.884 Å, respectively. The lattice parameters along a-axis measured for the samples before and after calcination at 700°C, 900°C and 1100 °C were 9.41 Å, 9.421 Å, 9.424 Å and 9.417 Å, respectively. Furthermore, the lattice parameters along c-axis measured for the samples before and
after calcination at 700°C, 900°C and 1100 °C were 6.891 Å, 6.886 Å, 6.884 Å and 6.9 Å, respectively. These values fall between those of standard HAP, suggesting that extracted HAP is phase pure. The HAP lattice parameters $a$ in all samples are slightly larger than that of standard HAP. Among them, the change in lattice parameters is one of the most conspicuous characteristics of the apatite structure. For example, the carbonate (CO$_3^{2-}$) can substitute either for the hydroxyl (OH$^{-1}$) or the phosphate (PO$_4^{3-}$) groups. The substitution of CO$_3^{2-}$ for OH$^{-1}$ causes a contraction in the $a$-axis without changing the c-axis and an increase in the crystallinity reflects in the increasing of crystallite size. These changes in crystal lattice parameters often induce changes in crystallinity, thermal stability, morphology, solubility, and other physicochemical and biological properties of the material (Shi, 2006).

In this study, the process of calcination causes an extension in the $a$-lattice parameter from 9.41 to 9.417 Å after that contraction took place whereas the $c$-axis value first decreased then increased. The slight alterations in the $a$- and $c$-axis of HAP can be attributed due to the substitution of carbonate at trace level at both positions OH$^{-1}$ and PO$_4^{2-}$. It was usually associated with an increase in the crystallinity, reflecting lattice rearrangement after the release of lattice water, lattice carbonate and an increase of crystal size that lead to greater stability to the structure. The removal of lattice water or carbonate could definitely stimulate the lattice readjustment and change the size of the HAP as reported by Hiller et al. (2003). The unit cell volumes of before and after calcination are presented in Fig.3 (b). The values of the volume of unit cell were 528.29, 529.41, 529.47 and 529.95 Å$^3$ before and after calcination at 700°C, 900°C and 1100 °C respectively. These values were comparable with the standard HAP that is 528.80 Å$^3$. This observation is in good agreement with the XRD patterns, which showed that the peaks shift to higher 2θ angles with an increase in calcination temperature as reported by other researchers (Fahami, Beall, & Betancourt, 2016).

![Fig. 3. XRD results of (A) lattice parameter ($a$- and c-axis), (B) unit cell volume (Å$^3$), (C) average crystallite size (D) and crystallinity degree (%) of HAP powdered samples before and after calcination](attachment:fig3.png)
Conferring to Fig. 3(c), the crystallite size of HAP samples were obtained in the range of 7.27 nm to 73.1 nm (Raw-HAP-HAP-1100). The consequences revealed that the calcination treatment of HAP powder samples caused the growth of crystallite size that resulted in rise of crystallite size and increases in lattice parameters. The volume of the hexagon also increases as compared with raw-HAP as stated in the literature (Murugan & Ramakrishna, 2005). Moreover, under the effect of calcination temperature, the nanocrystals tend to agglomerate to form larger crystals. According to the XRD patterns, the uncalcined HAP sample showed broad peaks which indicate the small crystal size of amorphous calcium phosphate, similar to the results reported in the literature (Niakan, et al., 2014). The crystallinity degree (Xc) after calcination of HAP powder samples was presented in Fig. 3(d). The results showed that the crystallinity degree of HAP powder samples increased from 57 to 99.29 (Raw-HAP-HAP-1100).

IV. Conclusion

The current research confirms the possibility of generating pure nanocrystalline HAP powder from bovine bone deprived of any impurities as shown by XRD analysis and their phase purity and crystallography changes observed were compared with respect to JCPDS standard. XRD data showed that the extracted HAP is pure, high crystalline and have a hexagonal crystal structure. Extracted HAP is A&B type carbonated as confirmed by XRD quantitative analysis. The FESEM micrographs showed the nano-rode like morphology that was changed to nano-flakes at higher calcination temperature. Hence this study paved the way to make value-added healthcare material 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, it is noteworthy mentioning that, the extraction processes have produced nanocrystalline HAP with considerably good yield and the average yields were found to be 66-68%. This outcome is obliging for future biomaterial design, preparation and application.

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Hatijah Binti Basri et al.
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