SYNTHESIS OF HYDROXYAPATITE MATERIAL FROM BLACK CARP BONE VIA THERMAL DECOMPOSITION

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TÓM TẮT

CHẾ TẠO VẬT LIỆU HYDROXYAPATITE TỪ XƯƠNG CÁ TRẮM ĐEN BẰNG PHƯƠNG PHÁP NHIỆT PHÂN

Vật liệu hydroxyapatite (HA) được chế tạo từ xương cá trắm đen bằng quy trình nhiệt phân một giai đoạn đơn giản. Các mẫu xương cá khô được nung ở các nhiệt độ khác nhau từ 500 °C đến 1200 °C trong 3 giờ để thu được vật liệu HA. Các phương pháp lý hóa học như XRD, FTIR, SEM và EDX được sử dụng để đánh giá các mẫu vật liệu HA được phân lập. Thực nghiệm ''in vitro'' trong dung dịch giả dịch thể người SBF và trong môi trường nuôi cấy tế bào nguyên bào sợi L929 đã được tiến hành để đánh giá hoạt tính sinh học và khả năng tương thích sinh học của vật liệu HA tổng hợp. Kết quả phân tích cho thấy mẫu xương cá trắm đen được nung ở 800 °C thể hiện vật liệu HA tinh khiết với tất cả các đỉnh đặc trưng của pha khoáng HA khi so sánh với mẫu chuẩn. Hoạt tính sinh học của mẫu HA tổng hợp đã được xác nhận qua sự hình thành một lớp khoáng apatite mới trên bề mặt vật liệu sau khi ngâm HA tổng hợp trong dung dịch SBF. Khả năng tương thích sinh học của vật liệu cũng được xác nhận trong môi trường nuôi cấy tế bào. Có thể thấy rằng vật liệu HA tổng hợp từ xương cá trắm đen trong nghiên cứu này có tiềm năng ứng dụng trong lĩnh vực y sinh học.

Từ khóa: *Xương cá trắm đen, hydroxyapatite, nhiệt phân, thực nghiệm ''in vitro'', hoạt tính sinh học, tính tương thích sinh học.*

1. INTRODUCTION

Hydroxyapatite (HA) is the main component of bones and teeth of humans and animals; specifically, it accounts for 65 - 70% of bone mass and 70 - 80% of teeth. Hydroxyapatite with the molecular formula was $Ca_{10}(PO_4)_6(OH)_2$, this material had applications in bone grafting and tooth filling surgery [1]. Studies showed that HA had bioactivity and biocompatibility. The bioactivity of HA was demonstrated when implanted into the human body, the material would gradually dissolve due to interaction with the environment, and then Ca^{2+}, PO_4^{3-} and OH^- ions would precipitate on the surface of the material to form a new HA mineral layer to bond, which used HA material and the natural bone. The biocompatibility of HA was the ability not to be rejected when HA material was implanted into the human body [1]. The literature review showed that the HA material could be synthesized by chemical routes, such as sol-gel, precipitation, hydrothermal, and solid-phase reaction methods [2]. Chemical methods could synthesize the HA material with high purity, and the structural morphology of the synthesized materials could be adjusted. However, chemical methods often required complex and quite expensive synthesis processes. The chemical methods were also

