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Collagen modulating crystallization of apatite in a biomimetic gel system

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Abstract

Apatite crystals were formed at 37 °C in a biomimetic gel system with the presence and absence of collagen under pH 6.5, 7.0 and 7.5 respectively. X-ray diffraction, transmission electron microscope and selected area electron diffraction pattern were applied to characterize the crystals. The results indicated that collagen modulate crystallization of apatite both in composition and morphology. With the absence of collagen, the obtained crystals were ribbon-shaped octacalcium phosphate (OCP) at pH 6.5, the mixture of OCP and nanosize rod-shaped hydroxyapatite (HAP) at pH 7.0 and 7.5 respectively. OCP would be a precursor of HAP with the absence of collagen. With the presence of collagen, collagen acted more effectively in controlling crystallization of HAP than pH did in the system. Ribbon-shaped HAP was the main phase at pH 6.5, kept a very thin structure at pH 7.0 while the needle-shaped HAP with several nanometers in diameter was obtained at pH 7.5. It was discussed amorphous calcium phosphate would be an intermediate phase of HAP with the presence of collagen. Such understanding of collagen and pH control on biomineralization gives new insights on the controlled synthesis of apatite.

Keywords: D. Apatite; Bio-crystallization; Biomaterials; Collagen

1. Introduction

Natural hard tissues usually own a good combination of stiffness and toughness due to their detailed composite micro-architectures, arising from the precisely controlled nucleation and growth of inorganic mineral crystals by organic matrices through specific protein-mediated processes [1]. Type I collagen, the predominant matrix protein in bone, possesses self-assembling properties and forms enclosed spaces in which the inorganic reinforcing phase hydroxyapatite (HAP) grows [2]. As the development of bone proceeds, HAP precipitates on the collagen framework via intermediate precursor polymorphs such as amorphous calcium phosphate (ACP), octacalcium phosphate (OCP), or dicalcium phosphate dehydrate (DCPD). It was supposed that type I collagen matrix does not have the capacity to induce matrix-specific mineral formation from metastable calcium phosphate solutions that do not spontaneously precipitate but merely provide the organizational framework and spatial constraint for crystal deposition [3]. Therefore, at the finer end of the hierarchical spectrum of bone, the self-assembled collagen fibril are impregnated with plate-shaped apatite nanocrystals of HAP that are 2–3 nm in thickness and tens of nanometers in length and width [1]. However, the stability of calcium phosphate crystals is pH-dependent no matter whether the biomacromolecular matrix exists [4,5]. Yang et al. mentioned the transformation from OCP to HAP was related to pH change induced by protein's hydrolysis *in vitro* [6]. Teng also reported that pH values influenced on the morphogenesis and species of calcium apatite [7]. Since the process of biomineralization is of high complexity, the crystallization of apatite *in vivo* is still unclear by now, especially the mechanism related to its formation in texture.

It was a consistent decision that restriction on a simulated research in a gel system can clearly reduce the level of complexity. The gel network is an excellent system for studying bio-crystallization due to the following reasons: (a) biomacro-

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molecular matrix is incorporated into the gel at room temperature and remains stable during the experiment; (b) the local concentration for crystallization is easy to afford; (c) the deposits are harvested with ease from the medium without contamination of gel [3]. In a gel system, the physicochemical nature more realistically mimics the mineralized tissue matrix environment, and more effectively controls apatite crystallization, in both composition and morphology. In the past decades, as a model system for the study of biological mineralization of bones and teeth, many gel systems have been successfully utilized by several researchers [3,8,9]. However, depending on the pH working conditions, calcium phosphates with different textures have been reported to be obtained in gel systems, although the applied protein keeps the same kind [9–13]. In addition, collagen showed no promotion on apatite crystallization in a gel system, which was different from what was observed in the solution system [4,14,15]. In this study, the experiment on deciphering the collagen dependence on biomineralization using a gel system to mimic the physiological environment under a pH of 6.5, 7.0 and 7.5 respectively was developed and collagen modulation crystallization of apatite was systematically studied. The final crystals were characterized by X-ray diffraction (XRD), transmission electron microscope (TEM) and selected area electron diffraction pattern (SAED). The study presents here has important implication for understanding apatite biomineralization process *in vivo* and controlling synthesis of apatite materials.

