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Calcium Phosphate Mineralization Induced by Synthetic Peptides Having Different Distributions in Simulated Body Fluids

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Synthetic peptides having hydroxyapatite binding ability can induce calcium phosphate (CaP) mineralization in simulated body fluids. The peptides freely dispersed in the solution induce formation of peptide–CaP hybrids having sheet-like morphologies, whereas immobilized on solid substrates they induce hydroxyapatite deposition on the substrate surfaces. The results clearly show that distribution of the peptides affects their mineralization properties.

One of the characteristics of biomineralization, a process forming mineral phase under physiological conditions, is that it is triggered by heterogeneous nucleation of the biominerals on organic scaffolds consisting of biomolecules, which results in formation of organic–inorganic hybrids. Therefore, biomimetic mineralization systems have been paid much attention from the viewpoints not only of understanding biomineral formation mechanisms in nature but also of obtaining novel organic–inorganic hybrid materials under mild conditions.[1] Calcium phosphates (CaP) are an attractive inorganic component for such studies because they are widely distributed in various living organisms as various forms such as hydroxyapatite (HAp, $Ca_{10}(PO_4)_6(OH)_2$), the major inorganic component in bone and teeth.[2] As for the biomimetic HAp mineralization, a system using simulated body fluid (SBF), a solution having similar inorganic ion concentrations to those of human plasma, has been widely used to deposit bone-like HAp on various shapes of scaffold materials made of organic, inorganic, and metallic substances.[3] In this system, it is believed that HAp deposition is triggered by heterogeneous nucleation of HAp on the scaffold surfaces displaying proper functional groups such as sulfuric acids, carboxylic acids, and silanol groups according to numerous related investigations.[4] Contrary to the HAp deposition on solid substrates, preparation of colloidal particles of HAp[5] and organic–HAp hybrids in SBFs has not been widely investigated although such particles have been prepared using various synthetic approach including aqueous solutions in the presence of organic molecules or their assemblies.[2] One reason for this might be that molecular sizes of organic components dispersed in SBF are thought to be ineffective to induce HAp nucleation because heterogeneous nucleation occurs at certain areas of solid–liquid interfaces. In this study, we investigated CaP mineralization induced by synthetic peptides having HAp binding ability in SBF. We evaluated the effect of peptide distribution in SBF, that is, freely dispersed in SBF or immobilized on solid substrates, on their mineralization abilities.

The peptide sequence was referenced to that reported as a HAp-binding peptide (HABP, CMLPHHGAC).[6] We also used a peptide that biotinylated at the *N*-terminal of HABP by introducing one ε-biotinylated lysine residue (B-HABP). They were synthesized using typical Fmoc solid-phase chemistry. SBF (pH 7.4) and 1.5SBF (a solution having 1.5 times higher ion concentrations than those of SBF, pH 7.4) were prepared according to Kokubo's method.[3,4] HABP or B-HABP (0.4 mM) were added in SBF and incubated at 36.5 °C. After proper incubation time, a portion of the solution was picked up and diluted with distilled water to stop the reaction. The samples were desalted by dialysis followed by lyophilization to be used for characterization. We also evaluated mineralization induced by B-HABP immobilized on avidin-coated glass substrates[7] using avidin–biotin interactions.[8] The resulting substrates were immersed in 1.5SBF and incubated at 36.5 °C. After the reaction, the substrates were rinsed with distilled water and dried by nitrogen flushing.

Results of scanning electron microscopy (SEM, S-5000; Hitachi Ltd.) showed that HABP molecules induced formation of deposits when they were dispersed in SBF (Figure 1). The morphology of the deposits was sheet-like and had several tenth–several hundreds micrometer lengths. No such deposits were found in the absence of the peptides (data not shown). It

