Calcium Carbonate Skeletal Material Is Synthesized via Phase Transition of the Calcium Carbonate Cartilaginous Material

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ABSTRACT: The formation mechanism of calcium carbonate (CC) skeletal tissues in biomineralization has remained poorly understood for a long time. Here, we propose an artificial CC biomineralization system equivalent to the natural one in terms of the primary physicochemical mechanism. Our system is constructed of a polymer gel and a CC solution unsaturated by a dissociated anionic polymer. The gel network consists of proton donor and proton acceptor polymers, which are analogues of polymers in the natural biomineralization system and have affinity for each other through hydrogen bonding interaction. Artificial biomineralization takes place within the polymer gel to produce a monolithic composite of the network and CC, whose powder X-ray diffraction pattern indicates calcite or calcite/ vaterite. Scanning electron microscopy and energy-dispersive X-ray spectroscopy observation of the composite during the mineralization process revealed a two-phase structure (network/CC solid solution phase and CC hypercomplex gel phase). As artificial biomineralization proceeds, the solid phase grows in size at the cost of the gel phase as if the latter is substituted with the former, until the solid phase occupies the whole depth of the composite. These results suggest that the hypercomplex gel is the precursor of the resultant network/CC solid solution, and its discontinuous change is a phase transition to the solid solution. Despite minute differences in higher-order structures between our model system and the natural system, the fundamental structure of CC skeletal tissues in the latter can be interpreted as a network/CC solid solution, whereas that of CC cartilaginous tissues as a CC hypercomplex gel. Then, it can be deduced that, in biomineralization, the CC skeletal tissue is in principle formed via a phase transition of the CC cartilaginous tissue.

INTRODUCTION

There are three major skeletal tissues in biological systems: hydroxyapatite (HA), calcium carbonate (CC), and silica skeletal tissues. Although the physicochemical mechanism of such biomineralization had been investigated for many years, the details remain unclear. The formation process of HA skeletal tissues has been studied both extensively and intensively probably owing to its medical importance. The formation mechanism of calcium carbonate (CC) skeletal tissues in biomineralization has remained poorly understood for a long time. Here, we propose an artificial CC biomineralization system equivalent to the natural one in terms of the primary physicochemical mechanism. Our system is constructed of a polymer gel and a CC solution unsaturated by a dissociated anionic polymer. The gel network consists of proton donor and proton acceptor polymers, which are analogues of polymers in the natural biomineralization system and have affinity for each other through hydrogen bonding interaction. Artificial biomineralization takes place within the polymer gel to produce a monolithic composite of the network and CC, whose powder X-ray diffraction pattern indicates calcite or calcite/ vaterite. Scanning electron microscopy and energy-dispersive X-ray spectroscopy observation of the composite during the mineralization process revealed a two-phase structure (network/CC solid solution phase and CC hypercomplex gel phase). As artificial biomineralization proceeds, the solid phase grows in size at the cost of the gel phase as if the latter is substituted with the former, until the solid phase occupies the whole depth of the composite. These results suggest that the hypercomplex gel is the precursor of the resultant network/CC solid solution, and its discontinuous change is a phase transition to the solid solution. Despite minute differences in higher-order structures between our model system and the natural system, the fundamental structure of CC skeletal tissues in the latter can be interpreted as a network/CC solid solution, whereas that of CC cartilaginous tissues as a CC hypercomplex gel. Then, it can be deduced that, in biomineralization, the CC skeletal tissue is in principle formed via a phase transition of the CC cartilaginous tissue.

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them was found to be suitable for forming the gel network. The proton donor polymer, for example poly(vinyl alcohol) (PVA) having −OH,
13 whereas the proton acceptor polymer, for example poly(acrylic acid) (PAA) having −CO2−, is an analogue of chondroitin sulfate that has acidic groups. Besides, an HA solution that is concentrated but unsaturated with respect to HA by adding the polyelectrolyte PAA was proven to be effective here (Table 1). The network immersed in this solution swelled and was continuously converted to an HA hypercomplex gel (Figure 1a), and further transformed discontinuously into a network/HA solid solution. These results indicate that the polymer network in bone biomineralization will consist of collagen and acidic proteins. Instead of the hole zone of fibril,14 the proton-donating function of collagen is important, because gelatin is also an effective network-forming polymer. The fibril structure is rather responsible for the bone strength. On the other hand, the polyelectrolyte PAA in an HA solution in the artificial system mimics the acidic proteins in the body fluid (e.g., blood plasma). In our model, the fundamental structure of cartilaginous tissue is interpreted as an HA hypercomplex gel, whereas that of the HA skeletal tissues as a network/HA solid solution. In this way, the change of the cartilaginous tissue into the bony tissue is naturally explained as a phase transition from an HA hypercomplex gel to a network/HA solid solution, from the perspective of material science.

