ABSTRACT: Diffusion-controlled crystallization in a hydrogel has been investigated to synthesize organic/inorganic hybrid composites and obtain a fundamental understanding of the detailed mechanism of biomineralization. Although calcium phosphate/hydrogel composites have been intensively studied and developed for the application of bone substitutes, the synthesis of homogeneous and integrated composites remains challenging. In this work, diffusion-controlled systems were optimized by manipulating the calcium ion flux at the interface, concentration gradient, and diffusion coefficient to synthesize homogeneous octacalcium phosphate/hydrogel composites with respect to the crystal morphology and density. The ion flux and local pH play an important role in determining the morphology, density, and phase of the crystals. This study suggests a model system that can reveal the relation between local conditions and the resulting crystal phase in diffusion-limited systems and provides a synthetic method for homogeneously organized organic/inorganic composites.

INTRODUCTION

Bone has unique structural and functional properties that originate from the synergistic combination of organic and inorganic materials. An integrated structure of the collagen fibrillary network and carbonated hydroxyapatite (cHAp) improves the stiffness and fracture resistance of bone. Complex hierarchical structures consisting of organic and inorganic parts result from deliberately controlled biomineralization, where calcium phosphate (CaP) is orderly crystallized in the collagen networks. In these contexts, synthetic biomaterials that mimic natural organic/inorganic composites have been extensively investigated to regenerate and substitute damaged bone structures.

Hydrogels have drawn widespread interest as organic scaffolds for synthesizing bone-mimicking composites because of their structural similarity with the extracellular matrix (ECM) in biogenic bones. Moreover, hydrogels have excellent biocompatibility, biodegradability, and availability in cell incorporation and proliferation. To mimic the structural and functional properties of natural bones, the intimate integration of a hydrogel and CaP is the most important issue. Three types of approaches have been studied to synthesize bone-mimicking composites with hydrogels. First, the simplest approach is mixing CaP particles with a pregel solution and subsequently gelating the mixture. Although a preferable amount of CaP particles can be directly incorporated into the composite, the resulting composite has poor integration between CaP particles and organic networks because CaP particles are physically trapped in the networks without chemical interactions. In fact, in vivo mineralization starts from constituent ions to complete bone crystals through the transient amorphous phase. The intimate interaction between amorphous precursors and organic templates forms the ordered orientation of bone nanocrystals, which results in a unique hierarchical structure. To synthesize more bone-like structures, it is better to start with synthesizing constituent ions instead of presynthesized particles. Second, a more biomimetic approach is simultaneous mineralization and gelation, which is conducted by mixing calcium and phosphate ions with the pregel solution and subsequently transforming the mixture into mature crystals by constructing a three-dimensional hydrogel. In situ mineralization and gelation are beneficial to prepare homogeneous bone-like composites because they are closer to the bone formation mechanism. Third, another biomimetic approach is diffusion-controlled
mineralization in hydrogels. In contrast to simultaneous mineralization and gelation, diffusion-controlled mineralization is performed by diffusing constituent ions into the hydrogel matrix. By immersing the matrix in the ionic solution, ions diffuse into the matrix, and mineralization occurs in the hydrogel containing counter ions. The approach effectively imitates a slow diffusion process in biological systems, where the mass transfer is slowly controlled by the cellular walls, membranes, or extracellular matrix. However, it is difficult to uniformly control the ion diffusion within the hydrogel matrix because the amount of diffusion or net ion flux directly depends on the length of the diffusion path. Consequently, the resulting composites are limited to two-dimensional films or have a gradient of crystal density along the diffusion direction.

To overcome these defects, more homogeneous composites have been synthesized by applying an external electric field, but the method requires a complicated instrument.

Bone regeneration using CaP composites depends on a crystal phase of incorporated CaP and the integration of organic and inorganic parts. Hydroxyapatite (HAp), β-tricalcium phosphate (β-TCP), octacalcium phosphate (OCP), and amorphous calcium phosphate (ACP) are commonly used for bone regeneration. HAp has gained much interest because of its structural similarity with bone apatite and its simple preparation method using a sintering process. However, implanted HAp is difficult to be resorbed and replaced by newly formed bone because of its thermodynamic stability. To improve the resorption of HAp, more soluble β-TCP, which is readily prepared by sintering, has been used together. In contrast, OCP and ACP can be easily resorbed for bone regeneration, although they must be prepared by a sophisticated wet process because of their thermal instability. Because they are considered an intermediate or a precursor of bone apatite, the regeneration process can be similar to biogenic bone formation. The existence of ACP in the initial bone formation process was supported by a peak at 1054 cm$^{-1}$ originating from the microstructures and crystal phases. The microstructures of DCPD and OCP. At higher

