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Preparation of hierarchically organized calcium phosphate–organic polymer composites by calcification of hydrogel

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Abstract

A novel type of calcium phosphate–organic polymer composite having a hierarchical structure was prepared by calcification of a poly(acrylic acid) hydrogel. Macroscopically, an organic gel containing phosphate ions was transformed into an opaque solid material by diffusion of calcium ions. We observed the formation of micrometer-scale layered structures consisting of nanoscale crystals of hydroxylapatite (HAp) in the opaque products. The laminated architecture resulting from the periodic precipitation of calcium phosphate varied with the reaction conditions, such as the concentrations of the precursor ions and the density of the gel. The nanoscopic structure of HAp crystals was modified by the addition of gelatin to the polymer matrix.

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1. Introduction

Hard tissues of vertebrates, such as bones and teeth, have a complex structure organized in hydroxylapatite (HAp) and organic matrices [1]. Natural bone, as a three-dimensionally ordered composite of nanoscale minerals and organic fractions, has unique mechanical properties, such as low stiffness, resistance to tensile and compressive forces, and high fracture toughness [2,3]. On the other hand, artificial materials currently used in bone grafts are far from ideal for bone substitutes because of the differences in their mechanical and biological properties. For example, titanium alloy affects the mechanical properties of normal bone [4]. Even when bioactive ceramics, such as sintered hydroxyapatite and β -tricalcium phosphate, are directly connected with bone, they are brittle and are easily crushed. Therefore, the production of artificial bone with a composition and nanostructure similar to natural bone is technologically important for medical and dental applications.

A biomimetic approach to the preparation of calcium phosphate–organic polymer nanocomposites has recently attracted great interest. HAp–organic polymer nanocomposites

have been studied as a structural analogue of HAp–collagen composites in real bone. Morphological control of calcium phosphate crystals has also been demonstrated by using various polymers. Bone-like HAp coatings on the surface of various organic substrates, such as collagen, cellulose and other polymers, have been achieved by mimicking biological processes in simulated body fluid (SBF) [5–17]. It has been proposed that an apatite–polymer fiber composite with a structure analogous to that of living bone can be synthesized by soaking, in SBF, organic fibers arranged in a three-dimensional structure with modifications of the surface for apatite nucleation. However, in past studies using these techniques, apatite was deposited only on the surface of the fibers exposed to the precursor solution. An alternative soaking process in calcium and phosphorous solutions provided the HAp formation in a three-dimensionally structured organic hydrogel matrix at ambient temperature [18,19]. An ordered composite powder of HAp and organic molecules was also prepared through self-organization by interplay at the organic–inorganic interface [20–28]. A composite material with a hierarchical structure was fabricated by the self-assembly of nanofibrils of mineralized collagen [29]. Recently, three-dimensional bone-like composites were fabricated by the calcification of hydrogels [30]. However, the highly tailored and hierarchically organized architecture of HAp–polymer composites has not been sufficiently realized in current technologies. The development of a fabrication technique for hierarchically

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layered HAp structures similar to natural bone would have a significant impact on medical applications.

In a recent study, we achieved hierarchically organized structures of nanocrystalline HAp using periodic precipitation in a non-equilibrium reaction system with diffusion of calcium ions into a poly(acrylic acid) (PAA) matrix containing phosphate ions [31]. This paper describes the preparation of a variety of hierarchical structures of HAp–polymer composites. The macroscopic and microscopic structures of the composites were systematically investigated as a function of the preparation conditions and by the addition of gelatin molecules. We investigated the roles of the organic polymers for the construction of the unique architectures and studied the similarities between the mechanical properties of the composite and those of natural bone.