### Table 1. Classification of Polymers Comprising Skeletal and Cartilaginous Tissues in Several Representative Biomineralization Systems

| mineral | system | polymer network | salt solution | refs |
|---------|--------|-----------------|---------------|------|
| Vertebrata | HA bone | collagen acidic protein | acidic proteins | 1 |
| cartilaginous tissue | collagen chondroitin sulfate | 2 |
| cartilage | collagen chondroitin sulfate | 2 |
| antler | collagen chondroitin sulfate | (unreported) | 3, 5, 6 |
| antler cartilage | collagen chondroitin sulfate | PAA | 7.5 | 10, 12 |
| Artificial System | HA solid solution | PVA, chitosan | PAA | PAA |
| hypercomplex gel | PVA, chitosan | PAA | 10, 12 |
| cartilaginous material | PVA, chitosan | PAA | 10, 12 |
| Mollusc | CC cuttle bone of squid | chitin sulfated glycan | (unreported) | 16 |
| precursor | chitin acidic protein | 37 |
| cranial cartilage | collagen chondroitin sulfate | (unreported) | 33, 34 |
| CC shell | conchoiulin anionic glycoprotein | (unreported) | 17 |
| chitin acidic polymer | 18 |
| silk-fibrin-like protein | 18 |
| noncrystalline tissue | 18 |
| jelly like substance | 38, 39 |
| Echinoderm | CC sea urchin spicule | collagen acidic glycoprotein | (unreported) | 19, 20 |
| noncrystalline tissue | (unreported) | 41, 42 |
| Artificial System | CC solid solution | PVA, chitosan | PAA | PAA | 7.5 | this work |
| hypercomplex gel | PVA, chitosan | PAA | 10, 12 |
| cartilaginous material | PVA, chitosan | PAA | 10, 12 |
| Porifera | silica spicule of sponge | spongion, chitin | (unreported) | 21, 43 |
| (unreported) | (unreported) | 21, 43 |
| Diatom | silica cell wall | glycoprotein sila | silaffin-2 | silaffin-1 | 22, 24 |
| (unreported) | (unreported) | 22, 24 |
| Artificial System | silica solid solution | PVA, chitosan | PAA | poly(allylamine) | 6.5 | next work |
| hypercomplex gel | PVA, chitosan | PAA | 10, 12 |
| cartilaginous material | PVA, chitosan | PAA | 10, 12 |

*The class of natural polymers reported in the references are estimated by analogy to those in the artificial system.*
In different animal phyla, mollusca squid,\textsuperscript{16,18} mollusca shell,\textsuperscript{17,18} and echinodermata echinoidea (sea urchin)\textsuperscript{19,20} among others have CC skeletal tissues. Furthermore, sponge\textsuperscript{21} in animalia and diatom\textsuperscript{22–24} in plantae both have silica skeletal tissues. All these tissues contain both mineral and organic polymers that can be regularly classified according to their physicochemical interactions (Table 1). Hence, the formation mechanism of network/HA solid solution can be theoretically generalized to the systems of network/CC and network/silica solid solutions. This way, the solid solution model may lead to a unified biomineralization mechanism for all three kinds of skeletal tissues.

To experimentally verify this hypothesis, in this study we manufactured a monolithic network/CC solid solution using proton donor and proton acceptor polymers and polyelectrolyte. Furthermore, we show that the obtained network/CC solid solution was formed via a phase transition of a hypercomplex gel. Then, we compare our artificial CC biomineralization system to natural examples reported in the literature, and discuss the similarity between the artificial and natural ones in not only the ions and polymers but also the formation process. Finally, the extension of our model to the biomineralization of silica skeletal tissues will be considered.