**RESULTS**

When Ca$^{2+}$ diffused into agarose hydrogels containing HPO$_4^{-}$, CaP crystals were gradually formed in the hydrogel. CaP/hydrogel composites were formed along the Ca$^{2+}$ diffusion pathway, and the composites were synthesized in 1–2 cm after 24 h (Figure 1a). Four different systems were tested to generate a homogeneous CaP/hydrogel composite by varying the Ca$^{2+}$ or HPO$_4^{-}$ concentration and the states (free solution or hydrogel medium) of the outer reservoirs. In all samples, the formed composites were opaque than the native hydrogel (Figure 1b). The distance of precipitation was measured as a function of time (Figure 1c). The precipitation gradually formed by up to ∼10 mm for 24 h, but the distance of Gel$_{0.5}$/Gel$_{0.3}$ was ∼5 mm because of slow diffusion from a gelated reservoir. Although each composite appeared homogeneous in a macroscopic view, microscopic and crystalline structures could be different along the diffusion direction because crystallization depends on the diffusion of Ca$^{2+}$. The composites were investigated along the Ca$^{2+}$ diffusion direction by focusing on the microstructures and crystal phases.

**Sol$_{0.5}$/Gel$_{0.3}$ Composite.** The Sol$_{0.5}$/Gel$_{0.3}$ composite appears homogeneous, but there are tiny transparent flakes at the interface of the solution and hydrogel (Figure 1a). Based on the SEM analysis, the composite can be divided into three typical regions according to the shapes and sizes of crystals, although the microstructures gradually changed along the diffusion direction. In region i, “plate-like” crystals, whose average length, width, and thickness were ∼40, ∼10, and ∼1 μm, respectively, were dominantly formed (Figure 2a). In region ii, radially aggregated “flower-like” crystals 10–15 μm in diameter were observed with plate-like crystals in region i (Figure 2b). However, the length, width, and thickness of the plate-like crystals decreased by ∼10, ∼4, and ∼1 μm on average, respectively. In region iii, only flower-like crystals were formed without plate-like crystals (Figure 2c). Thus, the microstructures gradually changed from plate-like to flower-like along the diffusion direction, with an intermediate region composed of both morphologies. The crystal phases of the respective regions were characterized by XRD (Figure 2d). In region i mainly consisted of DCPD with a small amount of OCP, which was confirmed by the relative intensity of XRD peaks. In region ii, the intensity of the DCPD peak decreased, but that of the OCP peaks obviously increased. Region iii was composed of only OCP without DCPD. In the FTIR spectra, peaks at 960 and 1020 cm$^{-1}$ were observed in the overall regions, which were assigned as $\nu_1$ and $\nu_2$ vibration modes of PO$_4^{3-}$ in OCP (Figure 2e). In region i, the presence of DCPD was supported by a peak at 1054 cm$^{-1}$ originating from HPO$_4^{2-}$ in DCPD, but the intensity was much lower than expected. Although the presence of DCPD in region ii was confirmed by XRD, a characteristic DCPD peak was not observed by FTIR, probably because of the low intensity of the peak at 1054 cm$^{-1}$. By integrating the SEM results with XRD and FTIR, the crystal phases of plate-like and flower-like structures are DCPD and OCP, respectively. Their morphologies are consistent with those observed in previous reports on the microstructures of DCPD and OCP. At a higher
concentration such as Sol\(^{0.083}/\)Gel\(^{0.05}\), agarose has not been gelated and stayed in a solution state.

In the Sol\(^{0.5}/\)Gel\(^{0.3}\) composite, a larger amount of DCPD was formed at the interface near the Ca\(^{2+}\) reservoir. The heterogeneity of crystal phases along the diffusion direction can be explained by Fick’s first law: Flux = \(-D\frac{dc}{dx}\).\(^{30}\) The flux is the amount of Ca\(^{2+}\) per unit area and unit time, \(c\) is the concentration, \(D\) is the diffusion coefficient of Ca\(^{2+}\), and \(x\) is the distance from the interface of the Ca\(^{2+}\) reservoir. The Ca\(^{2+}\) flux can directly influence the supersaturation (\(\sigma\)) of the reaction solution in the hydrogel because supersaturation is correlated with the concentration of constituent ions. The supersaturation (\(\sigma\)) is described as

\[
\ln \frac{IAP}{K_{sp}}
\]

where IAP is the ion activity product and \(K_{sp}\) is the solubility product. Changes in the Ca\(^{2+}\) flux can alter the pathway for CaP crystallization, which can be inferred from classical nucleation theory. According to classical nucleation theory, the rate of nucleation is a function of supersaturation (\(\gamma\) is the surface tension, \(\nu\) is the molecular volume).\(^{31}\)

\[
\frac{dN}{dt} = A \exp\left(-\frac{16\pi\gamma\nu^{2}}{3k_{B}T^{3}\sigma^{2}}\right)
\]

Variation in the kinetics of CaP crystallization can lead to different crystal phases and morphologies. The region closer to the interface of the Ca\(^{2+}\) reservoir encounters a higher Ca\(^{2+}\) flux because the flux is inversely proportional to the diffusion distance according to Fick’s law.\(^{38}\) In our diffusion system, a high Ca\(^{2+}\) flux induced the crystallization of DCPD, while a low Ca\(^{2+}\) flux induced the crystallization of OCP. From the results, we speculate that it is beneficial to maintain the homogeneity of the Ca\(^{2+}\) flux to generate homogeneous CaP/hydrogel composites. In addition, it is necessary to maintain a sufficiently low Ca\(^{2+}\) flux along the diffusion direction to dominantly generate OCP instead of DCPD.