2. Experimental section

2.1. Experimental procedure

PAA, a typical water-absorbing polymer, was used to provide an aqueous gel matrix for the precipitation reaction between calcium cations and phosphate anions. Calcification was achieved by the diffusion of calcium ions into a hydrogel of PAA containing a phosphate solution. A schematic illustration of the procedure is shown in Fig. 1. Organic polymer gels were prepared by mixing 5 g acrylic acid (Wako Pure Chemical), 0.1 g *N,N'*-methylene bis-acrylamide (Kanto Chemical) and 0.15 g ammonium peroxosulfate (Kanto Chemical) in 45 g purified water in polypropylene sample bottle (30 mm in diameter and 70 mm in height). In this step, we added diammonium hydrogenphosphate ($(\text{NH}_4)_2\text{HPO}_4$ (Junsei Chemical) into the precursor solution. Gelation of the mixture occurred after it had been kept at 60 °C for 2 h. The same volume of $\text{Ca}(\text{NO}_3)_2$ (Junsei Chemical) aqueous solution, which was adjusted to the physiological pH of 7.0 with α, α, α -tris(hydroxymethyl) methylamine–HCl, was loaded on the gel containing phosphate ions at 25 °C. The concentration of $(\text{NH}_4)_2\text{HPO}_4$ was varied in the range of 0.60–4.00 mol/dm³, and the concentration ratio of $\text{Ca}(\text{NO}_3)_2/(\text{NH}_4)_2\text{HPO}_4$ was changed from 1.67 to 8.35. In 72 h, the gel body, which was 10 mm thick, was solidified with the diffusion of calcium cations in exchange for protons. The organic gel was solidified

and converted into an opaque composite material through the precipitation of calcium phosphate in the gel matrix. The shape of the solidified composites with the calcification was slightly deformed after a drying process, although untreated gels without precipitation obviously shrank because of the evaporation of water. A certain amount of gelatin (Kanto Chemical) (0–40 mass%) was substituted for acrylic acid in the precursor solution in order to study the influence of the organic molecules on the crystal growth of calcium phosphate. Solidified gel was not obtained by the substitution of gelatin higher than 50 mass%. We also added DL-aspartic acid (Junsei Chemical) or L-glutamic acid (Junsei Chemical) to investigate the effect of amino acids in gelatin molecules on the morphology of the products.

2.2. Characterization

Morphological characterization was carried out using a field emission scanning electron microscope (FESEM, Hitachi S-4700). The cross-section of calcified sample (before firing) was cut using microtome and then the slice was observed by FESEM without further treatment. Structural and compositional determination of the products was performed with a Rigaku RAD-C X-ray powder diffractometer with Cu K α radiation (XRD) and a field emission transmission electron microscope (TEM, FEI TECNAI F20) equipped for energy-dispersive X-ray analysis (EDXA).

3. Results and discussion

3.1. Calcification of organic gel

We obtained a clear hydrogel of PAA containing a phosphate solution by gelation at 60 °C for 2 h. When a calcium solution was loaded on the gel, calcification immediately started on the interface between the gel and the solution (Fig. 2a and b). Calcium phosphate crystals were precipitated in the gel matrix with the diffusion of calcium

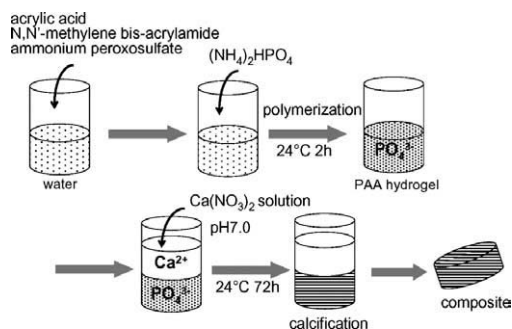


Fig. 1. A schematic illustration of the experimental procedure.

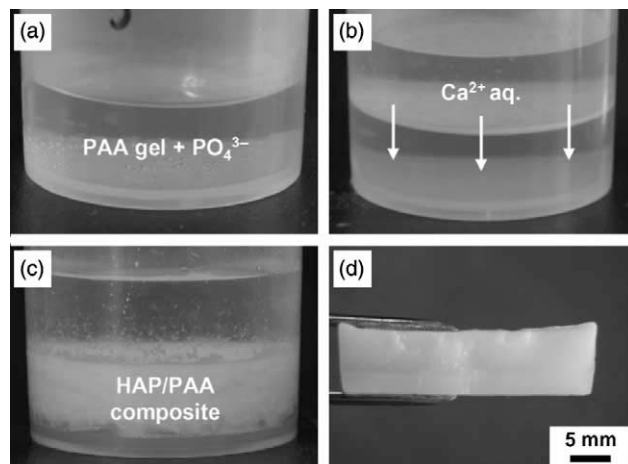


Fig. 2. Photographs for calcification of PAA hydrogel with the diffusion of calcium ions.