\section*{RESULTS AND DISCUSSION}

The occurrence of artificial biomineralization was checked for four combinations of the polymer network and the CC solution (Table 2). The immersed polymer network absorbed the CC solution and swelled to become a hydrogel. Then, the hydrogel solidified in the solution of $x = 30$. These solid composites (1–3) have high dry weight uptakes between 80

![Figure 1. Schematic illustration of the hypercomplex network formation via electrostatic interaction and hydrogen bonding ($\cdots$). (a) Artificial system and (b) natural system of HA biomineralization. (c) Artificial system and (d) natural system of CC biomineralization. (e) Artificial system and (f) natural system of silica biomineralization. The hypercomplex network forms a hypercomplex gel in the mineral solution and will further change discontinuously into a skeletal material or skeletal tissue via phase transition.](https://example.com/figure1.png)

\begin{table}[h]
\centering
\caption{Composites Synthesized under Different Conditions for Artificial CC Biomineralization}
\begin{tabular}{|l|l|l|l|}
\hline
composite & polymer network & $x$ in concentration [mM] & state & $W$: dry weight uptake [%] \\
\hline
1 & chitosan/PAA & 30 & solid & 80.2 \\
2 & PVA/PAA-p & 30 & solid & 90.0 \\
3 & PVA/PAA-c & 30 & solid & 80.7 \\
4 & PVA/PAA-c & 10 & hydrogel & 11.4 \\
\hline
\end{tabular}
\end{table}
and 90%, implying successful artificial biomineralization. Despite using the same network as composite (3), composite (4) with x = 10 remained as a hydrogel in the solution and had a low dry weight uptake of W = 11.4%. In this case, artificial biomineralization did not take place because the concentration of the CC solution was too low.

The powder X-ray diffraction (XRD) patterns of these composites are shown in Figure 2. Although the solution was unsaturated with respect to CC, composites (1–2) exhibit calcite structure that is the most stable CC polymorph, whereas composite (3) exhibits vaterite as well as calcite structures. Hence, composites (1–3) can be called artificial CC skeletal materials. As anticipated from its gel state in the solution, no characteristic peak of the CC lattice was observed in the diffraction pattern of composite (4), indicating that it is a noncrystalline material. In this composite, the low dry weight uptake is not from the CC skeletal material but probably from the ions absorbed onto the polymer network. Chitosan and PVA are proton donor polymers owing to the ions absorbed onto the polymer network. Chitosan and PVA in the CC solution were also proven here to be also useful for comprising the polymer network and CC have been synthesized.27,28 Wakayama et al. synthesized chitosan/PAA network and CC were formed uniformly. Crystallization of CC in gel media has been well studied, and many kinds of composites of the polymer network and CC have been synthesized.27–29

Figure 2. XRD patterns of composites (1–4) in Table 2. For clarity, the patterns are displaced upwards by 1000 × m cps units. (1) m = 5, (2) m = 2, (3) m = 1, (4) m = 0.

Energy-dispersive X-ray spectroscopy (EDX) analysis was carried out for another solid composite synthesized under the same condition as composite (1) and with W = 80.5%. This sample has a microporous structure (Figure S1, Supporting Information) because the original hydrogel was microporous. The line analysis of elements shows almost constant intensity in the cross section, except for dips at the X-ray absorpted vacuum microvoid spaces (Figure S2, Supporting Information). This observation means that mineralization took place throughout the hydrogel, and monolithic composites of chitosan/PAA network and CC were formed uniformly. Crystallization of CC in gel media has been well studied, and many kinds of composites of the polymer network and CC have been synthesized.30–31 Here, we formed the CC skeletal material under atmospheric pressure and room temperature instead. Also, the supercritical CO2 method seems to be applicable only to composites of a polymer network and carbonates, whereas our method is valid for the synthesis of composites of the polymer network and HA.

Next, we focus on the artificial CC biomineralization system utilizing the PVA/PAA-c network to examine the formation mechanism of the CC skeletal material. The swelling degree, Sw, was plotted against the dry weight uptake for composites (A–D) that underwent different mineralization periods of less than 3 weeks (Figure 3).

Naturally, Sw decreases whereas W increases as the CC mineralization proceeds. This tendency means the hydrogel swollen in the CC solution is converted to a solid composite in the solution. To clarify this change, scanning electron microscopy (SEM) images of the cross sections of dried composites (A–D) are shown in Figure 4.

Composite (A) was a hydrogel swollen in the CC solution. Accordingly, it can be regarded as a polymer network with absorbed ions (W = 14.8%). This noncrystalline material is assigned as the H phase. Understandably, this phase mainly contributes to Sw. In the subsequent composite (B) having W
=[56.2%], a new phase emerges from the surface in addition to the H phase. This new phase, assigned as the S phase, can be solid regions of the composite in the solution and hardly contributes to Sw of the composite, because Sw decreases with the increase of W as shown in Figure 3. Instead, the S phase is crystalline because the composite having high W has the same CC peaks as shown by composite (3) in Figure 2. As the artificial biomineralization proceeds, in composite (C) with W = 68.9%, the S phase grows at the cost of the H phase to occupy all the surface. In composite (D) with W = 81.4%, the S phase becomes thicker, whereas the H phase is thinner and disappears at the middle depth of the composite. Eventually, the whole sample will become a monolithic composite of the polymer network and CC. In this way, the decrease of Sw and the increase of W along with the progression of artificial biomineralization can be ascribed to the two-phase structure in the composite and their composition change, that is, the increase of the S phase at the cost of the H phase.