**Sol\(^{0.083}/\)Gel\(^{0.05}\) Composite.** To maintain a lower Ca\(^{2+}\) flux, Sol\(^{0.083}/\)Gel\(^{0.05}\) was prepared by reducing the concentrations of both the Ca\(^{2+}\) solution and the HPO\(_{4}\)^{2-} hydrogel. By decreasing the Ca\(^{2+}\) concentration, we decreased its flux according to its linear dependence on the concentration gradient. In the macroscopic observation, the Sol\(^{0.083}/\)Gel\(^{0.05}\) composite was less opaque than the Sol\(^{0.5}/\)Gel\(^{0.3}\) composite (Figure 1b). In addition, the composite had mostly uniform opacity except for the end of the bottom region. For a more detailed analysis, the microstructures of the composite were observed by SEM. Approximately two-third of the upper composite was covered by randomly distributed ribbon-shaped crystals in regions i and ii (Figure 3a,b). However, the crystals were less noticeable than those in Sol\(^{0.5}/\)Gel\(^{0.3}\), presumably because of their smaller size and lower density. During freeze-drying, the hydrogel readily collapsed because of dehydration when there were fewer crystals to support the hydrogel networks. In contrast to the Sol\(^{0.5}/\)Gel\(^{0.3}\) composite, only silhouettes of crystals were observed in the Sol\(^{0.083}/\)Gel\(^{0.05}\) composite. The lower one-third of the composite contained much fewer and smaller crystals than the other regions. Granules tens of nanometers in size were observed in region iii (Figure 3c). With XRD crystallographic analysis, the upper two-third of the composite could be further divided into regions i and ii because of their different crystal phases. Region i contained OCP and a small amount of HAp, while regions ii and iii were composed of only OCP (Figure 3d). Therefore, the Sol\(^{0.083}/\)Gel\(^{0.05}\) composite could be divided into three regions. The relatively broader peak of region iii confirmed that there were fewer crystals in that region. Moreover, the low intensity of XRD peaks from the Sol\(^{0.083}/\)Gel\(^{0.05}\) composite compared to those from the Sol\(^{0.5}/\)Gel\(^{0.3}\) composite could be explained by the decreased amount of crystals, which is consistent with the macro- and microscopic observations. The overall lower crystal density in the Sol\(^{0.083}/\)Gel\(^{0.05}\) composite resulted from a reduced concentration of constituent ions. At
the lower concentration such as Sol0.0167/Gel0.01, the amount of precipitation was considerably reduced. In Sol0.0083/Gel0.005, a precipitate was thinly formed only at the interface because the flux is not enough to supply ions further. The FTIR analysis confirmed the presence of OCP in every region, although HAp was not detected. The formation of DCPD was prevented by decreasing the concentration of the Ca²⁺ reservoir. However, the composite remained inhomogeneous mainly because of the sluggish ion transport. Especially in region iii, granular particles were formed instead of ribbon-shaped crystals because the Ca²⁺ flux was highly reduced in the region far from the Ca²⁺ reservoir. Therefore, to synthesize homogeneous composites, Ca²⁺ should be slowly but not deficiently transported. The reduced concentration of constituent ions inevitably resulted in the shortage of Ca²⁺ transport in the region distant from the Ca²⁺ reservoir (region iii). To make Ca²⁺ diffusion slow and sufficient, we manipulated the diffusion system to enhance the Ca²⁺ transport without reducing the ion concentration of the reservoir and the hydrogel.

**Gel0.5/Gel0.3 Composite.** To maintain the slow but sufficient supply of Ca²⁺, the Ca²⁺ reservoir changed to hydrogel media from a free solution, and the Ca²⁺ concentration was fixed to 0.5 M instead of 0.083 M. In addition, the HPO₄²⁻ concentration in the hydrogel was fixed to 0.3 M instead of 0.05 M to enhance the crystallization in the region far from the Ca²⁺ reservoir, which resulted in the Gel0.5/Gel0.3 system. Although the Ca²⁺ concentration gradients are identical in Sol0.5/Gel0.3 and Gel0.5/Gel0.3 because of the identical initial Ca²⁺ concentration, the diffusion coefficient of outer ions was manipulated in the Gel0.5/Gel0.3 system without changing their concentration gradient. On the macroscopic scale, the formed composite was more uniform and opaque than the other composites (Figure 1a). On the microscopic scale, the composite can be divided into two regions near the middle of the composite (Figure 4). In region I, most crystals had flower-like shapes ~17 μm in diameter, but crystals of plate-like shapes were also observed (Figure 4a). The crystals in region II had a similar morphology to those in region I, but plate-like crystals were not observed, and the petals of the flowers became sharper (Figure 4b). In contrast to the Sol0.083/Gel0.05 system.