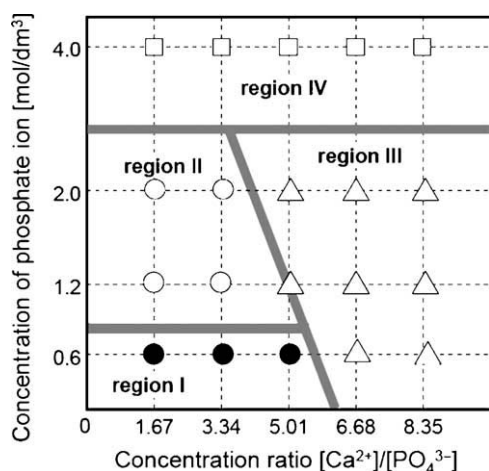


Fig. 3. The relationship between the sample preparation conditions and the whole shape of the resultant materials. The opaque composites that were not completely solidified (region I), totally solidified and opaque architecture (region II, suitable condition), the white solidified composite with shrinkage (region III), and the partially mineralized material (region IV) were obtained in the each condition.

cations by an ion exchange for protons. Then, the gel disk was totally solidified with the calcification for 72 h (Fig. 2c and d). We performed a series of experiments with phosphate anions and calcium cations at various concentrations. The schematic diagram in Fig. 3 shows the variation of the macroscopic form of the products with a change in concentrations of the reactants. When the concentrations of phosphate anions and calcium cations were relatively low (region I), an opaque product in the hydrogel was not completely solidified. The hydrogel was changed to a solidified opaque composite in a relatively high concentration of calcium cations (region III), even though the product was remarkably deformed, with shrinkage of the center of the disk. When the concentration of phosphate anions was high (region IV), the precipitation reaction and calcification immediately stopped in the surface region of the gel. Then, we found the most suitable concentration of the reactants (region II) for the preparation, and a totally solidified and opaque material was obtained without shrinkage. As a result of these experiments, we mainly characterized the products prepared at 1.2 mol/dm^3 $(\text{NH}_4)_2\text{HPO}_4$ and 4.0 mol/dm^3 $\text{Ca}(\text{NO}_3)_2$ (the molar ratio of $\text{Ca}^{2+}/\text{PO}_4^{3-}$: 3.34) as the standard condition.

3.2. The structure of the composites

Fig. 4 shows a cross-sectional view of the solidified gel prepared under the standard condition. We observed a laminated structure with a thickness of $\sim 10 \mu\text{m}$ in the product. Moreover, the presence of a submicron-scale thin plate in the micrometer-scale bands was recognized (Fig. 4c). These periodic bands were arranged perpendicularly to the direction of the diffusion of calcium cations in the gel matrix. In the course of the diffusion, sequential precipitation bands, denoted as Liesegang bands, formed in the gel matrix [32–36]. These laminated structures were ascribed to the periodic precipitation

with the diffusion of calcium cations in the hydrogel, although such a fine structure in micrometer-scale periodicity has rarely been reported. The mass ratio of the organic component (ca. 35 mass%) in the dried products was close to that of natural bone.

The TEM image of the products prepared with the standard concentrations (Fig. 4d) shows the presence of HAp nanocrystals (5–10 nm) in the organic matrix, which indicates that the laminated bands consisted of PAA and HAp nanoparticles without a specific crystallographic orientation. The Ca/P ratio of the mineral phase was estimated at 1.74 by EDXA, indicating the formation of HAp ($\text{Ca/P}=1.67$). Fig. 5 shows a typical XRD pattern of the solidified gel. Weak peaks for dicalcium phosphate anhydrate (DCP:CaHPO_4) were obtained with relatively strong, broad signals (Fig. 5a). Although we assume that DCP was formed during the drying process, the details of the formation mechanism are under investigation. The diffraction patterns were almost the same for all the products prepared with various concentrations of the reactants. However, the crystal phase of the products fired at 700°C depended on the concentration of phosphate ions in the gel matrix. After the products were fired at 700°C in air, inorganic structures of calcium phosphate appeared through the combustion of organic compounds. We obtained HAp at a relatively low concentration, below 1.2 mol/dm^3 (Fig. 5b). On the other hand, β -calcium pyrophosphate ($\text{Ca/P}=1.0$), which was easily transformed to DCP with calcination, was hardly observed in the diffraction pattern. This fact means that DCP was not a main component of the mineral phase in the composites prepared at a low phosphate concentration. Viewed from the TEM observation, HAp nanocrystals were mainly