Next, we elucidate the fundamental structures of H and S phases. Figure 5 shows the results of EDX analysis of elements Ca, C, and O along a line across the section of composites at several stages during artificial biomineralization. (A) W = 14.8%, (B) W = 56.2%, (C) W = 68.9%, (D) W = 81.4%. The white line corresponds to the thickness of the composite. The white squares indicate the areas in which the ZAF % of elements were analyzed.

Table 3. Concentration (at. %) of Element Ca, C, and O in the Square Area at the Cross Section of Composites as Measured by EDX**

| composite | A | B | C | D | PVA/PAA-c | CaCO3 |
|-----------|---|---|---|---|-----------|-------|
| phase     | H | H | S | H | S | S |
| [Ca]      | 3.4| 3.5| 17.6| 5.4| 18.0| 16.9| (0) (20) |
| [C]       | 66.8| 68.3| 37.7| 68.7| 37.5| 39.1| (64.4) (20) |
| [O]       | 29.8| 28.2| 44.7| 25.9| 44.5| 44.0| (35.6) (20) |

**The total content of Ca, C, and O in each area is normalized to 100%, eliminating H, which cannot be detected in EDX. The value in ( ) was calculated from the formula of the respective component, PVA/PAA-c or CaCO3.
Ca, C, and O (conducted in the area indicated by the yellow rectangles shown in Figure 4). The order of region of interest (ROI) intensity is O < Ca < C in every H phase, but C < O < Ca in every S phase. Phase H is obviously Ca-poor as can be recognized in Figure 5A–C, whereas phase S is Ca-rich as shown in Figure 5B–D. The intensity levels of each phase seem to be almost the same even in different composites at this resolution. In the S phase, there is no polymer domain where the ROI of Ca should be 0 [counts]. This means that the polymers do not aggregate and disperse inside the CC phase. The concentrations of elements change discontinuously at the interface between the H and S phases. For example, from phase H to phase S, the Ca concentration increases and the C concentration decreases sharply. In this way, these phases are clearly different in elemental composition. The ZAF atom % of elements Ca, C, and O in the white square in Figure 5 are listed in Table 3.

Certainly, the composition of the H phase is near that of the original network (PVA/PAA-c), whereas the composition of the S phase approaches that of CC.

Such a two-phase structure had also been observed in the artificial biomineralization of the HA (Ca₉(PO₄)₂(OH)) skeletal material. Along the cross-sectional line of those composites, the concentration profile of P as well as Ca could be measured by EDX, and therefore the profile of the [Ca]/[P] molar ratio was determined. ¹⁰,¹² This [P] was equivalent to the concentration of phosphate [P₄O₁₀] contributed from dry weight uptake W, [P] = [P₄O₁₀]. Therefore, the obtained values [Ca]/[P] ≈ 1.67 in the Ca-rich and high-W crystalline phase directly indicated that W was brought about by HA, which has [Ca]/[P] ≈ 1.67. In the Ca-poor and low-W noncrystalline phase, on the other hand, the content of P was low with [Ca]/[P] ≈ 3, indicating that the W came not from the HA but the adhesion of HPO₄²⁻ as well as Ca²⁺ ions. Here, it should be noted that the noncrystalline structure cannot be ascribed to amorphous HA, which should still have [Ca]/[P] ≈ 1.67. However, in the present case of artificial biomineralization of the CC skeletal material shown in Figure 5, the concentration of carbon, [C], is a sum from the polymer network, [C₉P₄O₁₀], and the carbonate in dry weight uptake, [C₉]. Namely, [C] ≈ [C₉] + [C₉]. Besides, this [C₉] cannot be distinguished from [C] in the composite by EDX, and therefore the existence of CC should not be judged using the molar ratio [Ca]/[C]. If [C₉] can be determined and [Ca]/[C₉] ≈ 1 in the S phase, this could directly indicate that the W is due to CC (CaCO₃). If [Ca]/[C₉] > 1 is obtained in the H phase, then the W is due to adhesion of HCO₃⁻ and Ca²⁺ ions. Nevertheless, whether the weight increase here is ascribed to artificial CC biomineralization or ion adhesion can be reasonably judged from the concentration profile of not [Ca]/[C₉] but [Ca] only, because the profile of [Ca] obtained here is quite similar to the one in the artificial biomineralization of HA skeletal materials. Both the HA and CC artificial biomineralization systems use the PVA/PAA network. In both systems, the [Ca] profile level was almost constant in each phase and changed discontinuously at the interface between the Ca-rich and Ca-poor phases. Furthermore, W was high in the Ca-rich crystalline phase, whereas W was low in the Ca-poor noncrystalline phase.