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**Figure 2.** (a–c) SEM micrographs of CaP crystals formed in regions i, ii, and iii of the Sol0.5/Gel0.3 composite. (d) XRD spectra and (e) FTIR of the Sol0.5/Gel0.3 composite.
composite, the Gel0.5/Gel0.3 composite contained a larger amount of crystals in all places. Intense XRD and FTIR peaks support that more crystals were formed in the Gel0.5/Gel0.3 composite than in the Sol0.5/Gel0.3 composite. XRD analyses confirm the presence of DCPD in region I, but the low intensity of the DCPD peak indicates that DCPD was not a major phase (Figure 4c). The FTIR spectra also confirm that the major phase of the composite was OCP (Figure 4d).

**Sol0.5/Gel/Gel0.3 Composite.** The Gel0.5/Gel0.3 composite had desirable homogeneity except for a small amount of DCPD. Because DCPD is formed when the Ca2+ flux is relatively high, the Ca2+ flux should be further slowly controlled, especially at the interface between the reservoir and the hydrogel, to synthesize a more homogeneous composite. To prevent a prompt supply of Ca2+ from the reservoir to the phosphate-containing hydrogel, an ion-free hydrogel layer was inserted between the 0.5 M Ca2+ solution and the 0.3 M HPO42− hydrogel. Sol0.5/Gel/Gel0.3 had the identical initial concentration of constituent ions to the Sol0.5/Gel0.3 system regardless of the inserted layer, although the state of the medium containing outer ions changed. It took approximately 30 min for Ca2+ to pass through the layer, and subsequent crystallization occurred in the HPO42− hydrogel. Crystallization also occurred in the inserted layer because of the diffusion of HPO42− from the inner hydrogel. Because the mobility of Ca2+ is highly limited while penetrating the layer, both the initial Ca2+ concentration and the Ca2+ flux decrease at the interface of the HPO42− hydrogel. The crystallization of DCPD could be prevented by the decreased factors while maintaining a sufficient flux at the distant region. The homogeneity of the Sol0.5/Gel/Gel0.3 composite was investigated by SEM. Only flower-like crystals 10−15 μm in diameter were observed in the entire composites, although the petals were sharper along the diffusion direction (Figure 5a,b). The DCPD formation was efficiently prevented by moderately decreasing the Ca2+ flux at the interface of the HPO42− hydrogel. The XRD and FTIR analyses confirmed that the Sol0.5/Gel/Gel0.3 composite consisted of only OCP (Figure 5c,d). As a result, the composite was homogeneous with respect to the overall morphology, distribution, and crystal phase except for a small deviation in the petal length.

![Figure 3. (a–c) SEM micrographs of CaP crystals formed in regions i, ii, and iii of the Sol0.083/Gel0.05 composite. (d) XRD spectra and (e) FTIR of the Sol0.083/Gel0.05 composite.](https://pubs.acs.org/journal/acsodf)
Figure 4. (a, b) SEM micrographs of CaP crystals formed in regions I and II of the Gel$_{0.5}$/Gel$_{0.3}$ composite. (c) XRD spectra and (d) FTIR of the Gel$_{0.5}$/Gel$_{0.3}$ composite (region I: upper half of the composite; region II: lower half of the composite).

Figure 5. (a, b) SEM micrographs of CaP crystals formed in regions I and II of the Sol$_{0.5}$/Gel$_{0.3}$ composite. (c) XRD spectra and (d) FTIR spectra of the Sol$_{0.5}$/Gel$_{0.3}$ composite (region I: upper half of the composite; region II: lower half of the composite).
In our system, the Ca\textsuperscript{2+} flux near the interface of each composite can be inferred by the time-dependent pH of the Ca\textsuperscript{2+} reservoir because protons are released during the CaP crystallization.\textsuperscript{43} When Ca\textsuperscript{2+} diffused from the reservoir, CaP crystallization occurred with the drop in pH in the hydrogel. The released H\textsuperscript{+} from crystallization successively migrated out of the hydrogel, and [H\textsuperscript{+}] in the Ca\textsuperscript{2+} reservoir continued to increase as a function of time (Figure 6b). The steep [H\textsuperscript{+}] gradients will be measured if the flux is high. The [H\textsuperscript{+}] gradients of Sol\textsuperscript{0.05}/Gel\textsuperscript{0.3} and Gel\textsuperscript{0.05}/Gel\textsuperscript{0.3} were relatively higher than that of Sol\textsuperscript{0.083}/Gel\textsuperscript{0.05}. Sol\textsuperscript{0.083}/Gel\textsuperscript{0.05} had extremely low [H\textsuperscript{+}] gradients. The gradients indicate that the crystallization of CaP rapidly proceeds because of the high Ca\textsuperscript{2+} flux in Sol\textsuperscript{0.05}/Gel\textsuperscript{0.3} and Gel\textsuperscript{0.05}/Gel\textsuperscript{0.3}, where the DCPD phase was formed at the top region. In contrast, the Ca\textsuperscript{2+} flux was relatively low in Sol\textsuperscript{0.083}/Gel\textsuperscript{0.05} and Sol\textsuperscript{0.083}/Gel\textsuperscript{0.05}, where the OCP phase was mainly observed at the top region. These results support that the final crystal phase strongly depends on the pH variation caused by the Ca\textsuperscript{2+} flux in the early stage of crystallization.

