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Synthesis of hydroxyapatite from cuttlebone under various pH conditions: An approach for medical materials

Sineenart KANGKAN[1], Premjit ARPORNMAEKLONG[2], Sarute UMMARTYOTIN[1,*]

[1] *Department of Materials and Textile Technology, Faculty of Science and Technology, Thammasat University, Patumtani, Thailand*
[2] *Oral Surgery Division, Faculty of Dentistry, Thammasat University, Patumtani, Thailand*

[*]**Corresponding author e-mail:** sarute@tu.ac.th

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Abstract

To support the bio-based economy, development on hydroxyapatite from cuttlebone was investigated. Cuttlebone is naturally occurring source of calcium. We calcined it at 700°C for 4 h to form a calcium oxide powder. Hydroxyapatite was coprecipitated with calcium oxide powder and phosphoric acid. The effect of the pH ranging from 6–10 was controlled by adding ammonium hydroxide. Fourier transform infrared and X-ray diffraction were employed to characterize the structural properties of the hydroxyapatite. Changes in pH did not affect the structural properties of the hydroxyapatite. Thermogravimetric analysis revealed that the hydroxyapatite was thermally stable up to 700°C. At a neutral pH, scanning electron microscopy revealed the presence of rod-like structures. However, at a basic pH, it had a spherical shape due to the high mobility of Ca^{2+} and PO_4^{3-}. The specific surface area, pore volume and pore size were estimated to be 5-15 $m^2 \cdot g^{-1}$, 0.05-0.1 cc·g and 15-25°A, respectively. It was tested for cytotoxicity, and cells were able to grow under all conditions used to produce the hydroxyapatite. Hydroxyapatite prepared from cuttlebone can be used as a medical material. The objective of this research work is to encourage the use of bio-based material in medical application.

1. Introduction

In recent years, with the exponential growth of the worldwide population, the development of materials science for medical technology has advanced both for naturally occurring materials and synthetic ones. Although the utilization of synthetic materials has various advantages such as purity and uniformity of the product, its high cost is an issue. From the viewpoint of medical research, the utilization of naturally occurring materials for bone and tissue engineering is preferred due to their nontoxicity and safety. In addition, the utilization of naturally occurring materials has been strongly encouraged by the Thai government. The BCG (Bio-Circular-Green) model [1] of the economy was announced in order to promote the utilization of biobased materials in a circular economy using green processes. This approach can achieve sustainability.

Up to the present time, to respond to the BCG economic policy, numerous types of biobased materials have been heavily utilized, such as cellulose and its derivatives, chitin-chitosan, polylactic acid, polycaprolactone, and hydroxyapatite. They were successfully prepared from naturally occurring resources and waste products. These materials have been employed in various medical applications, such as artificial bone replacement, regenerative medicine, drug delivery systems, and cosmeceutical research.

Here, one of the most interesting materials was focused on: hydroxyapatite [2,3]. It is a naturally occurring mineral form of calcium apatite with the formula $Ca_5(PO_4)_3(OH)$. It exhibits excellent properties, such as high dimensional stability, chemical resistance and high stiffness. Therefore, it is commonly employed as a bone replacement, a filler material for bone defects, artificial bone grafting, and as a scaffold material in prosthesis surgery.

Hydroxyapatite has been prepared by many research groups. In 2008, Javidi *et al.* [4] studied the electrophoretic deposition of hydroxyapatite on medical grade 316L stainless steel. In 2009, Descamps *et al.* [5] developed hydroxyapatite beads for medical applications. After that, in 2011, Yanovska *et al.* [6] studied a coating of hydroxyapatite on titanium-based alloy substrates. Recently, In 2016, Laranjeira *et al.* [7] studied the magnetic properties of Gd and Fe doped hydroxyapatite for medical imaging. Although hydroxyapatite has received much attention in research, the starting material for the synthetic process has previously always invol. ved chemical reagents in a laboratory.

Applying the concept of sustainability, hydroxyapatite needs to be obtained from municipal waste, agriculture residues, or natural resources. In 2015, Ummartyotin *et al.* [8] developed hydroxyapatite from eggshell waste. Ramesh *et al.* [9] developed hydroxyapatite from animal bone. The as-synthesized product could

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be prepared with high purity and homogeneity. Furthermore, hydroxyapatite prepared from eggshell waste was filled into a chitosan and polycaprolactone composite as reported by Trakoolwannachai *et al.* [10,11]. The design of a hydroxyapatite-based composite offered the ability of hydroxyapatite to be used in medical research with higher efficiency.