In the artificial biomineralization of HA skeletal materials, the Ca-poor, noncrystalline phase with low W did not contain amorphous HA. Instead, it was a hypercomplex network in the dry state and a hypercomplex gel in the HA solution.¹⁰⁻¹¹ The hypercomplex network was composed of the proton donor polymer PVA, the proton acceptor polymer PAA, and HPO₄²⁻ as well as Ca²⁺ ions joined by electrostatic and hydrogen bonding interactions (Figure 1a).

Therefore, it will be natural to also describe the H phase of the present system as a hypercomplex structure without amorphous CC. A schematic of the elemental structure of the hypercomplex network in CC artificial biomineralization is given in Figure 1c. As carbonic acid takes the dominant form of HCO₃⁻ in a pH = 7.5 solution, ³² it can be adsorbed on the polymer chain of the hydrogel. The −OH of HCO₃⁻ will be a proton donor to the −CO₂⁻ of PAA, and the other oxygen of HCO₃⁻ will accept the proton from −OH of PVA. When this adsorption of HCO₃⁻ occurs, Ca²⁺ can be concomitantly absorbed within the polymer gel by electrostatic interaction to neutralize 2 HCO₃⁻ ions. In addition to this adsorption of HCO₃⁻ and Ca²⁺, more Ca²⁺ can be taken into the network by electrostatic effect to neutralize two −CO₂⁻ of PAA. This hypercomplex network will correspond to the fundamental structure of the Ca-poor phase of the composite in the dry state. In the CC solution, the network swells to a hypercomplex gel to fulfill Donnan equilibrium by absorbing free Ca²⁺ and HCO₃⁻ from the external solution. This hypercomplex gel will correspond to the fundamental structure of the Ca-poor phase of the composite swollen in the CC solution.

In the artificial biomineralization of HA skeletal materials, the Ca-rich, high-W, and crystalline phase is not simply a solid dispersion of the polymer network and HA, but rather a solid solution of these two (i.e., a network/HA solid solution).¹¹ This model can be applied to the S phase of the present system. We can determine the equilibrium state of the S phase of composite immersed in a solution unsaturated with respect to CC, by examining the chemical potentials of CC at the interface between the solid composite and solution. For simplicity, we temporarily neglect the polymorphism of CC. At equilibrium, the chemical potential of CC in the solid composite equals that of CC in the unsaturated solution (designated as μ). Meanwhile, the chemical potential of CC in the saturated solution is equal to the standard chemical potential of CC (μ₀). As the concentration in the present system is unsaturated, μ < μ₀. This is satisfied when μ is expressed as μ = μ₀ + RT ln a, where R is the gas constant, T is the temperature, and a (a < 1) corresponds to the activity of CC in the solid composite. The relation a < 1 means that CC is miscible with the polymer network embedded in the solid composite, namely, forming a single solid solution. In other words, CC is a good solvent to swell the polymer network of PVA/PAA. In this sense, the solid composite forms a “solid gel phase”. In general, solid solutions are classified into two categories depending on their basic structure: the substitutional and interstitial solid solutions. For the network/CC solid solution, the model structures of substitutional and interstitial solid solutions are shown in Figure 6.

In a substitutional solid solution, the lattice points of CC are partially replaced by the repeating units of the network polymer. In an interstitial solid solution, the network polymer fits into the space between the CC lattice points instead. Figure 6 shows ideal model structures in which the main lattice points of CC are not dislocated. However, the presence of the network polymer will inevitably cause slight dislocation of the CC lattice points, no matter which structure the solid solution takes. This slight dislocation may have affected the XRD patterns and broadened the peaks located at Bragg angles.
corresponding to CC. In the solid solution model, the polymorphism can be explained not as a metastable state but a stable equilibrium state in which calcite-like and vaterite-like solid solutions coexist. The chemical potential of calcite in the solid solution, \( \mu_a \), is expressed as \( \mu_a = \mu_{a0} + RT \ln a_v \) where \( \mu_{a0} \) is the standard chemical potential of calcite and \( a_v \) is its activity in the solid solution. Similarly, the chemical potential of vaterite in the solid solution can be expressed as \( \mu_v = \mu_{v0} + RT \ln a_v \). The coexistence condition for CC, \( \mu_a = \mu_v \), means \( \mu_{a0} + RT \ln a_v < \mu_{v0} \) because calcite is more stable than vaterite. This leads to \( a_v = a_v^{\text{exp}} \left( \frac{\mu_{a0} - \mu_{v0}}{RT} \right) \), which means that vaterite is stabilized by its affinitive interaction with the polymer network to a larger extent than calcite does.