**DISCUSSION**

It is widely known that ACP acts as a transient precursor for CaP crystals in previous reports.\textsuperscript{19–21,31,33} CaP is crystallized by the aggregation of ACP particles, which further mature into more stable crystal phases such as DCPD, OCP, or HAp. Because the Ca-to-P atomic ratio varies with the CaP crystal phases, the atomic ratio in ACP is significant for determining the final phases.\textsuperscript{44} Despite its importance in mineralization, the exact chemical composition of ACP remains under debate. It has been suggested that ACP formation occurs due to the aggregation of spherical particles, named Posner’s clusters (Ca\textsubscript{3}(PO\textsubscript{4})\textsubscript{2}), which consist of only Ca\textsuperscript{2+} and PO\textsubscript{4}\textsuperscript{3–} ions.\textsuperscript{45} However, there is growing evidence that protonated phosphate ions (HPO\textsubscript{4}\textsuperscript{2–}) are included in an amorphous precursor of apatite in biogenic systems such as the mammalian skeleton and zebranohin rays.\textsuperscript{46} Gebauer et al. successfully synthesized two types of ACP phases in different chemical compositions by varying the experimental conditions such as pH.\textsuperscript{47} They found that acidic ACP (identified as CaHPO\textsubscript{4}·nH\textsubscript{2}O) formed at near neutral or slightly basic pH, while the classical Posner’s clusters (Ca\textsubscript{3}(PO\textsubscript{4})\textsubscript{2}·nH\textsubscript{2}O) named basic ACP formed at highly basic pH. Further analysis using \textsuperscript{31}P magic angle spinning (MAS) spectroscopy revealed that the chemical environment of the P atoms in acidic ACP was similar to that in DCPD. Structural similarities between the basic ACP and HAp were also reported.\textsuperscript{47} Furthermore, Renard et al. investigated the nucleation of DCPD and HAp in real-time using in situ Raman spectroscopy.\textsuperscript{44} The crystallization of CaP was controlled by the HPO\textsubscript{4}\textsuperscript{2–}·to-PO\textsubscript{4}\textsuperscript{3–} ratio, which was adjusted by the pH of the phosphate solution in three different ranges (from 9.8 to 6.8, pH > 12.2, from 12.4 to 11). The pathway for either DCPD or OCP/HAp varied with the type of ACP precursor determined by the environmental pH in the early crystallization step. Based on these reports, it is reasonable to propose that a decisive factor for determining the CaP crystal phase is not a final pH but a local pH at the initial process. Therefore, it is necessary to control the Ca\textsuperscript{2+} flux that affects the local pH variation at the reaction front to synthesize a homogeneous CaP/hydrogel composite.

The inhomogeneity of the Sol\textsuperscript{0.05}/Gel\textsuperscript{0.3} composite can be similarly explained by a mechanism suggested by Renard et al.\textsuperscript{44}
Ca\(^{2+}\) + HPO\(_4^{2-}\) + nH\(_2\)O → CaHPO\(_4\)·nH\(_2\)O  \hspace{1cm} (1)

8CaHPO\(_4\)·nH\(_2\)O + 5H\(_2\)O → Ca\(_6\)(HPO\(_4\))\(_4\)(PO\(_4\))\(_4\)·5H\(_2\)O + 2HPO\(_4^{2-}\) + 4H\(^+\) + 8nH\(_2\)O  \hspace{1cm} (2)

Ca\(_6\)(HPO\(_4\))\(_4\)(PO\(_4\))\(_4\)·5H\(_2\)O + 2HPO\(_4^{2-}\) + 4H\(^+\) + 11H\(_2\)O → 8CaHPO\(_4\)·2H\(_2\)O  \hspace{1cm} (3)

An acidic ACP is formed by mixing Ca\(^{2+}\) with HPO\(_4^{2-}\) in the pH range from 9.8 to 6.8 (eq 1). As crystallization proceeds, the acidic precursors transform into DCPD by dissolution and recrystallization processes (eq 3). The DCPD formation is driven by the released H\(^+\) in the course of OCP formation because DCPD is a preferred phase under acidic conditions. Considering the slightly alkaline condition of our hydrogel system (pH 8.89), acidic ACP will be dominantly formed at the interface or the top region in the initial crystallization stage regardless of the experimental settings. H\(^+\) is subsequently released when the ACP transforms into OCP, which results in a less acidic environment, which is unfavorable for DCPD formation.