To encourage the green concept policy, one of the most efficient sources of calcium is cuttlebone [12]. It is a hard, brittle internal structure found in all members of the family Sepiidae. It is well-known as a chambered and gas-filled shell used for buoyancy by the cephalopods. Cuttlebone is composed of calcium carbonate, which converts to calcium oxide after pyrolysis. Recently, it was studied as an organic matrix for catalytic applications and antimicrobial properties by Jia *et al.* [13] and Ramasamy *et al.* [14], respectively. In 2008, Poompradub *et al.* [15] developed a product made from cuttlebone that could be used as a reinforcing filler for natural rubber. Therefore, developing hydroxyapatite from cuttlebone seemed feasible.

To support the green concept policy, we wished to develop hydroxyapatite from cuttlebone by a precipitation technique. The effect of pH on the process was investigated. Its structural, thermal, and morphological properties were investigated by Fourier transform infrared, X-ray diffraction, thermogravimetric analysis, and scanning electron microscopy. Its specific surface area, pore size and pore vol. ume were evaluated by the BET technique. A preliminary investigation of its in vitro effects in a human osteoblast cell cytotoxic test was also performed.

2. Experimental

2.1 Materials

Cuttlebone was collected from a local beach in Pattani, located in the southern part of Thailand. It was employed as a starting material for hydroxyapatite synthesis. Analytical grade phosphoric acid was purchased from Qchemical Co., Ltd. Ammonium hydroxide was purchased from Macron™ Chemicals Co., Ltd. All chemical reagents were used as received without further purification.

2.2 Methods

2.2.1 Preparation of hydroxyapatite from cuttlebone by a coprecipitation technique

The cuttlebone was washed several times with DI water and ethanol in order to remove any odor and impurities. It was calcined at 700°C for 4 h. The powder was sieved in order to ensure uniformity of size. After that, DI water was poured into the calcined powder. The phosphoric acid was dripped into the mixture. To adjust the pH of the solution, ammonium hydroxide was added. The pH was adjusted to be 6, 7, 8, 9, or 10. The coprecipitant was found at the bottom of the beaker. It was adjusted to neutral pH by using DI water as a solvent exchange. The powders were stored in a desiccator in order to prevent moisture adsorption.

2.2.2 In vitro behavior of hydroxyapatite prepared from cuttlebone

In vitro cytotoxicity of the hydroxyapatite was tested with a human osteoblast cell line (hFOB1.19, ATCC, USA). The hydroxyapatite was sterilized by autoclaving. The cells were seeded in 96-well plates, 5×10^3 cells/well, and cultured overnight in the growth medium. After that, the culture medium was replaced with Group A: Control and B: Sample culture media.

Compositions of the culture media were as follows. For Group A: Control, the growth medium was DMEM-F12, 10% FBS, 1% penicillin-streptomycin and 0.1% fungizone (all from Gibco, USA), and for Group B (B1-B3), the same growth medium was supplemented with 2, 4 or 8 $mg\cdot ml^{-1}$ HA. Then, a cell viability assay (MTT) was performed on days 1 and 3 after incubation in the designated culture media (Groups A and B). In brief, the culture media was replaced with 20% MTT (CellTiter 96® AQueous One Solution Cell Proliferation Assay) in growth medium for 2 h. Subsequently, the optical density (OD) of the culture medium was measured using a microplate reader (TECAN, Männedorf, Zürich, Switzerland) at 490 nm and corrective optical density at 680 nm. Percent cell viability was calculated from the percentages of [(OD of sample/OD of control)*100] (n=5, mean ±SD).

2.3 Instruments

2.3.1 X-ray diffraction

The crystal structure was investigated by X-ray diffraction (XRD, Phillips P.W. 1830 diffractometer). It employed nickel-filtered CuKa radiation. Diffraction patterns were recorded over a range of 20-60°. Prior to the investigation, the sample was stored in a desiccator to prevent moisture absorption.

2.3.2 Scanning electron microscopy

The sample was investigated using SEM (SEM, Quanta 250 microscope, Japan). The specimens were gold-coated using a sputtering device (Jeol, JFC-1200) prior to the SEM observation. A magnification of 20 kX was used.