The change from the Ca-poor phase to the Ca-rich phase of the composite is discontinuous. Because of the aforementioned considerations of the phase structures, this change in the composite can be regarded as a phase transition from the hypercomplex gel, in which the polymer network is miscible with the CC solution, to the solid solution in which the polymer network is miscible with solid CC. Formally, this transition accompanies hydrolysis and can be written as

\[
\{ + Ca^{2+} + HCO_3^- \rightarrow [CaCO_3]^{\text{SS}} + H^+ \]  \( \text{(1)} \)

where \( \{ \) means the hypercomplex gel and \( [ \) means the solid solution. Moreover, this transition occurs in an open system, whose border is permeable to not only energy but also mass (ions and water). Although this transition model is identical to that of the HA artificial biomineralization system, the initiation point of phase transition is different between the two. In HA artificial biomineralization, it begins at the middle depth of the hypercomplex gel rather than on the surface. This difference is a problem to be solved in the future.

Then, we compare our artificial biomineralization system with the natural system. By substituting PVA in artificial HA biomineralization with collagen and PAA with chondroitin sulfate, Figure 1a gives the polymer network and hypercomplex network shown in Figure 1b. The polymer network is formed through hydrogen bonding, where the –OH of hydroxyproline residue on water-insoluble collagen acts as a proton donor, whereas the dissociated –OSO_3^- in chondroitin sulfate acts as a proton acceptor. In the hypercomplex network of (b), the –OH of HPO_4^{2-} will be a proton donor to the –OSO_3^- of chondroitin sulfate, and the other oxygen of HPO_4^{2-} will accept protons from the –OH of collagen. In addition to HPO_4^{2-}, Ca^{2+} can be adsorbed onto HPO_4^{2-} and –OSO_3^- in the hypercomplex network. We propose that this HA hypercomplex gel can represent the fundamental structure of the cartilaginous tissue, whereas the solid solution of this collagen/chondroitin sulfate polymer network and HA can represent the fundamental structure of HA skeletal tissue. This hypercomplex is the only structure model of the cartilaginous tissue at the molecular level as far as the author knows.

Hereafter, the cartilaginous tissue associated with the CC skeletal tissue will be referred to as CC cartilage, as distinct from the usual vertebrate HA cartilage that is associated with the HA skeletal tissue. The cranial cartilage of squid is a kind of CC cartilage and contains collagen and chondroitin sulfate. The squid pen, another CC cartilage, contains collagen, chitin, and acidic polymers. Some types of squids have CC skeletal tissue rather than the squid pen. This tissue, called the cuttlebone, also contains collagen, chitin, and acidic polymers as well as CC. These findings imply the presence of a polymer network in both CC cartilaginous and CC skeletal tissues of squids, similar to the presence of a polymer network in both HA cartilaginous and skeletal tissues (Table 1, Figure 6).}

![Schematic models of two types of network/CC solid solution: (a) substitutional solid solution and (b) interstitial solid solution.](image)

**Figure 6.** Schematic models of two types of network/CC solid solution: (a) substitutional solid solution and (b) interstitial solid solution. The structural unit of CC is located on lattice points with a long-range order.

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Then, the fundamental structure of CC cartilage of squid in its dry state can be regarded as a CC hypercomplex network (Figure 1d). Furthermore, the fundamental structure of cuttlebone can be regarded as a solid solution of this polymer network and CC. The precursor of the cuttlebone was found to contain a chitin–protein complex. We assume this precursor to be also a CC cartilaginous tissue, that is, a hypercomplex gel based on the polymer network that consists of chitin and acidic protein. Following this thinking, the formation of cuttlebone from its precursor can be explained as the phase transition of the CC hypercomplex gel to a network/CC solid solution.

