On the Crystallization of Hydroxyapatite under Hydrothermal Conditions: Role of Sebacic Acid as an Additive

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ABSTRACT: Hydroxyapatite (HAp) is a major inorganic component in bone minerals and is often used for bone tissue engineering. Herein, we synthesized HAp using sebacic acid as an additive at different pH values by a hydrothermal method. Sebacic acid, which has two carboxyl group ends of the carbonate chain, binds with Ca ions during the hydrothermal process to become a crystal nucleation site in (001) and at the same time could act as an inhibitor in a specific direction [i.e., (110)] for the HAp crystal growth. Sebacic acid and the hydroxyl ion (OH\(^{-}\)) are competitively attracted to the \(a(b)\)-plane of HAp. Depending on the pH condition, the crystal growth resulted in different morphologies depending on the ratio of sebacic acid and hydroxide ions. It was confirmed through Fourier-transform infrared spectroscopy and Raman spectroscopy that dicalcium phosphate anhydrous with HPO\(_4\) \(^{2-}\) was produced under acidic conditions and HAp was produced under neutral and basic conditions. The plate- and nanorod-HAp crystals’ preferential growth along the \(c\)-axis, which were obtained under neutral and basic conditions, was analyzed by transmission electron microscopy. Growth control in the \(c\)-axis direction of HAp is necessary for the understanding of crystallization of bone minerals because the mineral inside the collagen fibrils in bone tissue also shows a \(c\)-axis orientation.

INTRODUCTION

Hydroxyapatite \([\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]\), HAp) is a naturally occurring mineral. It is also one of the most investigated calcium phosphates (CaPs). Hydroxyapatite is a major inorganic component in hard tissue and can be found in about 60% of bones, 97% of tooth enamel, and 70% of tooth dentin. Bone tissues have a three-dimensional hierarchical structure, especially in the cortical bone section, with a lamella structure with a specific crystallographic orientation. The crystal growth direction of HAp is along with the collagen fibrils that serve as the substrate for bone tissue. The size of the HAp crystal was reported, with the range of 30–50 nm in length, 15–30 nm in width, and 2–10 nm in thickness. According to recent research results, the size of the HAp crystal is reported to be 21 ± 9 nm in length and 6 ± 1.5 nm in width. Moreover, the \(c\)-axis of HAp plates were often aligned parallel to the longitudinal direction of collagen fibrils. Many studies on bone tissue growth and regeneration are ongoing, and from a materials science point of view, the HAp crystal growth process occurs in the body in a specific morphology with micro/nanometer and atomic length scales. While many different CaPs are used as inorganic biomaterials, HAp is drawing attention for its excellent biocompatibility, bioactivity, and osteoconductivity. For this reason, the synthesized HAp is usually used as a material for bone tissue engineering. There are many different methods of HAp synthesis, for example, (1) dry methods including solid-state and mechanochemical methods and (2) wet methods including sol–gel, chemical precipitation, and hydrothermal methods. As various synthetic methods of HAp crystals have been developed, the morphology of crystals obtained according to the synthesis method can be adjusted in various ways. Many different synthesis methods result in various morphologies of HAp, for example, sphere, rod, and plate-like. Among them, the hydrothermal process provides the synthesis of specific shaped crystals such as plate and rod shapes at high temperatures and pressures in a controlled manner. In addition, the method enables the synthesis of HAp crystals with high crystallinity and controlled crystal shapes simply by adding additives. As additives, organic compounds are used, for example, ethylenediaminetetraacetic acid (C\(_{10}\)H\(_{16}\)N\(_2\)O\(_8\), EDTA) which forms chelates with calcium ions. In addition, among the fatty acids having a carboxylic acid group, naturally occurring fatty acids having a dicarboxylic acid group are used as additives. The dicarboxylic acid provides the nucleation site, and the hydrocarbon chain might induce oriented crystal growth of HAp during the hydrothermal process. In addition, organic acids with carboxyl groups are chemisorbed onto the surface of growing crystal grains under hydrothermal conditions.
conditions, which help the crystals grow in a specific orientation. These additives can also act as inhibitors of crystal growth for specific orientations in the synthesis of HAp, such as polyaspartic acid or fetuin, present in bone tissue.