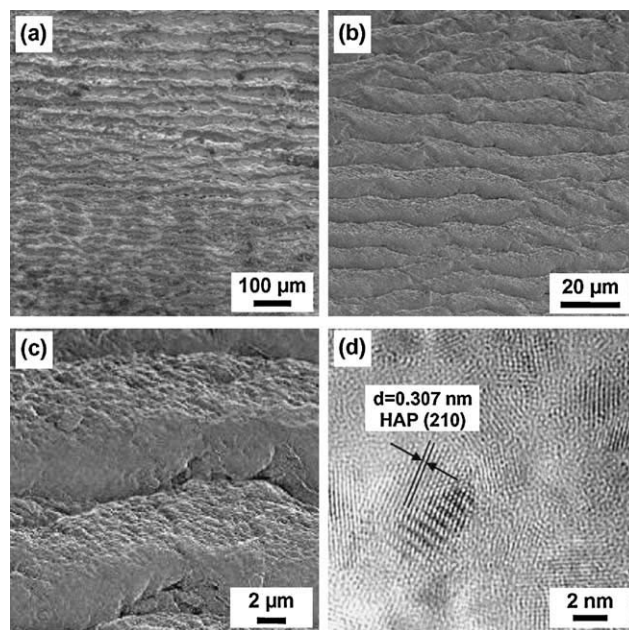


Fig. 4. Cross-sectional views of the composite prepared on the standard condition. (a and b) The macroscopic FESEM images of the laminated architecture, (c) FESEM image of the layered structure in submicron scale. (d) TEM image of HAp nanocrystals in this hierarchically organized architecture.

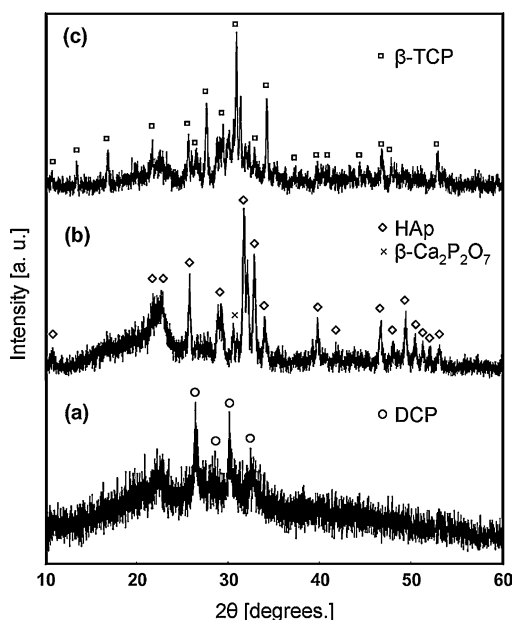


Fig. 5. Typical XRD patterns of as-deposited and calcined products at various concentrations of the precursors. (a) As-deposited sample in standard condition, (b and c) After calcined materials in phosphate concentration below 1.2 mol/dm^3 and above 2.0 mol/dm^3 , respectively.

contained in the gel matrix, even though the signals of HAp were not clearly obtained in the XRD patterns. Therefore, the white precipitation bands mainly consisted of nanocrystalline HAp, including a small amount of DCP and amorphous calcium phosphate. In the case of the preparation at a high concentration of phosphate above 2.0 mol/dm^3 , the formation of β -tricalcium phosphate (β -TCP) ($\text{Ca/P} = 1.5$) and β -calcium pyrophosphate ($\text{Ca/P} = 1.0$) was observed, suggesting that β -TCP and DCP with low crystallinity were formed. On the other hand, the crystal phase was not influenced by the concentration of calcium ions.

3.3. The porous architecture of fired products

We clearly observed the microstructures of calcium phosphate after the combustion of organic components by firing the composites at 700°C in air. As shown in Fig. 6, the layered porous structures of the thin plates, several micrometers in thickness, were obtained around the standard condition. The mineral laminated architecture was generated from the periodic precipitation in the gel matrix, even though the layer thickness and the interlayer distance slightly decreased with the removal of organic compounds. Since, submicron-scale thin plates were included in the each layer (inset of Fig. 6c), after firing, the porous products kept their hierarchically laminated morphologies. The presence of HAp nanocrystals realized the generation of the hierarchical structure in different scales.