considered unsafe because chemical residues might remain in the resulting HA product. Therefore, by-products in human daily life or the food processing industry were studied and used to manufacture HA material. The utilization of by-products brought environmental benefits as an approach to bio-waste management, especially those from the agricultural sector, such as cow bones [3], eggshells [4], snail shells [5], fish bones [6], and mussel shells [7]. On fish by-products that could be used for HA synthesis, some typical studies were published in recent years. A simple pyrolysis method successfully synthesized natural HA from tilapia bone [8]. Analysis showed that the best natural HA was obtained by heating fish bone at 900 ^{o}C, the synthetic sample had a Ca/P ratio of 1.699, closed to the ratio in the HA chemical formula [8]. Seabass bone samples were calcined at temperatures ranging from 200 to 1200 ^{o}C to synthesize natural HA. X-ray diffraction results showed that the main phase HA existed in all powder samples, and only a small amount of tricalcium phosphate was observed in the powder sample calcined above 800 ^{o}C. Notably, the crystallinity and crystal size of HA increased with increasing calcination temperature. Cell culture results showed that the synthesized HA samples were not toxic to cells [9]. Natural HA material was successfully synthesized by heating salmon bone at 850 ^{o}C. XRD results of the material samples showed that 100% of the phase composition was HA. In particular, the obtained HA sample had a nanostructure [10]. Carp bones were thermally heated to produce HA materials. The calcination process was carried out at different temperatures of 900 ^{o}C, 950 ^{o}C, 1000 ^{o}C, 1050 ^{o}C, and 1100 ^{o}C with a heating rate of 10 ^{o}C/min. The FTIR, XRD, and Raman analyses showed that organic components and proteins were completely removed when calcined at temperatures of 900 ^{o}C or higher. At 950 ^{o}C, the best natural HA was obtained; the Ca/P ratio reached a value of 1.6589, similar to the ratio in the HA formula of 1.67 [11]. Notably, the AFM observation showed that as the temperature increased, the HA crystallinity increased. The review of the literature found that there were few studies on the production of HA materials from black carp bones. Meanwhile, black carp was a very popular fish consumed in large quantities yearly in our area. HA material extracted from black carp was proven to be biocompatible due to its non-cytotoxicity [8, 11]. Additionally, studies on HA separation from black carp bones in Vietnam have not been reported, while studies worldwide often treated bone samples with chemicals [7-11]. Therefore, this study used the simple thermal decomposition to produce HA material from black carp bone. Carp bone samples were calcined at different temperatures, and their physicochemical properties were characterized by analytical methods such as XRD, FTIR, SEM, and EDX. The bioactivity and biocompatibility of HA materials were also evaluated.

2. EXPERIMENT

2.1. Synthesis process of HA material from black carp bone

The process of making HA material from black carp bone was carried out in the following steps. First, the black carp bones were boiled thoroughly to remove the remaining meat. Next, the black carp bones were dried at 100 ^{o}C for 6 hours.

Finally, the dried black carp bones were calcined at temperatures ranging from 500 ^{o}C to 1200 ^{o}C for 3 hours. After calcination, the black carp bone samples were crushed and sieved to obtain fine powders with sizes smaller than 40 µm.

2.2. In vitro experiment

To evaluate the bioactivity of HA synthesized from black carp bone, an in vitro experiment was conducted by soaking HA powder in a Simulated Body Fluid (SBF) solution [12]. The ratio of HA powder to SBF solution was set at 1:2 (mg/mL), with soaking durations of one and two weeks. After the soaking periods, the powder was separated and dried for the assessment of its physicochemical properties. The biocompatibility of the synthesized HA material was evaluated using fibroblast cells (L-929) under ISO 10993-5 standard [13]. The percentage of viable cells was determined using an MTT assay. According to the regulations, the material was considered non-toxic if the percentage of viable cells was greater than 70% compared to the control sample (cell sample not in contact with the material).

2.3. Evaluation methods

Various analytical methods were employed to evaluate the physical and chemical properties of the HA material synthesized from black carp bones. X-ray Diffraction (XRD) was utilized to analyze the phase composition of the material. Fourier Transform Infrared Spectroscopy (FTIR) was conducted to examine the structural groups. Scouldning Electron Microscopy (SEM) was used to observe the structural morphology of the synthesized HA material. Additionally, Energy Dispersive X-ray Spectroscopy (EDX) was employed to analyze the elemental composition of the synthesized HA.

