Novel Biomineralization for Hydrogels: Electrophoresis Approach Accelerates Hydroxyapatite Formation in Hydrogels

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As a first step toward hydroxyapatite (HAp) formation in agarose hydrogels, we have tailored the internal chemistry using an electrophoresis approach. HAp was formed using aqueous solutions of calcium chloride and disodium hydrophosphate, which were set in a conventional agarose electrophoresis apparatus. Calcium and phosphate ions provided cations and anions, respectively, and were shown to migrate into the agarose hydrogel toward the corresponding electrode side. HAp was formed after colliding with each ion. The time needed to reach complete HAp formation was 30 min, and 130 ng of HAp was formed in 1 mg of agarose hydrogel when the equilibrium swelling state was reached. The electrophoresis approach accelerated the HAp formation, and the linear velocity of 1 mm/min was shown to be roughly 15 times larger than that of simple diffusion (0.06 mm/min).

Introduction

Polymer hydrogels are attractive materials that exhibit interconnectivity within their 3-dimensional space. In general, hydrogels contain a large amount of free water that provides an environment capable of hosting biological functions. Hydrogels have frequently been used to host and release drugs in which the release is often triggered by stimuli response.[1−6] Furthermore, hydrogels are utilized for analytical chemistry; in particular, agarose and poly(acrylamide)-based hydrogels have often been used in electrophoresis. On the other hand, hybrid materials composed of polymer hydrogels and inorganic compounds have been investigated as advanced biomaterials.[7−11] Hydroxyapatite (HAp) and calcium carbonate are major inorganic compounds and are common in nature. For example, vertebrate animals have bones and teeth, and coral reefs and shells consist of mineral calcification. In particular, human natural bone is composed of 70% HAp and 30% collagen. HAp powder itself has been prepared by precipitation from aqueous solutions and mechanochemical−hydrothermal methods.[12−13]

In 1990, Kokubo reported a pioneering biomineralization study that provided a desired thickness of HAp onto polymer films, ceramics, and metals using a biomimetic process in simulated body fluid (SBF).[14] The research regarding the SBF process has recently been summarized.[15] Although SBF is diverse and simple, the static preparative condition requires a prolonged time to reach complete HAp formation (2−3 months), which often is not suited for industrial applications. In 1997, Decher reported the fundamental concept regarding the build-up of layered nanoassemblies composed of polycations and polyanions.[16] The polyelectrolyte assemblies were prepared by an alternate adsorption process. Taguchi et al. discovered an alternative process to prepare HAp in hydrogels.[17−20] In this process, the hydrogel was alternately soaked with calcium chloride and disodium hydrogen phosphate solutions. The time needed to reach HAp formation was normally within 1 day, about 100 times faster than that of the SBF process. Furthermore, calcium carbonate formation in hydrogels could be prepared by changing sodium carbonate and disodium hydrogen phosphate solutions. Thus, the polyelectrolyte approach is capable of biomineralization and able to provide solutions that better fit industrial demands. Recently, HAp hydrogel composites were evaluated as an implantable material for regenerative medicine.[21−22]

Although faster, the polyelectrolyte process involves numerous problems. For example, the formation of HAp is regulated by the diffusion of ions from each solution. The diffusion is given by the following equation:[23]

$$t = r^2/6D$$

in which t is the time to reach complete diffusion, r is the diffusion distance, and D is the diffusion coefficient (diffusivity of glucose in agarose,[24] 10^{-6} cm^2/sec). For example, a thick hydrogel (2 mm) needs roughly 30 min to diffuse in each solution. The linear velocity is roughly 0.06 mm/min. If 10 cycles were carried out by polyelectrolyte adsorption, roughly 10 h is needed to prepare HAp in the agarose hydrogel.