2. Experimental

Calcium phosphates were crystallized by controlled chemical reaction between calcium and phosphate ions (with and without collagen) in a gel medium at 37 °C and physiological pH of 6.5, 7.0 and 7.5 respectively. The reaction system is called a single diffusion system [8]. The gel medium was prepared as following: agar (2 wt.%) was added to deionized water under stirring in a water bath of 90 °C, then the solution kept stirring till agar dissolved completely. $NH_4H_2PO_4$ and $(NH_4)_2HPO_4$ with a concentration of $[NH_4H_2PO_4] + [(NH_4)_2HPO_4] = 30$ mM, altogether with a molar ratio of $[NH_4H_2PO_4]$ to $[(NH_4)_2HPO_4]$ in 2:1, 1:1 and 1:2 respectively, were introduced to the 2 wt.% agar solution, while NaCl was added in a concentration of [NaCl] = 150.0 mM. Collagen I (collagen, from calf skin, Sigma) in acetic acid solution was then added at 37 °C till the concentration in the agar solution arrived to 4 mg/ml; the pH of mixture was adjusted to 6.5, 7.0 and 7.5 using concentrated ammonia and HCl aqueous. The solution was stored at 4 °C over night till the gel with PO_4^{3-} (30 mM) and collagen was solidified. The other gel without collagen was the control. 50 mM Ca^{2+} solution were prepared by dissolving $Ca(CH_3COO)_2$ into deionized water (containing NaCl 150.0 mM), then the pH of Ca^{2+} solution was adjusted to 6.5, 7.0 and 7.5 using concentrated ammonia and HCl aqueous. All chemicals were analytical grade. After solidified completely, the agar gels with PO_4^{3-} were put into 37 °C water bath. The Ca solution was added over the crystallizing gel medium. After reaction for three days, a layer of white product was seen at the interface of Ca solution and PO_4^{3-} gel with or without collagen. For XRD and TEM, the products were heated under about 50 °C with deionized water, centrifuged while they were warm. In order to remove agar, the products were washed several times till the upper solution was clear.

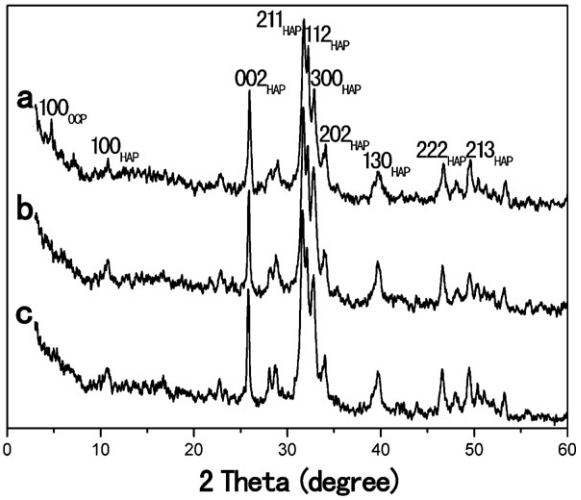

Fig. 1. XRD patterns of the products in the gel system with the presence of collagen under pH 6.5 (a), 7.0 (b) and 7.5 (c).

XRD measurements (XRD, MSALXD-2) were conducted using CuKα at 40 kV and 20 mA at 2θ degree range from 2 to 60° employing at a step size of 0.02. Crystals' size and shape were studied by TEM in a JEM-200CX microscope where SAED patterns were also recorded at accelerated voltage of 100 kV and camera constant of 0.80 m. The samples for TEM were dispersed by ultrasonic treatment in ethanol medium before they dropped on Cu grids covered previously with carbon films. The pH was measured by Model PHS-3B Meter Instruction Manual (Shanghai Precision & Scientific Instrument Co. Ltd.).