It has also been reported to affect the final product by various phosphate ions generated depending on the pH of the solution used for HAp synthesis. The effect of pH on the crystalline formation of CaPs thus obtained is also related to the properties of the solution containing phosphate ions. Triprotic ions in aqueous H₃PO₄ are not easy to characterize the concentration differences between H₂PO₄⁻, HPO₄²⁻, and PO₄³⁻. Many studies have been conducted to synthesize CaP crystals by adjusting pH of the solution. These complexes in equilibrium make their control and prediction difficult in the nucleation and crystal growth process of CaP crystals. Therefore, in the process of HAp-based bone tissue formation, it can be said that pH plays a very important role in determining the properties of CaPs.

Based on this, in this study, various forms of HAp were synthesized by adding sebacic acid (C₁₀H₁₈O₄) as an additive in dicarboxylic acid. Sebacic acid with calcium ions has been used as a precursor, and under hydrothermal conditions, they help to grow HAp crystals in the form of plates. In addition, the HAp synthesis was performed through a hydrothermal process of a solution containing phosphate and calcium ions with different pH values. The pH affects the chemical composition and crystal growth orientation of the final product. It is confirmed that how the sebacic acid influenced the crystal formation in the crystal growth process by hydrothermal synthesis. In addition, at each pH, by analyzing the results on crystallinity and the chemical composition of the final products, it is possible to see what variables affect the growth of HAp crystals.

### RESULTS AND DISCUSSION

Figure 1 shows scanning emission microscopy (SEM) images of dicalcium phosphate (DCPA, CaHPO₄) and HAp synthesized under different conditions of pH 4, 7, and 12 and with/without sebacic acid. It is noted at different magnifications according to pH of 4, 7, and 12 (see, different scales of 20, 2, and 0.2 μm, respectively).

Figure 2. XRD patterns of CaP crystals synthesized with the additive (sebacic acid) at different pH values. (a) DCPA synthesized at pH 4 used ICDD 70-0359 as a reference and (b) HAp synthesized at pH 7 and pH 12 using ICDD 09-0432 as a reference. The XRD result of HAp synthesized without the additive at pH 7 is different from the result of HAp synthesized with the additive (Figure S1).
synthesized at pH 12 show the smallest crystal size with a nanorod shape. Comparing the addition of sebacic acid, the size of CaP crystals synthesized without the addition of sebacic acid is smaller than that of the crystals synthesized with its addition. It can be inferred from the chemical formation of sebacic acid, which has a chemical structure of dicarboxylic acid (HO₂C−R−CO₂H) and negatively charged in solution (COO⁻), on a surface that is positively charged by Ca²⁺ ions in the CaP crystal, that it can be grown in a long axis.27 As the pH increased, that is, the high concentration of OH⁻ ions influenced the growth of primary unit particles, and under dicarboxylic acid-free conditions, this also seemed to block the chemical binding reactions between CaP crystal surfaces.34

XRD analysis of CaP crystals hydrothermally synthesized at different pH values is shown in Figure 2. XRD patterns of hydrothermally synthesized CaP crystals under pH 4 can be assigned to DCPA (CaHPO₄), which is in good agreement with ICDD 70-0359 (Figure 2a). The sharp and strong intensities of the XRD peaks indicated a highly crystallized CaP crystal, that it can be grown in a long axis. Comparing the addition of sebacic acid, the size of CaP crystals synthesized without the addition of sebacic acid is smaller than that of the crystals synthesized with its addition.35 As shown in the results of the XRD pattern of hydrothermally synthesized CaP crystals under pH 7 and pH 12, all diffraction peaks can be indexed and assigned to a pure HAp using ICDD 09-0432 (Figure 2b). No peaks for any other phases were detected for these samples. The main peaks of HAp can be identified at 2θ of 31.7°, 32.2°, and 32.9°. The XRD results at pH 7 and pH 12 also show the highest intensity peak of the (211) plane, which corresponds to the peak at 31.7°. The structure of the HAp crystals hydrothermally synthesized at pH 7 and 12 is a hexagonal crystal system with the P6₃/m space group, which is in good agreement with the reported ones.36