3. RESULTS AND DISCUSSION

3.1. Physical-chemical characterization of the synthesized HA material

Fig. 1 showed the XRD diagrams of black carp bone samples calcined at temperatures ranging from 500 °C to 1200 °C. The bone sample calcined at 500 °C showed the formation of some HA broad peaks, characterizing the poor crystallinity of the synthesized HA material. When the calcination temperature increased to 600 °C and 700 °C, the number of peaks characteristic of the HA phase increased; however, the observed peaks were still quite low and broad, indicating that the HA crystalline phase was not completely formed. When the calcination temperature was from 800 °C to 1200 °C, the XRD diagrams of the calcined bone samples showed stable and almost identical shapes. Observation showed that all the peaks characteristic of the HA phase were present [JCPDS 90432]. The peaks are sharp and pointed, confirming the crystalline nature of the achieved HA phase. Additionally, the XRD pattern of calcined fish bone did not show any phases other than HA. This result was significantly different from previous studies, where the XRD diagrams of the fish-bone calcined at high temperatures often showed small peaks characteristic of the $Ca_3(PO_4)_2$ phase [14-15]. The HA synthesized from black carp bone showed to be a material with high crystallinity and purity when the black carp bone samples were calcined from 800 °C to 1200 °C. Thus, pure HA material with all formed characteristic peaks was formed at a fairly high temperature range. According to previous references, the increase in temperature not only decomposed the organic matter in the bone but also sintered the inorganic mineral part into a

crystalline phase [10-13]. To save energy, 800 °C was considered a suitable temperature for preparing HA material from black carp bone, and the HA sample synthesized at this temperature was selected for further evaluation.

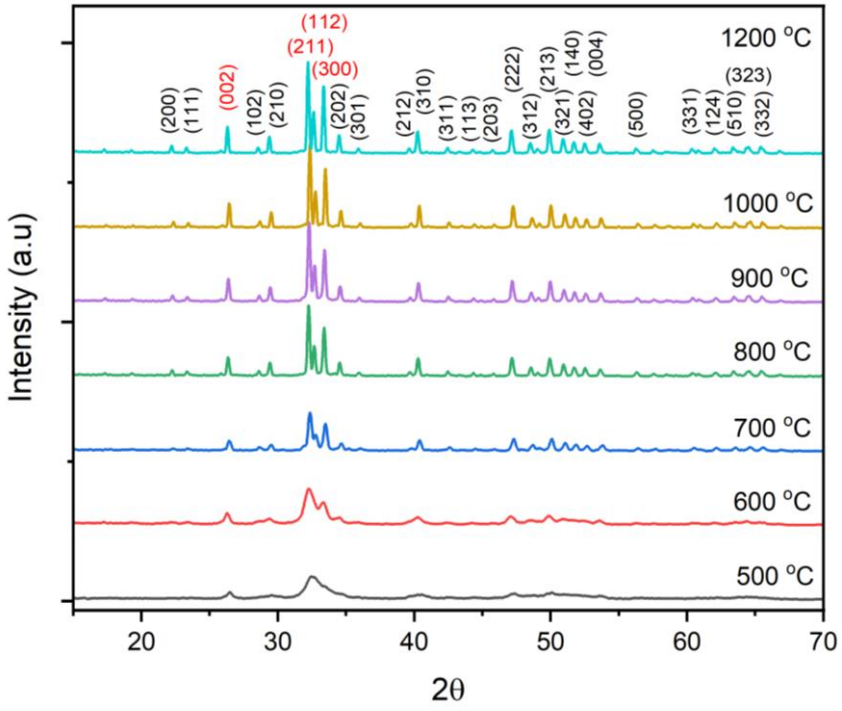

Fig. 1. XRD diagrams of black carp bone samples annealed at different temperatures