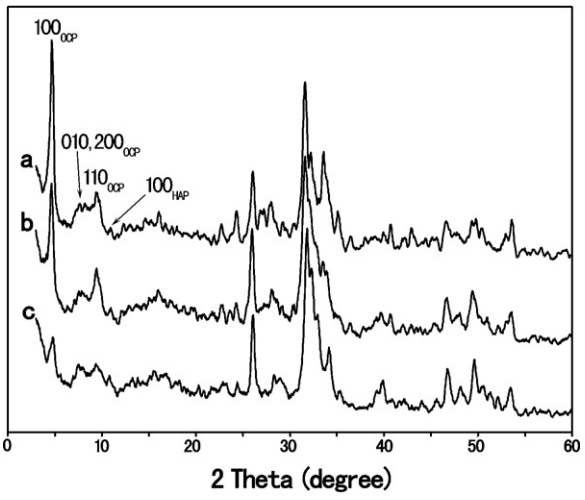

Fig. 2. XRD patterns of the products in the gel system with the absence of collagen under pH 6.5 (a), 7.0 (b) and 7.5 (c).

3. Results

Fig. 1 shows XRD patterns of the products obtained with the presence of collagen at pH 6.5, 7.0 and 7.5 respectively. The characteristic XRD peaks at 25.9° and 31.8° in 2θ attributed to HAP were observed, indicating that the major precipitations were HAP in the gel system with collagen. The (1 0 0) reflection at 4.7° in 2θ of OCP is very weak at pH 6.5, and disappears at pH 7.0 and 7.5. It means that less OCP can be detected at low pH 6.5, and the as-obtained products at pH 7.0 and 7.5 were composed of single-phase HAP. For the samples with the absence of collagen (Fig. 2), at the 2θ of 4.7, 9.4 and 9.7°, the characteristic reflections of OCP were observed, which could be distinguished from (1 0 0) reflection of HAP at 10.8° in 2θ. The intensity of the characteristic peak at 4.7° in 2θ obviously decreases when a high pH 7.0 and 7.5 was applied. This suggests the content of OCP phase in the gel system without collagen decreases as the increase in pH. Overall, the