In this study, we present a technique using electrophoresis to deliver ions to the interior of the hydrogel. Particularly, we have focused on HAp formation using calcium chloride and disodium hydrogen phosphate solutions in combination with an agarose hydrogel. Agarose hydrogels are commonly used for electrophoresis, and agarose is a widely used polysaccharide in the biomaterials field, for example, when encapsulating Langerhans' islets.[25] As shown in Scheme 1, agarose hydrogels were applied between the anode and cathode Pt electrodes. Calcium chloride and disodium hydrogen phosphate aqueous solutions were poured into the anode and cathode cells, respectively, and each ion could diffuse into the agarose hydrogel by electrophoresis. The ions were mixed in the center of the agarose hydrogel such that HAp was formed. The electrophoresis approach greatly increased the rate of ion delivery relative to diffusion.

Experimental Section

Materials. Agarose (NuSieve) was purchased from Cambrex Bio Science Rockland, Inc., Rockland, ME. The gel strength was over

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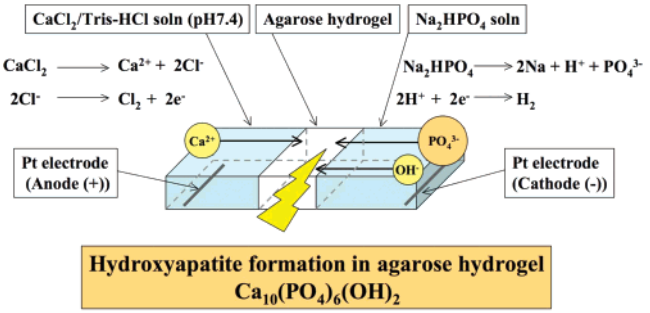
Scheme 1. Design of HAp Formation in Agarose Gel[a]



[a] The diffusion coefficient would be larger under the electrophoresis conditions.

1,400 g/cm^2 with a 4% gel concentration. Anhydrous calcium chloride was purchased from Kishida Chemical Co., Osaka, Japan. Disodium hydrogen phosphate, Tris(hydroxymethyl)aminomethane (Tris), and Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. All reagent were extra pure grade and were used as-received. Ultrapure water was used through the experiment.

Biomineralization of Agarose Hydrogel by the Electrophoresis Approach. A submarine-type electrophoresis apparatus (Mupid, Advance Co., Ltd., Tokyo, Japan) with a Pt electrode was utilized for the HAp formation. A 3 wt % agarose hydrogel was prepared in 10 mmol/L Tris-HCl (pH 4.8), and was molded by the gel tray (6 × 11 × 0.8 cm). The hydrogel was then set in the electrophoresis apparatus. Typically, the calcium chloride aqueous solution was prepared at 40 mmol/L, and was adjusted to pH 7.4 using Tris-HCl buffer. The disodium hydrogen phosphate aqueous solution was prepared at 40 mmol/L. The calcium and phosphate solutions were then set in the apparatus on the cathode and anode sides, respectively (Figure 1). The electrophoresis was carried out for 30 min at 100 V. After the electrophoresis, the biomineralized agarose hydrogel was immersed into ultrapure water to remove excess calcium and phosphate solutions.

Characterization of Agarose Hydrogel-Formed HAp. The formed HAp was evaluated in terms of the total amount of calcium. First of all, the HAp composite was cut into small pieces, and the specimen was immersed into 1 mL of hydrochloric acid aqueous solution (1 mol/L) to dissolve the HAp in the agarose hydrogel (overnight). A small amount of the supernatant was used for further evaluation, and the concentration of calcium ion was evaluated using a Calcium E-Test Wako (Wako Pure Chemical Industries, Ltd.). The fundamental mechanism was formation of chelate compound. The calcium ions in the supernatant were captured by the chelating reagent (methyl xylenol blue), which was contained in the detection kit. After the formation of the chelate compound, the change in absorbance at 610 nm was monitored. Calcium standard solution (10 mg/dL) was used for making the calibration curve.