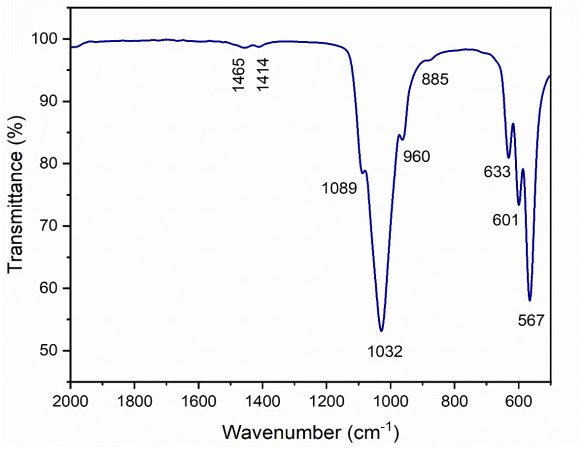

Fig. 2. FTIR spectrum of HA material synthesized from black carp bone

The functional groups of the selected HA material were determined by FTIR spectroscopy as shown in Fig. 2. It was observed that the characteristic spectral bands of hydroxyapatite material such as the bands located at 567, 601, 960, 1032, and 1089 cm^{-1}, were characteristic of P-O vibrations from PO_4^{3-} groups [16]. The spectral band at 633 cm^{-1} corresponds to O-H bonds in the structure of the hydroxyapatite phase [17]. The presence of the CO_3^{2-} group was mentioned by the weak intensity characteristic bands at 885, 1414, and 1465 cm^{-1} [16-17]. Carbonate ions were common impurities in the FTIR measurements. Thus, the analytical results obtained from FTIR spectroscopy confirmed the functional groups of HA material extracted from black carp bones. Fig. 3a presented an SEM micrograph of the HA sample synthesized from black carp bone. The HA particles were quite uniform in spherical shape, with the particle size ranging from 40 to 150 nm, and tended to adhere together into large aggregates. The observed aggregates were interwoven, creating porosity for the synthesized HA material. EDX analysis showed that the synthesized HA included

the main elements Ca, P, and O (Fig. 3b). The calculated value of Ca/P ratio is 1.662, which is close to 1.667 in the HA mineral formula [18].

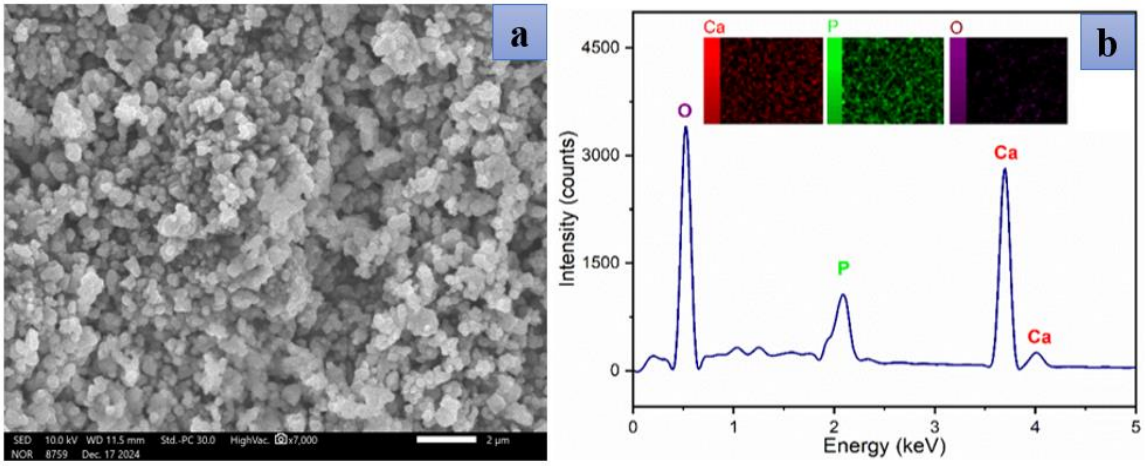

Fig. 3. SEM image (a) and EDX spectrum (b) of HA material synthesized from black carp bone

3.2. Bioactivity

Fig. 4a presented the XRD diagrams of HA material synthesized from black carp bone after the in vitro experiment in an SBF solution. The XRD diagram of the HA sample after 7 days of soaking showed a decrease in intensity and a broadening of characteristic peaks, indicating the reduction of the crystalline degree of the initial HA material. This phenomenon was characteristic of the dissolution of the HA sample in the SBF solution. However, when the soaking time increased to 14 days, the XRD diagram showed sharper peaks and higher intensity, indicating an increase in crystallinity of the material. This observation suggested the gradual crystallization of ions in the solution over time to form a new HA crystal layer. After in vitro experiments in SBF solution, the formation of a new HA layer on the initial HA material confirmed the bioactivity of the HA sample synthesized from black carp bone. In addition, after being immersed in the SBF solution, the XRD spectrum of the HA sample still retained the number and position of the characteristic peaks, and there was no appearance of any strange peaks. If the graft material transforms into another material that was not similar to the natural bone structure, it would lead to rejection from the body, meaning that the material was not biocompatible [19]. SEM image of the HA sample synthesized from black carp bone after 7 days of in vitro testing is shown in Fig. 4b. The HA surface had obvious changes compared to before immersion due to the formation of tiny and dense particles on the initial HA material. After 14 days of immersion, the particles gradually grew larger, covering and filling the voids, making the material having a dense surface. The above XRD analysis results confirmed the formation of a new HA layer on the surface of the old material and no other foreign phases were detected. Therefore, the newly formed crystalline layer observed was the HA layer due to the gradual precipitation of Ca^{2+}, $PO_4^{3-,}$ and OH^- ions from the SBF medium onto the material surface. Thanks to the formation of this new HA layer, the implant material was firmly attached to the natural bone, thereby repairing and filling the damaged bone [19-20]. As the immersion time increased to 14 days, the new HA layer grew and became denser, containing new fibrous crystals (Fig. 4c).