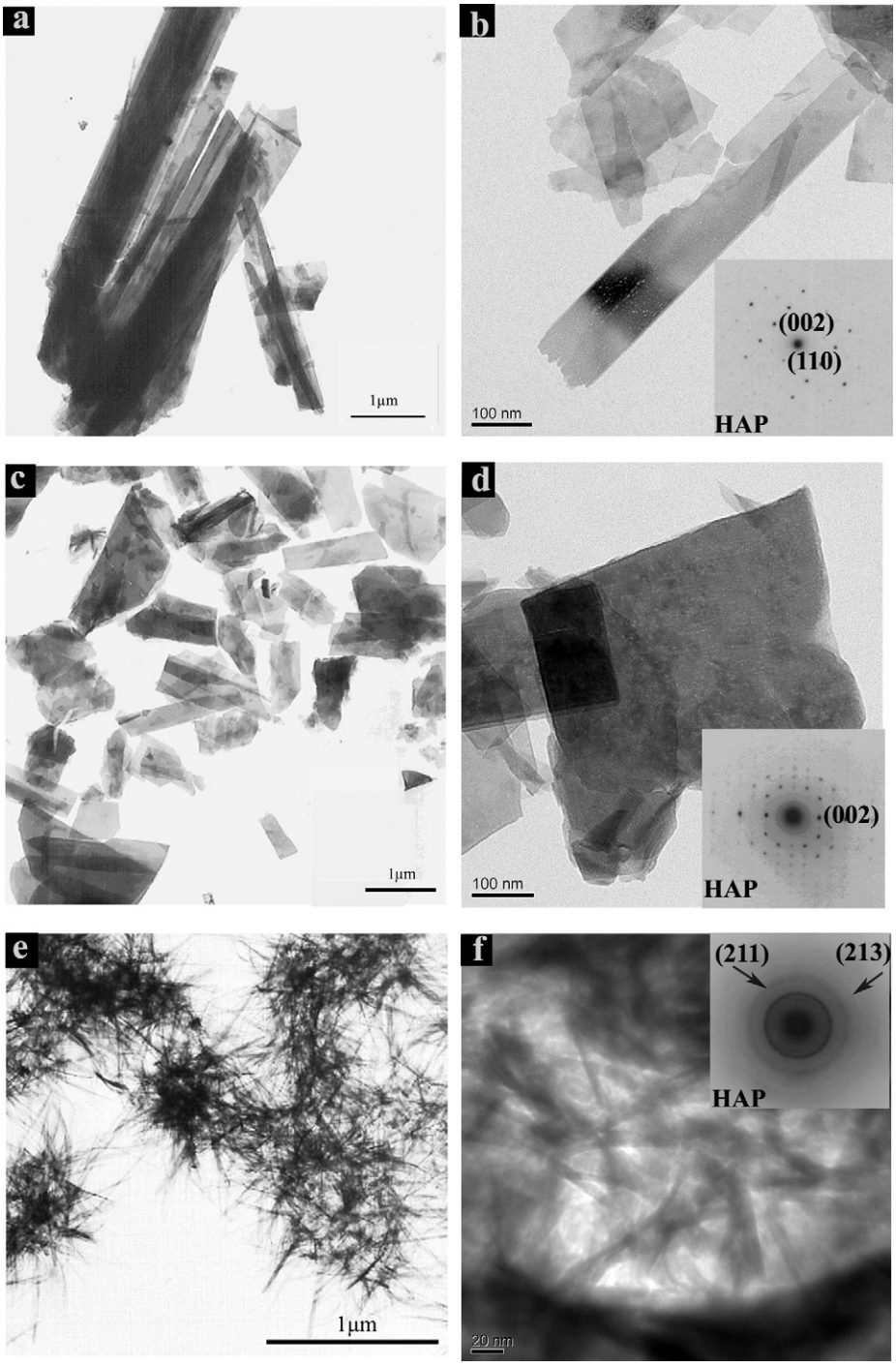

Fig. 3. TEM images together with the corresponding ED patterns (right insets) of the crystals in the gel system with the presence of collagen under pH 6.5 (a and b), 7.0 (c and d), and 7.5 (e and f).

products in the gel system with the absence of collagen under different pH are mixture of HAP and OCP.

Fig. 3 shows TEM images of the as-obtained crystals in the gel system with the presence of collagen. At low pH 6.5, the crystals present ribbon-like shape with large length of 5.0–10.0 μm and width of several hundreds of nanometer. Those ribbon-shaped crystals aligned very well according to Fig. 3a. The crystal phase is mainly HAP according to Figs. 1 and 3b, which an inserted SAED pattern of HAP corresponds to ribbon-like crystals. We refer these ribbon-like crystals as two-dimensional structure as the thickness is much less than the lateral dimensions. At pH 7.0, the crystals also present plate-like shape with about 2.0 μm in length and 1.0 μm in width, altogether with a very thin structure; and no other obvious crystals are observed in Fig. 3c. This very thin structure is similar to that in bone's crystal. The inserted diffraction pattern of the plate-shaped crystals was indexed as that of HAP crystals in Fig. 3d. At pH 7.5, the morphology is different from any of the others. Needle-like crystals with several nanometers in diameter are shown in Fig. 3e. The inserted electron diffraction pattern reveals the polycrystalline structure in nature and two electron spots which were the brightest and closest to the