In a low-flux system (Sol\(^{0.083}/\text{Gel}^{0.05}\)), a small amount of HAp formed in region i. HAp is formed through a basic ACP-mediated process.\(^{44,47}\)

3Ca\(^{2+}\) + 2HPO\(_4^{2-}\) + nH\(_2\)O → Ca\(_3\)(PO\(_4\))\(_2\)·nH\(_2\)O + 2H\(^+\)  \hspace{1cm} (4)

3Ca\(_3\)(PO\(_4\))\(_2\)·nH\(_2\)O + Ca\(^{2+}\) + 2OH\(^-\) → Ca\(_{10}\)(PO\(_4\))\(_6\)·(OH)\(_2\) + 3nH\(_2\)O  \hspace{1cm} (5)

The basic ACP formed, and H\(^+\) was simultaneously released (eq 4). The ACPs further aggregated and transformed into HAp (eq 5). In the previous reports, the in vitro formation of basic ACP requires highly alkaline conditions (pH > 12.2).\(^{44,47}\) Considering the pH of our system (8.89), it is reasonable that OCP was formed through the acidic ACP-mediated pathway.
A tiny amount of H+ may have been slowly released during crystallization because of the lowered Ca2+ flux. If the released H+ was rapidly neutralized by the alkaline medium, the local pH could remain suitable for the formation of basic ACP at the early crystallization stage. This hypothesis is supported by a previous report; an alkaline Ca(OH)2 solution (pH > 12.2) was used to synthesize transient basic ACP and HAp to neutralize the H+ released by the CaP formation. Although neutralization assisted by alkaline media is a feasible scenario in our system, it is still questionable because of the huge pH gap between previous reports and our experimental conditions. Considering that a tiny amount of HAp was formed only at the top region, limited diffusion could explain the unexpected formation of HAp. In contrast to other reports conducted in free media, the mobility of ions is highly restricted in hydrogels, which retards the overall process. Although H+ can be locally accumulated at the first moment, an alkaline medium has a sufficient buffer capacity to neutralize the small amount of H+. The rapid elimination of H+ turns on the basic ACP-mediated process (eq 4). However, the pathway cannot last sufficiently long to produce a considerable amount of HAp because the transformation from basic ACP to HAp requires additional OH− (eq 5). During the transformation, H+ continuously accumulates and subsequently changes the dominant pathway to an acidic ACP-mediated process. Because both Ca2+ ions and accumulated H+ further diffuse, the main mechanism is acidic ACP-mediated processes in the middle and bottom regions despite the lowered Ca2+ flux. Although the final pH condition of the top region in Sol0.083/Gel0.05 becomes slightly acidic (pH ~ 5.1), HAp can remain for 24 h because of its thermodynamic stability.19 Regardless of the interesting formation of HAp, the main problem of this composite is the low and inhomogeneous density of OCPs.

Based on the CaP formation mechanisms, the system was technically manipulated to overcome the inhomogeneity and low density of CaP in the composite. The situation is starkly contradictory; the high flux intensifies the inhomogeneity of the crystal phase, but the low flux reduces the crystal density. Under the high Ca2+ flux (Figure 8a), a large amount of acidic ACP is formed and transforms into OCP with rapid deprotonation, which induces an acidic environment at the reaction front. The acidic environment enhances the transformation of OCP into DCPD. In the case of a low Ca2+ flux, crystal inhomogeneity can also be observed, and a relatively alkaline environment can be maintained to form the basic ACP during early crystallization, which results in the HAp formation (Figure 8b). Accumulated H+ subsequently acidifies the reaction front and facilitates OCP formation through the acidic ACP-mediated process. Moreover, only small amounts of crystals are produced because of lowered concentrations of both Ca2+ and HPO42−.

When the Ca2+ flux was technically controlled lower in high-concentration systems such as Gel0.5/Gel0.3 and Sol0.5/Gel/Gel0.3, H+ was moderately released during the OCP crystallization (eq 2). It was beneficial to restrict the transformation of OCP into DCPD (eq 3). Consequently, OCP was mainly formed under a controlled Ca2+ flux (Figure 8c). In the distant region, the flux was gradually reduced but remained sufficiently high to induce a considerable amount of crystals. As a result, only OCP formed in the entire region of the composites. Although DCPD is thermodynamically preferred at the final pH (<5.0), OCP remained as a final phase, presumably because OCP is kinetically favorable under the controlled Ca2+ flux for 24 h.

The main finding of this work is that homogeneous OCP composites can be synthesized on centimeter scales by controlling the Ca2+ flux. A highly regulated Ca2+ flux is required to produce homogeneous OCPs because the CaP