FTIR analysis confirmed the functional groups of CaP crystals hydrothermally synthesized at different pH values in Figure 3a. Unlike HAp, DCPA has hydrogen phosphate (HPO₄²⁻) functional groups, and the difference can be observed in FTIR results. First, as per the results of the hydrothermal synthesis of CaP crystals at pH 7 and 12, that is, the group corresponding to HAp, it was confirmed that carbonate ions (CO₃²⁻) were substituted. It can be inferred that during the synthesis process, carbonate ions are not present in the reactant, but carbonate-substituted HAp was produced by dissolved CO₂ in the ambient.37 The region of 1550–1280 cm⁻¹ in wavenumber is represented by the ν₃ vibrational mode of carbonate ions. The carbonate ions can be substituted with the sites of hydroxyl (OH⁻) and phosphate (PO₄³⁻) ions in HAp. In detail, it is known that when the carbonate ions are substituted at the hydroxyl ion position (A type), IR peaks appear at 1544 and 879 cm⁻¹, and IR peaks at 1455 and 1409 cm⁻¹ appear when substituted at the phosphate ion position (B type).38 Theoretically, IR spectroscopy identifies four vibration modes for phosphate ions, ν₁, ν₂, ν₃, and ν₄. As shown in Figure 3a, band peaks at 1091 and 1041 cm⁻¹ in the range of 1190–900 cm⁻¹ are associated with the triply degenerate asymmetric stretching mode (ν₁), and one at 961 cm⁻¹ was related to the symmetric stretching mode (ν₁) of P–O bonds. The peaks appearing at 601 and 571 cm⁻¹ in the lower range are assigned to the triply degenerate bending (ν₂) mode of O–P–O bonds. It was observed that the phosphate ν₂ band was assigned to a doubly degenerate bending mode of O–P–O bonds at 466 cm⁻¹. Furthermore, two unique peaks observed at 3570 and 632 cm⁻¹ were assigned to stretching (ν₃) and vibrational (ν₄) modes of the structural hydroxyl anion in HAp, respectively (Figure S3).39

As a result of Raman spectroscopy of DCPA crystals obtained by hydrothermal synthesis under pH 4, it was confirmed that there was a peak characteristic different from HAp results obtained by synthesis at pH 7 and pH 12. DCPA shows a peak at 891 cm⁻¹, which can be said to be related to the P–O(H) stretching mode.40 As a result of the Raman spectrum of HAp obtained at pH 7 and pH 12, the characteristic tetragonal PO₄³⁻ (ν₁) group peak is observed at 961 cm⁻¹, and the PO₄³⁻ (ν₂) vibrational mode at 430 cm⁻¹ indicates the O–P–O bending modes. Moreover, the band appearing at 1041 cm⁻¹ is because of the asymmetric stretching of P–O (ν₃), and the peak at 587 cm⁻¹ can be applied as PO₄³⁻ (ν₄). The Raman spectrum of DCPA showed slightly different results from HAp. We can see that the spectrum assigned to DCPA and HAp phases are visibly distinguishable. The spectrum collected for DCPA particles can be assigned to the DCPA phase according to the available literature data.41
From the DCPA spectrum, we observed the distinct shifts and splitting of the PO₄³⁻ vibrational frequencies. Raman spectrum results of HAp and DCPA are blue-shifted or split compared to the internal modes of the free PO₄³⁻ group. It is indicated that the strong crystal field in the structure leads to distortion of the spectral lines. When suggested through more complex DCPA spectrum results, there are additional effects on the vibrational characteristic of DCPA crystals. In addition, reinterpreting the Raman spectra results of HAp crystals synthesized at pH 7 and 12 were grown in the c-axis direction (b,c). (The d-spacing of the (001) plane of HAp is 0.34 nm.)