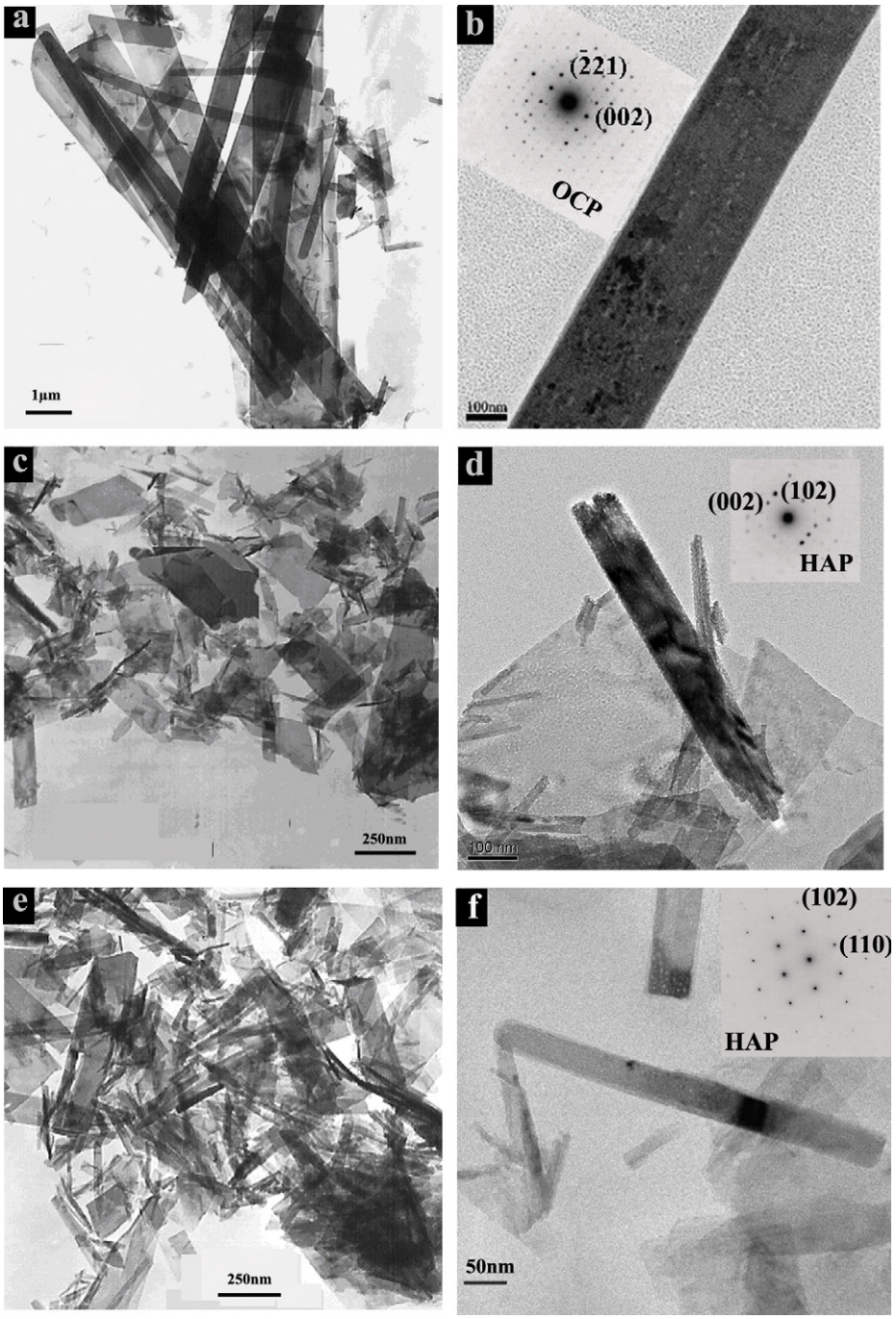

Fig. 4. TEM images together with the corresponding ED patterns (right insets) of the crystals in the gel system with the absence of collagen under pH 6.5 (a and b), 7.0 (c and d) and 7.5 (e and f). Note the inserted ED pattern in (d) is corresponding to the rod-shape crystals splitting from the plate-shaped crystal.

incident beam were calculated and revealed d_1 = 2.81 Å(2 1 1) and d_2 = 1.84 Å (2 1 3) of HAP.