The morphology of HAp was observed using a scanning electron microscope (SEM, JSM-6700FE, JEOL, Tokyo, Japan). The biomineralized agarose hydrogel was first lyophilized, and osmium tetraoxide was coated to suppress charging for 30 s. The SEM observation was carried out using 3000× and 15 000× magnification. In the case of cross-sectional observation, the lyophilized specimen was cut into two pieces before osmium coating.

The HAp was also characterized in terms of the X-ray diffraction (XRD) patterns of the crystals (RINT In Plane UltraX18, Rigaku, Tokyo, Japan). The X-ray source was Cu Kα, and 40k V and 200 mA was used for the measurement. The scan speed was 2°/min, and 10−40° was monitored.

Results and Discussion

Biomineralization of Agarose Hydrogel. To respond to an applied electric field, agarose was added to the Tris-HCl buffer

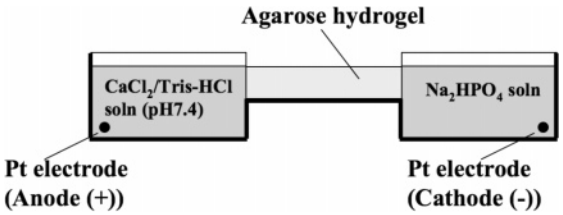


Figure 1. Schematic illustration of the electrophoresis setup for the formation of HAp in agarose hydrogel. Each solution was separated by agarose hydrogel, and Pt electrodes were set in each cell.

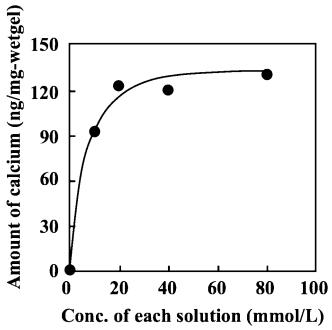


Figure 2. Total amount of calcium content in HAp-formed agarose hydrogel with an equilibrium swelling state.

(10 mmol/L), which was added to the agarose hydrogel as an electrolyte. Furthermore, the concentration was optimized to prevent short-circuit of the electrophoresis process. Typically, the calcium chloride and disodium hydrogen phosphate concentration was adjusted to 40 mmol/L. The time-course of biomineralization was quite unique. No significant optical change was observed after 20 min of electrophoresis. Subsequently, a turbid band appeared at the center of the agarose hydrogel, and the width of the turbidity band spread until a total operation time of 30 min. The linear velocity of each ion was 1 mm/min, which was estimated by electrophoresis of bromophenol blue. Therefore, the velocity of the ions was roughly 15 times higher in comparison with that of the alternate soaking process (0.06 mm/min). If we operate for longer than 30 min, an exotherm is formed due to the resistance of the formed HAp. Thus, the electrophoresis operation must finish as soon as possible (until roughly 30 min). A plausible electrochemical reaction is shown in Scheme 1; calcium, phosphate, and hydroxyl ions were delivered and concentrated in the agarose hydrogel. The ionic mobility is regulated by the following equations through the electrophoresis process:

$$V_{ep} = \mu_e \times E$$

$$\mu_e = q/6\pi\eta r$$

where V_{ep} is the electrophoresis velocity, μ_e is the mobility of the ions, E is the intensity of the electric field, q is the total charge of the ions, η is the density of the media, and r is the Stokes radius of the ion. From this equation, the ionic mobility of calcium and phosphate ions is changeable by the electric field; for example, a 2 × 11 cm HAp composite with 8 mm thickness can be prepared in only 30 min at 100 V. If the biomineralization was carried out at 50 V, the time to complete biomineralization was 60 min. From this, the intensity of the electric field is inversely proportional to the biomineralization. In the case of hydrogels with a 20 mm thickness, 2.7 × 10^3 min (45 h) was necessary in each step for biomineralization by the alternate soaking process.