TEM images were observed by adjusting the beam spot size to reduce the damage caused by the electron beam of the HAp crystal. TEM images of the HAp crystals synthesized at pH 7 and pH 12 are shown in Figure 4. As shown in Figure 4a, the HAp crystals synthesized at pH 7 exhibited a plate-shaped morphology with a few μm in length, and the width is less than about 200 nm. The area of the high-resolution TEM (HRTEM) lattice image is the circle in Figure 4a. An interplanar distance of 0.34 nm corresponds to a lattice spacing of [002] plane in HAp perpendicular to the c-axis. The selected area electron diffraction (SAED) pattern of the crystal synthesized at pH 7 (Figure 4c) was also measured at the same area as a circle in Figure 4a. In the SAED pattern with the [110] zone axis, the growth direction was confirmed as the c-axis, which is [002] perpendicular to the (002) plane. The SAED pattern presents the only forbidden spots along the c-axis (00l spots with odd l) by the P6₃/m space group. As a result, it was confirmed that the synthesized HAp plates were grown along with the c-axis in the longitudinal direction as shown by the arrow in the HRTEM image. The TEM image of HAp nanocrystals synthesized at pH 12 (Figure 4d) shows that the size of the nanocrystals is about 50–200 nm in length, similar to the observation in SEM (Figure 1c). The HRTEM image (Figure 4e) showed an interplanar distance of 0.34 nm and can be inferred to grow along with the c-axis as the plates synthesized at pH 7. All reflections from the (210), (211), (112), (300), and (002) planes can be identified as crystalline HAp. According to the hexagonal HAp structure, three lattice planes of (300), (112), and (211) are quite close each other with d = 2.72, 2.77, and 2.81 Å, respectively; therefore, they can be observed as almost one ring in the SAED pattern (Figure 4f). The growth orientation of nanocrystals can be seen in the SAED patterns. Real bone minerals have also been reported to grow in the c-axis.

In the bone mineralization process, there are two types of mineralization: intra- and extrafibrillar. Intrafibrillar mineralization is the nucleation and growth of HAp between the collagen triple helix. As shown in Figure 5a, the bone tissue is composed of CaPs, organic molecules, mainly type I collagen, and mineralized collagen containing water. In addition to collagen, calcium-binding proteins such as osteocalcin are
known to mediate the nucleation and growth of platelet-shaped HAp crystals. The most suitable for monomer molecules bound to the HAp surface is the plane parallel to the c-axis. As discussed earlier, the results of TEM and XRD confirmed that the HAp crystal growth is along with the c-axis; it can be parallel to the collagen fibrils in real bone tissue, as shown in Figure 5b,c.45,46

The schematic diagrams of the growth mechanism of a HAp crystal with sebacic acid are described in Figure 6. According to the crystal structure of HAp, which is hexagonal, the hydroxide ions are located in the channel parallel to the c-axis. The corresponding c- and a(b)-planes differ in composition and atomic arrangement. For example, the a(b)-planes are positively charged because it is rich in calcium ions, whereas the c-planes are rich in hydroxide ions and are mostly negatively charged. Therefore, the chemical composition and ions of the solution used in the hydrothermal process shown in this study can be arranged under the influence of the electric charge of each side of the nucleus initially generated. As shown in Figures 1b,e and 5b, the effect of sebacic acid used as an additive had a great influence on the growth of the hydrothermally synthesized HAp crystals in the plate form under pH 7. The presence of dicarboxylic acids in solution during the crystal growth of HAp affects the morphology of the resulting crystals (a(b)-planes) as well as their growth direction (c-axis). The dicarboxylic acid group in sebacic acid was able to bind to the a(b)-planes between crystals, which can be interpreted as inducing crystal growth in a platelet shape. However, as a result of the hydrothermal synthesis of HAp crystals at pH 12 (Figure 5c), it can be determined that the hydroxide ion concentration was affected. In other words, it can be inferred that if the hydroxide ion concentration is high enough, it can also gather on the polar faces such as (002) and inhibit the extension.44,46