Fig. 4 shows TEM images of the as-obtained crystals in the gel system with the absence of collagen at different pH values. At low pH 6.50, the crystals show ribbon-shaped crystals with length of 5.0–10.0 μm and width of about 1.0 μm in Fig. 4a, which is similar to the one with the presence of collagen. The SAED pattern (in Fig. 4b) is in agreement with a typical structure of OCP, which is also consistent with XRD pattern in Fig. 2. At pH 7.0, the plate-like crystals present about 500 nm in length and width, which are obviously shorter that those at pH 6.5 (shown in Fig. 4c). Some of fine rod-like crystals can be seen in Fig. 4c. From Fig. 4d, a rod-like crystal with a SAED pattern of HAP was splitting from plate-like crystals along a defined axe. According to computer-assisted lattice matching and DQNMR data, OCP transforms to HAP topotaxially, where the $[0\,0\,0\,1]_{HAP}$ and $[2\,1\,1\,0]_{HAP}$ axes are the same direction as the [0 0 1]ocp and [0 1 0]ocp axes respectively[7]. At pH 7.5, the morphology that plate-like and rod-like crystals were mixed together, is similar to that of pH 7.0, but the rod-like crystals predominately occur according to Fig. 4e and f. From TEM result in Fig. 4f, the rod-like crystal indexed as that of HAP crystal is about 50 nm in diameter and 400 nm in length, which keeps the same diameter as the splitting rod-like crystal in Fig. 4d. It means that the increase in pH promoted the hydrolysis of OCP and led to more HAP formation.

4. Discussion

The experiment was designed to study the apatite crystallization modulated by collagen at pH 6.5, 7.0 and 7.5 respectively in a gel system, which is closer to physiological environment than a solution system. Most studies use collagen in a solution system to induce bone remodeling process [4,16–20]. Those studies to mimic the composition and structure of bone focused on using calcium phosphate system via solution precipitation with reconstituted collagen, and HAP nucleated in the collagen fibril was reported [16,21–23]. However, collagen I showed no effect to nucleation of HAP in a gel system [14], and micrometer sized DCPD (Dicalcium Phosphate Dihydrate) crystals with a platy morphology were precipitated in the presence of type I collagen in a gel system at pH 7.4 [3]. While OCP or DCPD forms due to kinetic reasons under physiological condition, it is by no means a prerequisite for the formation of ribbon-like HAP at 37 °C, although a plate-shaped Ca-deficient HAP was obtained at pH 6.0 under 150 °C [24]. Here, the ribbon-shaped morphology of HAP crystals in presence of collagen was obtained at pH 6.5 under 37 °C. The plate-shaped morphology in a very thin structure kept less changed even at pH 7.0. As we know, this morphology resembles bone apatite in a very thin structure. The present study provides evidence that an enhanced HAP formed in a bone-like structure throughout collagen matrix. Usually, calcium phosphate precipitated on the collagen under condition (physiology temperature with a high concentration of calcium and phosphate ions over 20 mM) [17,20]. In a solution condition, the crystalline phases identified in the presence of collagen were DCPD, OCP and HAP depending on the pH range [3,17]. According to Honda et al., OCP-collagen plate-like structure was predominantly precipitated at pH 6.99–7.20 in simulated body fluid (SBF) [17], which is different from our results. In our gel system, it is likely the phosphate ions affect the nucleation event of the crystals in the presence of collagen when phosphate ions were firstly introduced into the gel [25,26], result to the modification of the degree of supersaturation adjacent to the surface of collagen; and then further induce the precipitation of HAP with different texture depending on pH. Due to the limitation of existing experiment's approaches for monitoring *in situ* nucleation evolution in a gel system, we cannot get information about the initial crystallization. However, Kniep et al. showed some evidence that the dipole field influences the growth development of fluoroapatite–gelatine crystal seeds, it monitors the fractal morphologenesis of the crystals [11]. The results in the study also imply that collagen conform the morphology of apatite in some degree and keeps apatite in a very thin structure *in vivo*. With the regarding, the nano-sized needle-like crystal at pH 7.5 also reveals that collagen acts as a template during apatite crystallization. Although a needle-like structure is usual for apatite in a solution system, but it was less shown in a gel system with a presence of biomacromolecular. According to Cui's study, the carboxyl groups on the outside of the collagen threefold spiral, which favor chelation of calcium ions, are one kind of site for collagen mineralization in a neutral solution and the needle-like crystals with nano-size forms along with the fibril of collagen [4,27,28]. In addition, ACP mostly could be the precursor of the plate-shaped HAP, otherwise the transformation from OCP to HAP might lead to the split of the plate-shaped crystals, and then, not plate-shaped but rod-shaped apatite would have produced at pH 7.0.