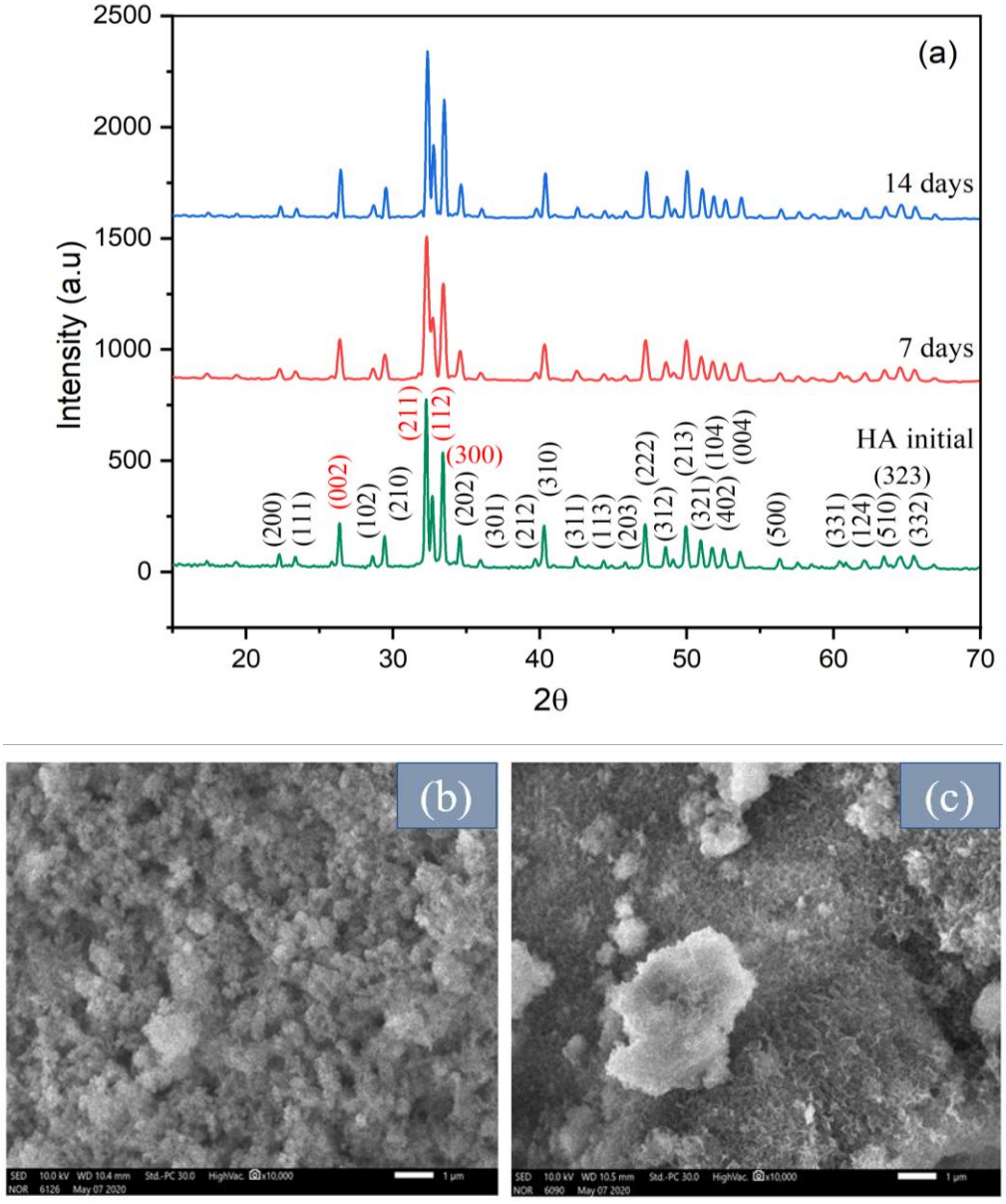

Fig. 4. XRD diagrams and SEM images of synthetic HA after in vitro experiment in SBF solution

3.2. Bioactivity

The MTT colorimetric assay method was used to quantify cell viability when exposed to the HA material synthesized from black carp bone for 24 hours. The cytotoxicity test on HA material was effectuated with different extracted concentrations, such as 0 (control sample without material extract), 12.5, 25, 50, and 100%, as shown in Fig. 5. The analysis data showed that the cell survival rate for all extracts was higher than that of the control sample even at high concentrations of the extract. The obtained result confirmed the biocompatibility of the synthesis HA material to L-929 fibroblast cells [13, 21].

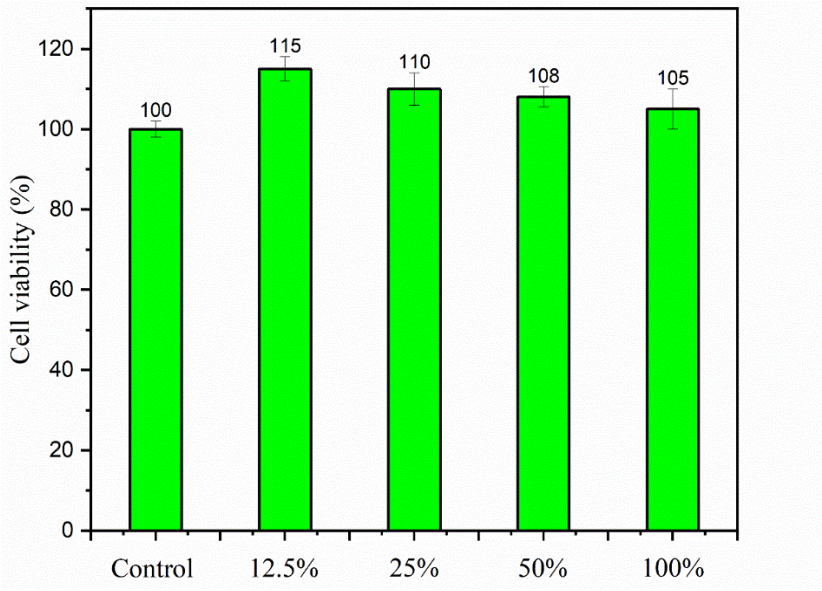

Fig. 5. Cytotoxicity of the synthesized HA on L-929 fibroblast cells at different extract concentrations

4. CONCLUSION

HA biomaterial was successfully synthesized from black carp bones by the thermal decomposition method. XRD and FTIR analyses showed that 800 °C was a suitable calcination temperature to obtain pure HA materials with high crystallinity. SEM image showed that the HA material consisted of nearly spherical particles, which tended to adhere to form larger particle clusters. EDX analysis showed that the Ca/P ratio was 1.662, close to 1.67 for standard HA. After soaking in SBF medium for 7 and 14 days at a ratio of 1/2 (mg/mL), XRD and SEM analyses confirmed the formation of a newly formed HA mineral layer on the initial HA material. The new HA particles were in the form of flake-like particles, evenly distributed on the material surface, and filled the voids to form a dense material surface. MTT cytotoxicity test with L929 fibroblast cells showed that the cell survival rate at all concentrations was higher than the control value and all values exceeded the limit of 70%. Thus, the HA material synthesized from black carp bone has good bioactivity and biocompatibility, suitable for application in the biomedical field as artificial bone material.

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