Figure 8. Scheme of crystallization pathways near the Ca2+ reservoir under (a) high, (b) low, and (c) moderate Ca2+ flux.
crystallization simultaneously induces the local pH variation, which is a critical factor in determining the final crystal phase (Figure 9). Under an excessive Ca\(^{2+}\) flux (Figure 9, Sol\(^{0.5}/\)Gel\(^{0.3}\)), DCPD was formed at the interface because a large amount of H\(^+\) was suddenly released by rapid crystallization. In addition, a crystal phase gradually changed to OCP with a decreased flux along the diffusion direction. To control the Ca\(^{2+}\) flux at the interface, we manipulated two major parameters in various systems: the concentration gradient and the diffusion coefficient based on Fick’s law. To obtain a low Ca\(^{2+}\) flux by reducing the concentration gradient of Ca\(^{2+}\) at the interface, the concentration of a Ca\(^{2+}\) solution was reduced (Figure 9, Sol\(^{0.083}/\)Gel\(^{0.05}\)). Although this composite contained OCP in the entire region, one-third of the region had a barren structure, and the amount of the precipitate was too small. Furthermore, a tiny amount of HAp precipitated near the interface because alkaline conditions were maintained in the early crystallization step because the reduced supply of Ca\(^{2+}\) caused slow deprotonation. A lower diffusion coefficient of Ca\(^{2+}\) at the interface, we manipulated two major parameters in various systems: the concentration gradient and the diffusion coefficient based on Fick’s law. To obtain a low Ca\(^{2+}\) flux by reducing the concentration gradient of Ca\(^{2+}\) at the interface, the concentration of a Ca\(^{2+}\) solution was reduced (Figure 9, Sol\(^{0.083}/\)Gel\(^{0.05}\)). Although this composite contained OCP in the entire region, one-third of the region had a barren structure, and the amount of the precipitate was too small. Furthermore, a tiny amount of HAp precipitated near the interface because alkaline conditions were maintained in the early crystallization step because the reduced supply of Ca\(^{2+}\) caused slow deprotonation. A lower diffusion coefficient of Ca\(^{2+}\) at the interface can be achieved by substituting the Ca\(^{2+}\) hydrogel for the Ca\(^{2+}\) solution (Figure 9, Gel\(^{0.5}/\)Gel\(^{0.3}\)). However, the Gel\(^{0.5}/\)Gel\(^{0.3}\) composite had a slightly higher Ca\(^{2+}\) flux than needed, which results in coprecipitation of a small amount of DCPD at the top region. We finally found the optimal Ca\(^{2+}\) flux, which led to a homogeneous crystal morphology and phase in the entire region by decreasing both the concentration and the diffusion coefficient of Ca\(^{2+}\) at the interface by inserting an ion-free hydrogel layer between the Ca\(^{2+}\) solution and the hydrogel (Figure 9, Sol\(^{0.5}/\)Gel/ Gel\(^{0.3}\)). The mobility of Ca\(^{2+}\) ions can be moderately controlled in the inserted gel by interfering with the diffusion of ions in Sol\(^{0.5}/\)Gel/Gel\(^{0.3}\). Under the controlled Ca\(^{2+}\) flux, homogeneous OCP/hydrogel composites were successfully synthesized with respect to the crystal distribution and crystal morphology.

### CONCLUSIONS

OCP/hydrogel composites with homogeneous distribution and morphology were synthesized in the entire region of the hydrogel by manipulating the Ca\(^{2+}\) flux. Based on Fick’s law, the Ca\(^{2+}\) flux can be systematically controlled by varying two variables: the concentration gradient and the diffusion coefficient. It was impossible to obtain homogeneous and highly crystallized OCP/hydrogel composites by simply decreasing the Ca\(^{2+}\) concentration in the reservoir to reduce the concentration gradient or by replacing the Ca\(^{2+}\) reservoir solution with the Ca\(^{2+}\) hydrogel to decrease the diffusion coefficient. By inserting an ion-free hydrogel between the Ca\(^{2+}\) solution and the HPO\(_4^{2-}\) hydrogel, the Ca\(^{2+}\) flux was optimized to synthesize homogeneous OCP/hydrogel composites. In other words, the accumulated Ca\(^{2+}\) flux, which is directly related to the final density of crystals, should be controlled homogeneously across the entire composite region. If the Ca\(^{2+}\) flux is too high or low at certain instances, the accumulated Ca\(^{2+}\) flux cannot be uniform. Therefore, the Ca\(^{2+}\) flux should be moderately controlled by manipulating a
diffusion system. When CaP is crystallized by the Ca\(^{2+}\) delivery, the surrounding pH varies with the H\(^+\) released from hydrogen phosphate ions, which can be the main factor in determining the final CaP phase. Thus, the controlled Ca\(^{2+}\) flux at the interface is critically responsible for the formation of uniform composites. To control the crystallization precisely, it is necessary to quantitatively reveal the flux by measuring or calculating concentrations of constituent ions and intermediates as a function of time. Considering a technical difficulty in measuring concentrations of ions and intermediates in a hydrogel, it is more feasible to calculate them based on simulation, as we reported in recent work.\(^{48}\) Although it still remains a challenging issue, the quantitative analysis of crystallization in a hydrogel will be useful for fundamentally understanding the slow diffusion systems as well as precisely synthesizing organic–inorganic composites.