2.3.3 Fourier transform infrared (FTIR)

The sample was investigated using FTIR. The spectra were recorded using a Nicolet Impact 410 FTIR, and a diamond crystal was used as the reflection element. The spectra were measured at room

temperature in the spectral range of 4000 to 650 cm^{-1} with a resolution of ±4 cm^{-1} and a scan frequency of 32 times.

2.3.4 Thermogravimetric analysis

The thermal degradation behavior of the powder was characterized using TGA (TGA Q500, TA Instruments). A 20 mg sample was heated from room temperature to 700°C in N_2 at a heating rate and flow rate of 5°C·min^{-1} and 70 ml·min^{-1}, respectively.

2.3.5 BET analysis

The BET surface area, pore size and pore vol. ume of the powder were analyzed by using a N_2 adsorption-desorption analyzer (Quantrachome, Autosorb IQC). A 10 mg sample was inserted into the system. The samples were pretreated to remove moisture and then allowed to adsorb N_2 at -196°C. Five measurements were tested. The data are reported as the statistical average.

3. Results and discussion

Hydroxyapatite was successfully synthesized from cuttlebone. The product was found to be a fine white powder. It should be stored in desiccators in order to prevent moisture adsorption. In the reaction, hydroxyapatite was typically prepared by a coprecipitated technique using calcium oxide and phosphoric acid. The formation of hydroxyapatite was due to the immediate growth of the particle around the nucleate after mixing the salt solution, as suggested by Parakhonskiy *et al.* [16]. The hydroxyapatite was formed by an ion-exchange reaction between the calcium and phosphate ions.

Figure 1 presents the FTIR spectra of the hydroxyapatite prepared from cuttlebone. The effects of a pH ranging from 6-10 during the coprecipitation was investigated. All of the spectra presented similar features. No significant change was observed with variation of the pH. The wavelength of 3500 cm^{-1} was assigned to the OH stretching mode of the hydrogen bond. The characteristic peaks at 560 cm^{-1} and 1030 cm^{-1} were related to the fundamental vibration mode of the asymmetric O-P-O bending mode and asymmetric stretching vibration of the P-O bond. They are referred to as the vibration modes of PO_4^{3-} of hydroxyapatite. They may involve a ν_{3a} triply degenerate asymmetric stretching mode, a ν_{3c} triply degenerate asymmetric stretching mode and a ν_1 nondegenerate symmetric stretching mode, as suggested by Tautkus *et al.* [17]. The characteristic peaks at 1150 cm^{-1} and 1500 cm^{-1} were attributed to Ca-O stretching and C-O stretching, respectively.

Figure 2 exhibits the XRD pattern of the hydroxyapatite prepared from cuttlebone. All of the pH variations produced a pure and homogeneous product. No impurity can be observed within the scanned range. The 2θ of 22°, 22.5°, 27.5°, 31°, 31.5°, 32.5°, 34.5°, 40°, 41°, 45°, 47°, 48°, and 53° correspond to (200), (002), (210), (211), (112), (300), (202), (301), (310), (311), (113), (222), (312), and (004), respectively. The single phase of hydroxyapatite was successfully synthesized similar to a previous work [18]. This is consistent with JCPDS no. 00-064-0738. The trend of crystallinity was increased at the higher pH values. This increase probably occurred because in a basic solution, a coprecipitant was obtained. At a basic pH, the Ca^{2+} and PO_4^{3-} ions mobility rate is high, so they are attracted to each other rapidly, which leads to the formation of hydroxyapatite crystals.

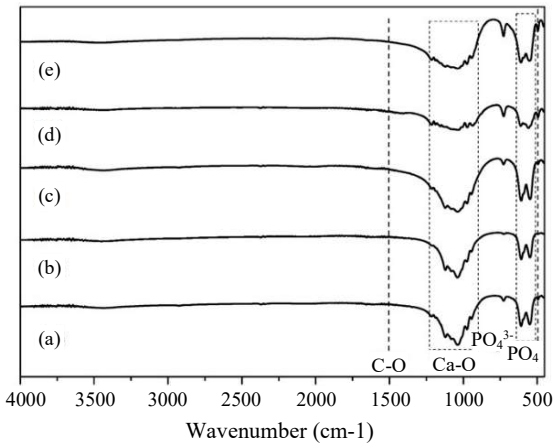

Figure 1. FTIR spectra of hydroxyapatite prepared from cuttlebone: effect of pH of precursor solution (a) pH = 6, (b) pH =7, (c) pH =8, (d) pH = 9, and (e) pH = 10.