Why did the biomineralization start in the center region of agarose hydrogel? We checked the hydrogel interior pH using

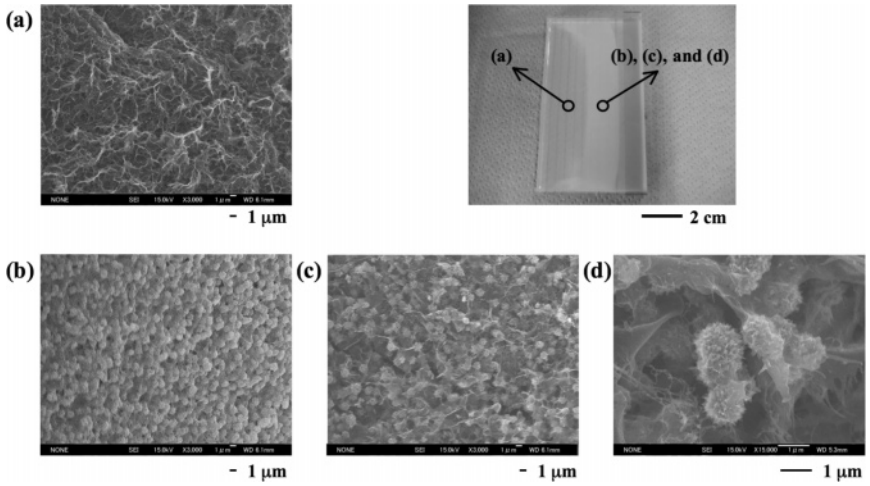


Figure 3. SEM observations of HAp: (a) agarose hydrogel, (b) surface of HAp-formed agarose, (c) interior of HAp-formed agarose, and (d) high magnification.

pH indicators such as methyl orange (red-yellow, pH 3.0−4.4) and phenol red (yellow-red, pH 6.8−8.4). In general, HAp and calcium carbonate were easily dissolved in acidic solution, and never precipitated. In the case of methyl orange, the red color (pH 3.0) turned to yellow (over pH 4.4) from the anode to the cathode side, showing acidic to neutral pH. On the other hand, phenol red made a color change from yellow (below pH 6.8) to red (pH 8.4) from the anode to the cathode side, showing acidic to alkaline pH. This result indicated that a pH gradient was induced in the agarose hydrogel throughout the electrophoresis. Therefore, HAp was formed in the neutral and/or alkaline region in the agarose hydrogel, thus explaining the build-up of a 2 cm width turbid band after the electrophoresis operation.

HAp Formation by a Change in the Preparative Conditions. In the electrophoresis approach, the formation of HAp was dependent on the concentration of calcium and phosphate ions, summarized in Figure 2. The formed HAp in the agarose gel was dissolved using hydrochloric acid, and the soluble calcium ions were evaluated using a calcium detection kit. In the case of 10 mmol/L, the total amount of calcium was roughly 90 ng per 1 mg of HAp-formed agarose hydrogel when the equilibrium swelling state was reached. The swelling ratio of the agarose hydrogel was roughly 32, calculated by the following equation:

$$\text{Swelling ratio} = (W_s - W_d)/W_d$$

where W_s is the weight of the hydrogel at the equilibrium swelling state, and W_d is the hydrogel weight at the dry state. The swollen agarose hydrogel (1 mg) initially contained 31 μg of dry agarose, which contained 90 ng of HAp. From this, the percentage of the formed HAp to dry gel is about 0.3 wt %. The formed HAp increased with increasing concentration of each solution. If the concentration was over 20 mmol/L, the calcium content was almost the same at 130 ng/mg wet gel. In this case, the percentage of formed HAp was 0.42 wt % to dry gel. This result indicates that the HAp formation was dependent on the concentration of each solution because the formed HAp showed resistance in the electrophoresis circuit. Thus, HAp formation was limited to 130ng/mg wet gel.