OCP is a possible precursor in a biological system because it is easy to nucleate according to thermodynamic theory [29–31], while it is also unstable relative to HAP and tends to hydrolyzed according to the reaction [32]:

$$5Ca_8H_2(PO_4)_6 \cdot 5H_2O \xrightarrow{H_2O} 4Ca_{10}(PO_4)_6(OH)_2 + 18H^+ + 6(PO_4)^{3-} \quad (1)$$

As shown by the reaction equation, the increase of pH value leads to more products of HAP. Usually, the critical pH range of apatite formations is 6.55–6.65 at 40 °C in SBF [17]. The plate-like OCP crystals transform into hexagonal rod-shaped HAP crystals as pH increases slowly from 4.35 to 6.69 in a solution system at 100 °C [33]. Our experiment was carried out at 37 °C in a gel system with the absence of collagen; the transition from plate-like OCP to rod-like HAP seems to happen at pH 7.0 of which plate-like OCP and rod-like HAP coexist. In the typical transforming process of OCP to HAP, a changed morphology from the plate-like shape with several micrometers in width for the OCP precursor to the nanosize rod-like HAP crystals was clearly identified in Fig. 4d. The close similarity between the OCP and HAP crystal structures leads to the transformation happen easily. Crystal structure of OCP is composed of a portion of apatitic structure and a portion where H_2O molecules are concentrated. When OCP crystal split into small portions, small portions with apatitic structure were formed in the

process of conversion [32,34]. Although the transit has been well established in theory, the direct transformation process is less reported. The presence of a splitting crystal in Fig. 4d in the study provides evidence for the transformation.

The higher the reaction pH was, the more HAP formed according to the reaction (1). As pH reached 7.5, rod-like HAP was the main phase. The result indicates the transformation process as an OCP intermediate or precursor of HAP in the gel system with the absence of collagen.

5. Conclusion

In the gel system, crystallization of apatite modulated by pH and collagen was studied. With the presence of collagen, ribbon-shaped and plate-shaped HAP crystals in micrometer size formed under pH 6.5, 7.0; while needle-shaped HAP in nanosize produced at pH 7.5. With the absence of collagen, a mixture of plate-shaped OCP and nanosized rod-shaped HAP occurred at pH 7.0 and 7.5, while ribbon-shaped OCP crystals existed at pH 6.5. The rod-shaped HAP nanocrystal splitting from the plate-shaped OCP crystals clearly was observed at pH 7.0. It was discussed that HAP originated from the hydrolysis of OCP intermediate precursor in the gel system with the absence of collagen. With the presence of collagen, collagen promoted rod-like HAP formation at a lower pH in the near physiological system.

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