The CC skeletal tissue of mollusk shells contains chitin, silk-fibroin-like protein, and collagen-like conchiolin as water-nonsoluble polymers, and acidic polymers as water-soluble polymers (Table 1). In this system, the water-nonsoluble polymers will act as proton donors, and are bound by hydrogen bonding interaction with water-soluble polymers that act as a proton acceptor to form a polymer network. The formation process of the mollusk shell has been investigated in detail by microscopic analysis. Nacre comprises thick layers of tablets that have an aragonite structure. Weiss et al. reported that the tablets are formed in larval bivalves from a noncrystalline precursor. They found that the aragonite fraction grew larger.
via transformation of the noncrystalline fraction in a hydrogel that contained chitin and silk-fibroin-like protein. The Raman spectrum of the noncrystalline precursor shows the peak of stretching mode of carbonate at 1087 cm$^{-1}$ but not the lattice mode of carbonate in CC. The hypercomplex network shown here in Figure 1d is consistent with the result of their Raman spectrum analysis. Hence, the precursor will be regarded as a CC-based hypercomplex gel. In this model, transformation of the noncrystalline fraction into the aragonite fraction can be explained as the phase transition of the CC hypercomplex gel into a network/CC solid solution. The CC hypercomplex gel will appear as the CC cartilaginous tissue. In fact, the presence of jelly-like substance in shells certainly has been reported in conjunction with the thickening process of shells.

The embryo of sea urchin contains collagen evolutionarily homologous to vertebrate collagen and acidic glycoproteins. These facts also imply the existence of a polymer network for mineralization that contains collagen (proton donor) and acidic glycoprotein (proton acceptor) combined with each other by hydrogen bonding interaction (Figure 1d and Table 1). During the generation or regeneration of sea urchin spine, precursor formation has been observed by SEM. The precursor, which contains calcium, carbonate, and water, shows no spot in the electron diffraction pattern, indicating that it is not crystalline. The precursor is transformed into the calcite skeletal tissue of sea urchin spine. Assuming this noncrystalline tissue to be a CC cartilage composed of the polymer network with both Ca$^{2+}$ and HCO$_3^-$ as in the shell system (Figure 1d), the formation of sea urchin spine can be explained as the phase transition of the CC hypercomplex gel (cartilaginous tissue) into the network/CC solid solution (skeletal tissue).

These examples indicate that, in different kinds of organisms, the CC skeletal tissues are formed from the cartilaginous precursor and the transformation of the cartilaginous tissue into the CC skeletal tissue is a phase transition of the CC hypercomplex gel to a network/CC solid solution. It is natural to suppose this mechanism in other CC biomineralization systems. Furthermore, we would like to extend our model to the silica biomineralization system. The spicule of sponge contains spongin (a collagenous protein), chitin, or amorphous silica, which is regarded as a CC-based hypercomplex gel (Figure 1f). It will be worthwhile to search for such tissues during the silica biomineralization process of diatoms and sponge. So far, we have proven that the phase transition of the hypercomplex gel to the solid solution of a polymer network/mineral can be considered the mechanism of both HA biomineralization and CC biomineralization. If this phase transition mechanism can be extended to silica biomineralization with experimental proofs, it would become a unified mechanism for all three biomineralization systems.

**CONCLUSIONS**

Solid solution systems of metal/metal (alloy), polymer/polymer (polymer alloy), and mineral/mineral (mixed crystal) are well known. In this study, we have introduced a new category of important solid solution systems, namely, the polymer network/mineral solid solution, to describe the fundamental structures of a polymer network/mineral composite synthesized in a solution unsaturated with respect to the mineral. As CC crystal cannot be formed in its unsaturated solution, our solid composite cannot be a dispersion of a polymer network in CC phases. This is a reason why we introduced the solid solution model. This dense solid solution is formed throughout the original gel via the phase transition of a hypercomplex gel that is a polymer network swollen in the mineral solution. This system can explain the components, structure, and formation process of skeletal and cartilaginous tissues in biological systems. At least, the HA and CC skeletal tissues have noncrystalline precursors, and these skeletal tissues and their precursors contain a network of proton donor and proton acceptor polymers. These facts are reflected in our artificial biomineralization system. Therefore, the fundamental structure of skeletal tissues will be regarded as a polymer network/mineral solid solution, whereas that of cartilaginous noncrystalline tissue will be regarded as a hypercomplex gel that is a polymer network swollen in the mineral solution. We believe that our model can be extended...
to cover the silica biomineralization system. This unified model, if experimentally validated, may provide a new insight into the evolution of skeletal and cartilaginous tissues. Furthermore, it will offer a guiding principle to develop exotic skeletal materials with mineral components other than HA, CC, or silica. Functional polymers also can be adopted in these exotic skeletal materials. For examples, when a double-network gel is adopted as the polymer matrix, a very tough skeletal or cartilaginous material can be manufactured; and utilizing electroconductive gel as the polymer matrix can result in electroconductive skeletal or cartilaginous materials. Thus, many fascinating problems remain to be explored.