Characterization of HAp in Agarose Hydrogel. The hydrogel obtained was washed in a large amount of distilled water to remove any surplus ionic solution, and the pieces were lyophilized. Scanning electron microscopy (SEM) was used after the vapor deposition of osmium tetraoxide to observe the interior of the hydrogel. Figure 3 shows the SEM micrographs of HAp. The preparative condition was 40 mmol/L of calcium and phosphate ion solutions, and the electrophoresis was operated for 30 min. The biomineralized agarose hydrogel contained three regions: a transparent anode side, a center turbid part, and a transparent cathode side. In the transparent region, only the agarose polymer network was observed, and no HAp (Figure 3a). In the anode region, an acidic pH was observed using the pH indicator, thus HAp did not exist in the agarose hydrogel. On the other hand, the HAp in the center turbid region was well observed, as shown in Figure 3b−d. On the surface, numerous monodisperse HAp particles were observed, with an average diameter of roughly 1 μm (Figure 3b). A cross-sectional view of the HAp-formed agarose hydrogel (Figure 3c) revealed that many particles were entangled with the agarose network. The morphology was also similar between the surface and the cross-sectional region, indicative of a homogeneous biomineralization process, even though a pH gradient was observed. However, the pH gradient was not observed in the turbid region (2 cm), showing similar color by pH indicator. Zooming in on the particles, a fine morphology was observed (Figure 3d) as the surface was covered with needle-shaped crystals, much like a sea urchin and/or spherical moss. Ma et al. reported HAp crystalline particles with a similar morphology, which were prepared in a poly(lactic acid) porous matrix by an SBF process.[26−27]

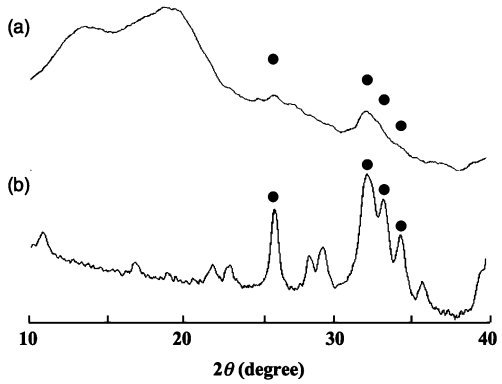


Figure 4. XRD patterns of (a) HAp-formed agarose hydrogel and (b) high crystalline HAp.

Figure 4 shows the XRD patterns of the crystals. The obtained patterns were broad because of the lower content of HAp crystals in the agarose hydrogel. However, typical peaks were observed

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at 25.8, 31.7, 32.9, and 34.1° by peak search treatment, attributed to an HAp crystal structure (002), (211), (300), and (202) (Figure 4a). In the case of pure HAp, four characteristic peaks were observed (Figure 4b). Taguchi et al. also reported XRD patterns of formed HAp polyelectrolyte adsorption using a poly(vinyl alcohol) hydrogel.[17,19] The total amount of HAp was 150−460 times higher than that of the electrophoresis approach. Thus, the typical XRD patterns were also observed at 25.9, 31.8, 32.2, and 32.9°, which were assigned to the main peaks of the HAp crystals.[28] The peak intensity increased with a number of adsorption processes. Therefore, the typical XRD patterns were dependent on the total amount of formed HAp. Although the obtained XRD pattern was relatively broad, the typical XRD patterns indicate that HAp was formed inside the agarose hydrogel.

Conclusions

We have developed a novel HAp formation technique that employs an agarose hydrogel and electrophoresis. HAp was formed homogeneously in the agarose hydrogel within 30 min for dimensions as large as a 2 × 11 cm width with an 8 mm thickness. The obtained hybrid material contained HAp crystals with a spherical morphology and needle-shaped crystals on/in the agarose hydrogel. Furthermore, calcium carbonate was also formed in the hydrogel by the electrophoresis approach using calcium chloride and sodium carbonate solutions. The biomineralization process using electrophoresis is a more efficient route to synthetically derived HAp for biomaterials application.

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