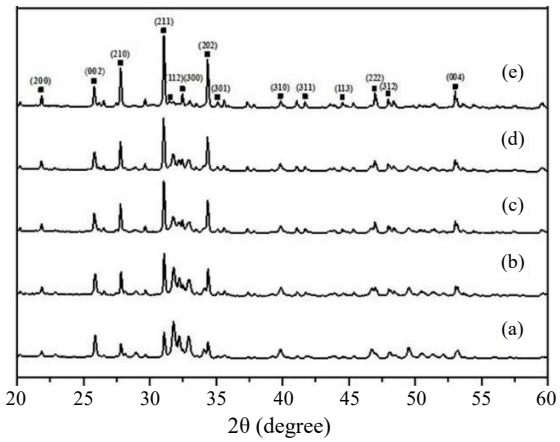

Figure 2. XRD pattern of hydroxyapatite prepared from cuttlebone (a) pH = 6, (b) pH =7, (c) pH =8, (d) pH = 9, and (e) pH = 10.

Figure 3 presents the thermal degradation behavior of the hydroxyapatite prepared from cuttlebone. No significant weight loss can be observed with the variation of pH. Only 2 wt% of weight loss was

observed due to the existence of humidity. This was almost certainly due to water adsorption as suggested by Ibrahim *et al.* [19]. The hydroxyapatite should be stored in a desiccator prior to any usage. Moreover, it was notable that the thermal stability of hydroxyapatite from cuttlebone is very high. Only one region of temperature degradation can be observed. The process of utilization of hydroxyapatite in medical research, which involves sterilization under thermal and UV irradiation, can, therefore, be performed to ensure it is free of pathogenic agents and bacteria [20].

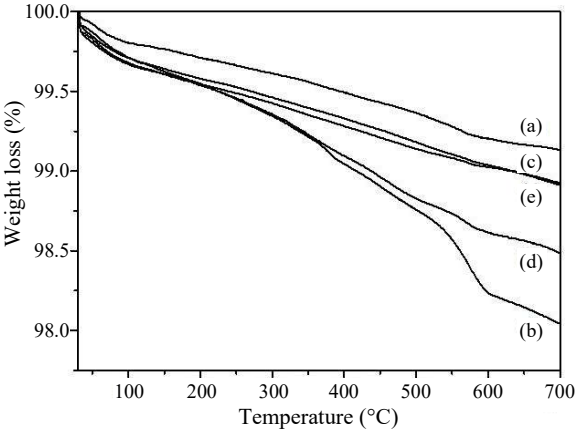

Figure 3. Thermal decomposition behavior of hydroxyapatite prepared from cuttlebone: effect of the pH of the precursor solution (a) pH = 6, (b) pH =7, (c) pH =8, (d) pH = 9, and (e) pH = 10.

The SEM micrographs exhibit an important influence of the pH of the solution during the precipitation reaction. Figure 4 presents the morphological properties of the hydroxyapatite from cuttlebone. At pH = 6 and 7, the micrograph of the sample reveals the presence of rod-like structures. Whereas, at pH = 8, 9 and 10, it exhibits the presence of spherical shapes in the nano range. It was remarkable to note that at neutral pH, the reaction medium was inactive, probably because the Ca^{2+} and PO_4^{3-} ions releasing rate was too slow to form hydroxyapatite. There was consequently less agglomeration of particles. However, at a high pH, the Ca^{2+} and PO_4^{3-} ions mobility rate was high. This can lead to a higher agglomeration rate. These findings are consistent with a previous article by Palanivelu *et al.* [21]. A porous structure was observed between the particles. Agglomeration was observed.

Table 1 illustrates the BET analysis of the hydroxyapatite prepared from cuttlebone. It was remarkable to note that the surface properties of the hydroxyapatite may affect the ability of cell adhesion [2]. The specific surface area, pore vol. ume and pore size were estimated to be 5-15 $m^2 \cdot g^{-1}$, 0.05-0.1 $cc \cdot g^{-1}$ and 15-25°A, respectively. The pH ranging from 6-10 did not significantly alter the surface properties. All of the conditions exhibited a microporous region. This is optimal for its use in medical research.