The morphology of the porous architectures varied with a change in the initial concentration of phosphate anions and acrylic acid. As the concentration of phosphate ions decreased, the discrete precipitation bands transformed blurred

lamination. Then, the layered morphologies changed into porous and open cellular structures composed of curved thin films with cross-linkages (Fig. 6a–c). The structural variation with an increase in the concentration of calcium cations was relatively small in comparison with the phosphate concentration (Fig. 6c–e). The density of PAA gel also influenced the morphology of the layered structure. The gel body was densified by an increase in the amount of acrylic acid added to the precursor solution. As shown in Fig. 7, porous cellular structures were formed in a high-density gel (10 g PAA/45 cm^3 water), while the densely packed laminated structures were observed in a low-density matrix (5 g PAA/45 cm^3 water).

In gel media, the diffusion of reactants is the rate-controlling factor of crystal growth [37]. When the ionic concentration product of calcium and phosphate exceeds a critical saturation value K_C by the diffusion of calcium cations in a gel matrix containing phosphate anions, the precipitation starts at the top of the gel. However, the consumption of phosphate anions around the top of the gel results in the formation of the first band and the subsequent suppression of the reaction. Far from the first band, the next precipitation band would be formed

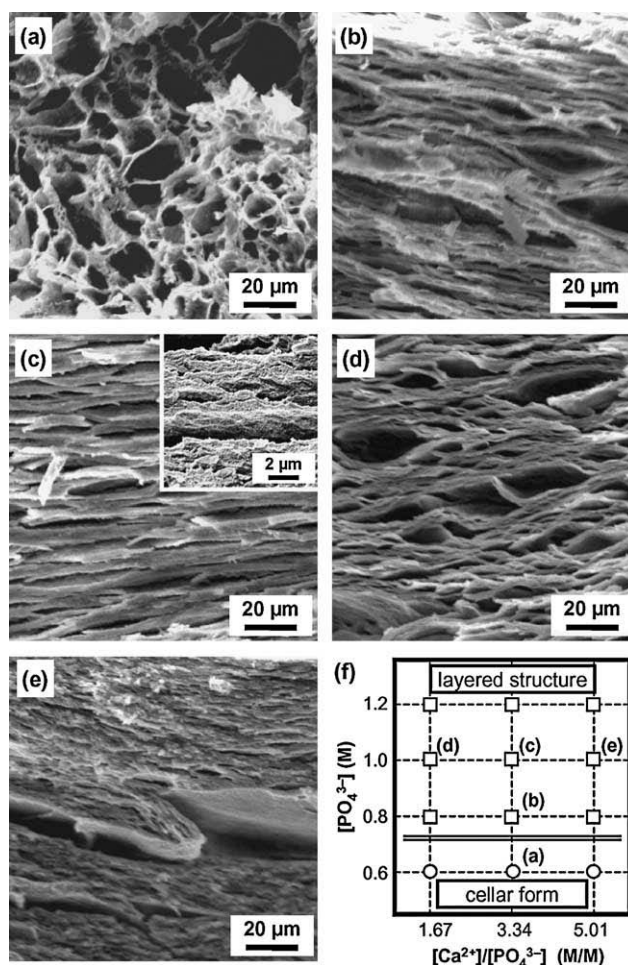


Fig. 6. Structural variation of the porous architecture after calcinations. (a–e) FESEM images of the cross-section and (f) the schematic diagram of the morphologies.

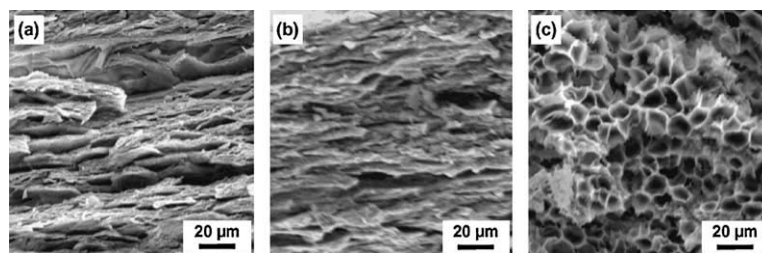


Fig. 7. FESEM images of cross-sectional view of the calcined porous structures produced on the standard condition at various gel densities. (a) PAA 5 g, (b) 7 g, and (c) 10 g in 45 cm³ water.

where the concentration of phosphate becomes sufficient. In this way, the variation of the concentration in the gel matrix leads to the generation of the sequential precipitation bands. The layered morphology was changed into a cellular structure by a decrease in the concentration of phosphate. This morphological variation is ascribed to the weakening periodicity of precipitation bands with a decrease in the degree of supersaturation on a Liesegang ring phenomenon. Because, the diffusion of ions is suppressed in a dense gel matrix, the fluctuation of the concentration is amplified [33]. Thus, the porous cellular structures were produced with the weakening periodicity through the amplified fluctuation in a high gel density.