**CONCLUSIONS**

This study describes the successful use of dicarboxylic acids (i.e., sebacic acid) in the synthesis of HAp through a facile hydrothermal process. It has been confirmed that sebacic acid can accelerate nucleation and act as an inhibitor in the specific direction of CaP crystal growth. In addition, it has been demonstrated that as the pH increases during the synthesis process, hydroxide ions decrease in particle size because of the difference in concentration from the dicarboxylic acid provided by sebacic acid. DCPA was synthesized under acidic conditions, and HAp was synthesized under neutral and basic conditions, characterized by XRD, FTIR, and Raman spectroscopy. DCPA and HAp were basically able to be distinguished using the XRD diffraction pattern, and it can be confirmed that they are distinguished by the P—O(H) stretching vibration in FTIR and Raman spectroscopy results. In addition, TEM analysis confirmed that the synthetic HAp grows in the same c-axis direction as bone minerals. This result suggests that different forms of CaPs could be synthesized depending on pH and using an additive is a strategy for controlling the morphologies and orientation of the crystal of HAp.

**EXPERIMENTAL SECTION**

Calcium chloride (CaCl₂, anhydrous powder, ≥ 97%), sodium dihydrogen phosphate dihydrate (NaH₂PO₄·2H₂O, 99%), and sebacic acid (99%) were purchased from Sigma-Aldrich. Ethanol (99.5%) and sodium hydroxide (NaOH, 97%) were purchased from Samchun Chemical.

A typical synthetic procedure is described as follows. First, sebacic acid solution (100 mM) was prepared by dissolving sebacic acid powder in ethanol. Aqueous solution of CaCl₂ (200 mM) was then added to the prepared sebacic acid solution, followed by the addition of NaOH solution (1.2 M). After stirring for further 30 min, the aqueous solution of NaH₂PO₄·2H₂O (400 mM) was added dropwise, and then, the pH was adjusted with prepared NaOH solution (pH 4−12). The resulting suspension was then transferred into a polytetrafluoroethylene-lined stainless-steel pressure vessel and heated at 180 °C for 12 h. After this time, the precipitate was separated from the suspension by centrifugation (3000 rpm for 5 min at room temperature), washed two times with ethanol/distilled water, and dried at 60 °C overnight.

The morphology and size of the obtained crystals, which was coated with Pt sputter, were then observed by field-emission scanning electron microscopy (FE-SEM; JSM-7600F, JEOL) operating at 15 kV. The crystalline structure of synthesized CaPs was confirmed by XRD (SmartLab, Rigaku) with Cu Kα radiation (λ = 1.5406 Å), operating at 200 mA and 45 kV. The crystalline phase of the synthesized CaPs was confirmed by comparison with the data reported in the International Centre for Diffraction Data (ICDD) database. The samples of each group were pelletized with KBr, and the molecular structural properties of the synthesized CaPs were analyzed using an FTIR (Bruker, IFS-66) spectrometer. The Raman spectrum of CaPs functional groups was taken at room temperature using a confocal Raman microscope (inVia, Renishaw) with linear polarized laser light (λ = 532 nm) as the excitation source and a 100X objective. In order to observe the micrograph acquisition of HAp obtained under the conditions of pH 7 and pH 12 in the synthesized CaPs more precisely, the analysis was performed using transmission electron microscopy (TEM; JEM-2100F/JEM ARM200F, JEOl) operating at an acceleration voltage of 200 kV equipped with a CCD camera. The SAED mode was also conducted to analyze the crystallinity of CaPs synthesized under different pH levels. The dried CaPs
were dispersed in ethanol and deposited on a lacy carbon-filmed copper-mesh grid and dried at room temperature.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c03297.

XRD results of synthesized HAp without sebacic acid; XRD results of synthesized CaP with sebacic acid at various pH values; FTIR results based on a wide range of x-axis (wavelength; 4000–400 cm⁻¹); TGA results (temperature; 50–1300 °C); and zeta potential of CaP crystals synthesized at different pH values (PDF)

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**Notes**

The authors declare no competing financial interest.

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