**EXPERIMENTAL SECTION**

Chitosan (a degree of deacetylation of 75−85% and a viscosity of 5−20 cps by the Brookfield method using 1% solution in 1% acetic acid) as a proton donor component of a polymer network, and two types of PAA (molecular weight Mw = ca. 250 kDa as a proton acceptor component of a polymer network and 2 kDa as a polyelectrolyte component of salt solution) were purchased from Sigma-Aldrich Corporation. Two network components, namely, PVA (degree of polymerization around 2000) as a proton donor and PAA (Mw = ca. 5 kDa) as a proton acceptor, sodium chloride (NaCl), calcium chloride dihydrate (CaCl2·2H2O), and sodium hydrogen carbonate (NaHCO3) were purchased from WAKO Pure Chemical Industries, Ltd.

The chitosan/PAA network (chitosan/PAA), PVA/PAA physically cross-linked network (PVA/PAA-p), and PVA/PAA chemically cross-linked network (PVA/PAA-c) were prepared by the method described in detail in previous reports. Chitosan/PAA was prepared by mixing water-insoluble chitosan and water-soluble PAA (Mw = ca. 5 kDa) solutions at the polymer weight ratio of 2:1 under an acidic condition. After drying, this network film turned out to be insoluble without further treatment in the CC solution. PVA/PAA-p was prepared by repeated freezing−thawing of a mixed solution of water-soluble PVA and PAA (Mw ≈ 250 kDa), introducing fine-crystalline PVA to avoid the dissolution of PVA or the network. In the preparation of PVA/PAA-c, PVA and PAA (Mw ≈ 250 kDa) solutions were mixed in the polymer weight ratio of 3:2. Then, after drying, the polymer film was cross-linked by heat treatment under reduced pressure to introduce esterification to stabilize the network.

We prepared aqueous CC solutions (200 mL) containing CaCl2 at x mM, NaHCO3 at x mM (x ≤ 30), PAA (Mw ≈ 2 kDa) at 0.14 mM in terms of the repeating unit, and NaCl at 140 mM. The pH of this solution was around 7.5. The high concentrations of source ions Ca2+ and HCO3− in the solution were maintained by the PAA: precipitation occurred in the absence of PAA, whereas in its presence the solution remained transparent for more than 1 week even at 30 °C. As mineralization proceeded, the concentrations of source ions Ca2+ and HCO3− in the solution were aperiodically adjusted to keep them as constant as possible by renewing the solution. After an appropriate interval, the polymer network changed to an organic/inorganic composite. The composite was taken out of the solution, and rinsed three times in pure water for 10 min to remove residual free soluble ions.

The dry weight uptake, W, corresponds to the weight percent of inorganic constituents in the obtained organic/inorganic composite: W = 100(M − M0)/M, where M0 and M are the dry weights of the initial network and obtained composite, respectively. The swelling degree of the composite, Sw, was determined as Sw = 100(M′ − M)/M, where M′ is the weight of the composite swollen in water at 30 °C.

XRD patterns of the composites were obtained with a MiniFex II diffractometer (Rigaku Co.). The composite based on chitosan/PAA-p was embedded in curable resin, and the ones based on PVA/PAA-c were sandwiched with silicon wafers for support. The composites were cut together with the support medium by ion beam polishing to make their cross section as flat as possible. An electroconductive coating was carried out on the composites, with a carbon layer of ca. 2 nm for the composite based on chitosan/PAA-p and a platinum layer of ca. 3 nm for that based on PVA/PAA-c. The coated samples were used for the SEM observation, and the subsequent analysis of the distribution profile of principal elements (Ca, C, and O) across a representative cross-sectional line was carried out by EDX.

It is difficult to directly evaluate the state of composites in situ, that is, in the CC solution. Instead, the obtained composites were evaluated in their dry state. From the evaluation results and the swelling degree, we will estimate the state of composites under their artificial biomineralization conditions, which are expected to be close to the natural biomineralization condition.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b01420.

Cross-sectional images and EDX line analysis of elements obtained for the chitosan/PAA-p based composite having W = 80.5%; schematic models of solid solution of polymer network and amorphous silica (PDF)

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