Furthermore, this synthetic strategy using a regulated Ca\(^{2+}\) flux can be an alternative method to produce homogeneous OCP/hydrogel composites, which produce integrated bone-like composites for biomedical applications. Since controlled diffusion is a fundamental principle of the biomineralization process, an elaborate understanding and control of diffusion will yield a more desirable solution to bone defects. A three-dimensionally organized organic/inorganic composite can be used as a therapeutic material for the effective regeneration of large bone defects.\(^{49}\) With respect to the small bone flaws, complete recovery could be accomplished by a nonsurgical operation. The hydrogel scaffold containing osteogenic stem cells and growth factors could be injected into defect sites by minimally invasive manners, which has gained growing interest recently.\(^{50}\) In contrast with small flaws, large ones are difficult to heal completely by authorizing osteogenetic cells to make perfect bone without any structural basis. Biological requirements are insufficient to fill the large volume of defects, therefore, the structural basis is required for large defect regeneration. Until now, autologous bone grafting has been considered as a standard therapy despite several drawbacks such as donor site morbidity, infection, pain, and volume restriction limit.\(^{51,52}\) To treat large defects more safely, it is necessary to quantitatively reveal the crystal phase of the composites was analyzed with a high-resolution X-ray diffractometer (XRD, X'Pert PRO MRD, Cu Ka = 1.54 Å, PAN Analytical, Netherlands). For the XRD analysis, the crystal was prepared by dissolving agarose with hot water and subsequently purifying with centrifugation. The pH was measured from three independent samples by a pH meter (SevenCompact pH/Ionometers S220, Mettler Toledo, Switzerland) with an electrode of a 3 mm diameter (InLab Ultra-Micro, Mettler Toledo, Switzerland).

**EXPERIMENTAL SECTION**

**Materials.** CaCl\(_2\)-2H\(_2\)O (≥99.0% purity) and Na\(_2\)HPO\(_4\) (≥99.0% purity) were purchased from Sigma-Aldrich, and agarose (guaranteed grade) was provided by Becton-Dickinson. Purified water with a resistivity higher than 18 M\(\Omega\) was used.

**Preparation of Different Diffusion Systems.** Four different systems were used to make homogeneous CaP/hydrogel composites on a centimeter scale. The mineralization of CaP in a hydrogel was induced by diffusion of Ca\(^{2+}\) (outer ion) into the hydrogel containing HPO\(_4^{2-}\) (inner ion). Depending on the concentrations of the outer and inner ions, states (free solution or hydrogel medium) of the outer reservoirs, and the presence of an interfacial hydrogel layer between them, four different diffusion systems were prepared: Sol\(_{0.5}\) and Sol\(_{0.083}\) hydrogel (Gel\(_{0.5}\)) and a 0.3 M Na\(_2\)HPO\(_4\) agarose hydrogel, and the subsequent reaction was observed for 24 h at room temperature.

**Sol\(_{0.5}\)/Gel\(_{0.3}\) Composite System.** A 0.3 M or 0.05 M Na\(_2\)HPO\(_4\) in a 1 w/v % agarose aqueous solution was prepared by adding each compound to the purified water and subsequently heating them in a microwave oven. Then, 10 mL of the prepared solution was transferred to a 20 mL glass vial (25 mm in diameters, 50 mm in a height) and cooled for 2 h at room temperature. Afterward, 10 mL of a 0.5 M or 0.083 M CaCl\(_2\) solution was added to the top of the agarose hydrogel, and the subsequent reaction was observed for 24 h at room temperature.

**Sol\(_{0.5}\)/Gel\(_{0.3}\) Composite System.** A 0.5 M CaCl\(_2\) in a 1 w/v % agarose aqueous solution was prepared by adding each compound to the purified water and subsequently heating them in a microwave oven. After cooling to 60 °C, 10 mL of the CaCl\(_2\) hydrogel solution was poured on top of a 0.3 M Na\(_2\)HPO\(_4\) agarose hydrogel, and the subsequent reaction was observed for 24 h at room temperature.

**Characterization.** Microstructures in the respective systems were investigated by field-emission scanning electron microscopy (SEM, FEI Co., Netherlands) with an accelerating voltage of 10 kV after sputter-coating with platinum. The composites were analyzed by Fourier transformed infrared spectroscopy (FTIR, Alpha eco-ATR, Bruker Optik Co., Germany). For SEM and FTIR analysis, CaP–hydrogel composites were prepared by rinsing with pure water for 1 h twice and freeze-drying (TFD 8503, ILShinBioBase, Korea). The crystal phase of the composites was analyzed with a high-resolution X-ray diffractometer (XRD, X'Pert PRO MRD, Cu Ka = 1.54 Å, PAN Analytical, Netherlands). For the XRD analysis, the crystal was prepared by dissolving agarose with hot water and subsequently purifying with centrifugation. The pH was measured from three independent samples by a pH meter (SevenCompact pH/Ionometers S220, Mettler Toledo, Switzerland) with an electrode of a 3 mm diameter (InLab Ultra-Micro, Mettler Toledo, Switzerland).
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Notes

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ABBREVIATIONS USED

CaP, calcium phosphate; DCPD, dicalcium phosphate dihydrate; OCP, octacalcium phosphate; HAp, hydroxyapatite; CaP, calcium phosphate; DCPD, dicalcium phosphate

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