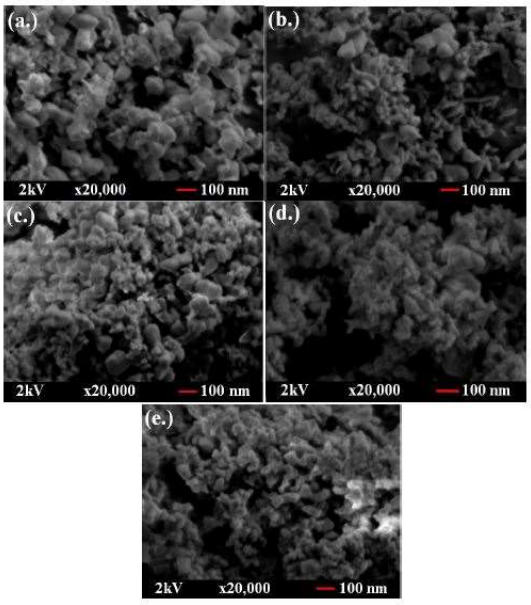

Figure 4. Morphological properties of hydroxyapatite prepared from cuttlebone: effect of the pH of the precursor solution (a) pH = 6, (b) pH =7, (c) pH =8, (d) pH = 9, and (e) pH = 10.

Table 1. BET analysis of hydroxyapatite prepared from cuttlebone.

pH	Surface area ($m^2 \cdot g^{-1}$)	Pore volume ($cc \cdot g^{-1}$)	Pore radius (Å)
6	6.71	0.08	17.06
7	5.71	0.08	15.27
8	10.69	0.09	24.46
9	9.26	0.07	17.04
10	10.32	0.09	17.06

To investigate the feasibility of using hydroxyapatite synthesized from cuttlebone as a medical material, an in vitro cell cytotoxic test was performed. Figure 5 presents the cell cytotoxic test of hydroxyapatite prepared at different pH values. The cells were able to grow for all conditions of hydroxyapatite synthesis. It did not affect cell growth between 36 h and 5 days. Application of 2%w/v of hydroxyapatite showed better cell growth compared to 4 and 8%w/v, suggesting that a lower concentration of the particles did not inhibit cell growth, but cell viability was affected by a high concentration or density of particles. The effect of the particles on osteogenic differentiation should be further investigated as suggested by Sun *et al.* [22]. It should be noticed that at a high pH, the hydroxyapatite particles were quite large, but at lower pH values, it only formed small particles that became a slurry in the culture medium. For the pH ranging from 7-10, the growth behavior showed a similar trend. However, at pH = 6, there was optimal cell growth and the least

adverse effects from the particle density, particle size and its agglomeration. Thus, pH =6 was considered to have the most favorable effect on cell growth, and it actually promoted cell growth. The condition of pH = 6 should be further investigated if hydroxyapatite prepared from cuttlebone will be employed as a medical material.

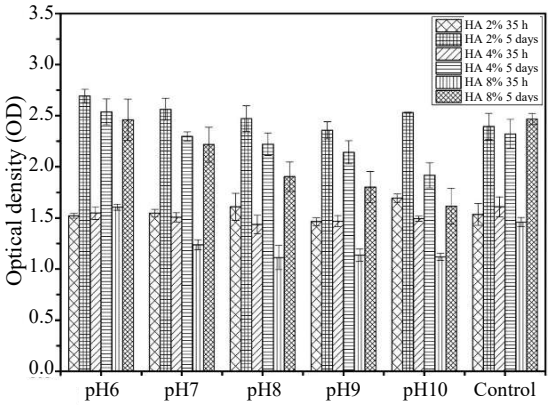

Figure 5. Cell cytotoxicity of hydroxyapatite prepared from cuttlebone: effect of the pH of the precursor solution.

4. Conclusion

Hydroxyapatite was successfully synthesized from cuttlebone, a well-known calcium source. A coprecipitation technique was employed using calcium derived from cuttlebone and phosphoric acid. The pH ranging from 6-10 was adjusted by adding an ammonium hydroxide solution. High purity and homogeneity of the powder were found for all pH values. At neutral pH, scanning electron microscopy revealed the presence of rod-like structures. However, at basic pH values, it had a spherical shape due to the high mobility of Ca^{2+} and PO_4^{3-}. The specific surface area, pore vol. ume and pore size were estimated to be 5-15 $m^2 \cdot g^{-1}$, 0.05-0.1 $cc \cdot g^{-1}$ and 15-25°A, respectively. A preliminary investigation of the hydroxyapatite in a cell cytotoxicity test was conducted. The hydroxyapatite was compatible with cell growth. This project has successfully supported the design of a biobased economy.

5. Acknowledgments

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