3.4. Influence of the addition of gelatin on the architecture of the composites

Gelatin was substituted for PAA in the gel matrix in order to modify the microscopic structure of HAp. According to the XRD profiles, the substitution of gelatin for 40 mass% PAA inhibited the formation of DCP (data not shown). While the macroscopic opaque form was closely similar to that formed in pure PAA hydrogel (Fig. 8a), the micrometer-scale layered structure was composed of porous plates (Fig. 8b). Nanoscopic fibrous structures consisting of HAp nanocrystals were observed in TEM images (Fig. 8c and d). The substitution of aspartic acid and glutamic acid for 40 mass% PAA gel exhibited almost the same effect on the morphology in the nanoscopic and microscopic scales. Thus, the amino acids in gelatin molecules induced the formation of HAp and its one-dimensional arrangement in the fibrous morphologies. It has been reported that collagen and gelatin molecules induced the alignment of HAp nanocrystals on a *c*-axis through the specific interaction between carboxy groups and the crystal surface [20]. However, the orientation of the nanocrystals in the composite was not determined in this study because the architectures were damaged by the electron beam. The hierarchically organized architecture was associated with the two respective roles of polymers: (1) gelatin molecules control the nanoscopic structure of HAp, and (2) the PAA matrix realizes the formation of macroscopic morphologies. Therefore, the architecture was closely similar to that of natural bone.

3.5. Mechanical properties of the composites

A three-point bending test (ASTM E855-90) was performed on the HAp/PAA composites prepared under the standard condition. From a load displacement diagram, we estimated that Young's modulus and tensile strength were 3.7 and 73.1 MPa, respectively. These values are close to those of natural bone (Young's modulus: 0.3–24 GPa; tensile strength: 100–220 MPa) [3,38]. The results suggest that the composite was about as flexible as natural bone. On the other hand, sintered HAp had a relatively high Young's modulus, although the tensile strength was sufficiently high. The composite had mechanical properties similar to those of natural bone in comparison with a sintered body of HAp particles.

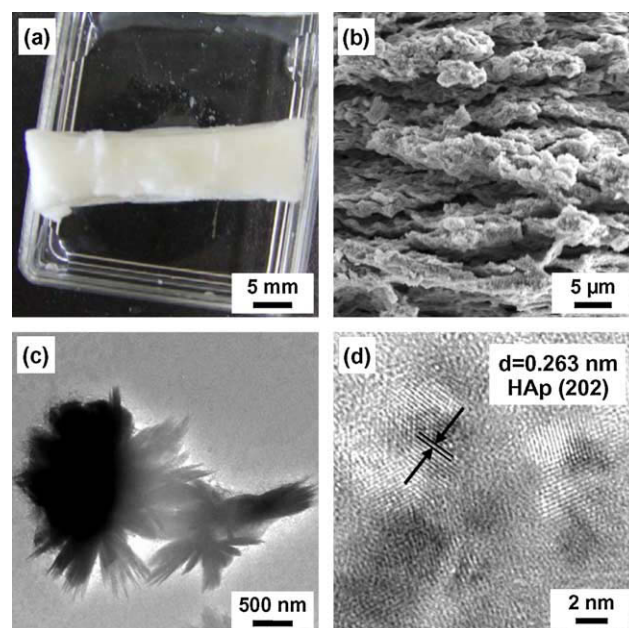


Fig. 8. Morphological variation of the composite with substitution of gelatin (60 PAA: 40 gelatin). (a) macroscopic image, (b) FESEM image of the porous layered structure, (c) TEM image of the fibrous aggregation consisting of HAp nanocrystals, and (d) the high-resolution image exhibiting lattice spacing of HAp.

4. Conclusions

We developed a new type of HAp–PAA composite having a hierarchically organized architecture similar to that of natural bone. Furthermore, a porous HAp architecture with a hierarchically layered structure was also obtained by firing the composite. The macroscopic periodic structure of HAp was successfully controlled from laminated layers to a cellular porous form by the reaction condition. The microscopic structure of HAp was modified by the addition of gelatin. The laminated HAp–polymer composites have potential for new types of biocompatible materials, because their mechanical properties are similar to those of natural bone. The hierarchical porous HAp architectures could be useful as an adsorbent and as a scaffold for tissue engineering.

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