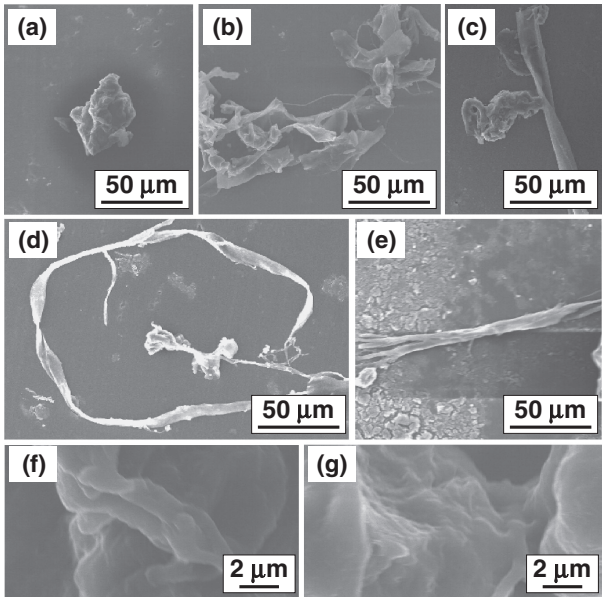

Figure 1. (a)–(e) SEM images of the deposits formed in the presence of HABP after incubation of 10 (a), 20 (b), 30 (c), 60 (d), and 120 min (e) in SBF. (f, g) Magnified images of 1a (f) and 1b (g).

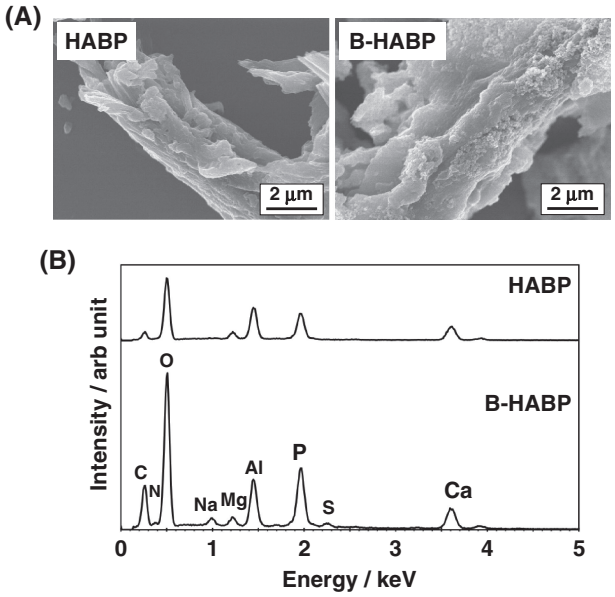

Figure 2. SEM images (A) and EDX spectra (B) of the deposits induced by addition of HABP and B-HABP in SBF (24 h incubation). In the EDX spectra, the Al peak comes from specimens that sample applied.

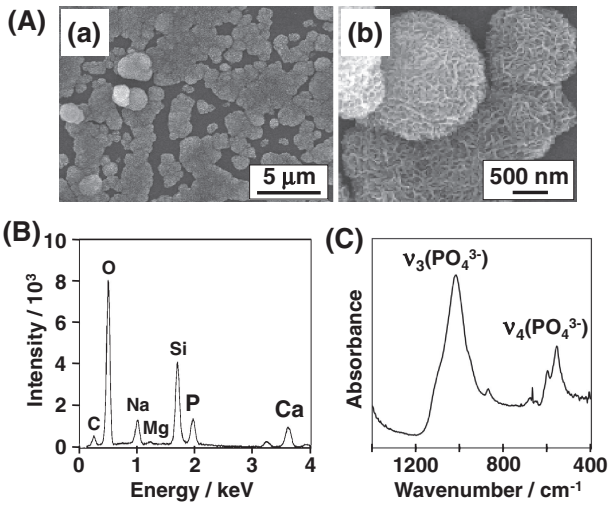

Figure 3. SEM images (A), EDX spectra (B), and FT-IR spectra (C) of the deposits formed on B-HABP-immobilized surface after 1 week incubation in 1.5SBF. In (A), (b) is a mignified image of (a). In (B), the Si peak comes from the glass substrate.

was also found that more than about 20 min of incubation was required to obtain deposits with sheet-like morphologies (Figures 1a–1e). Further increase of incubation time seemed less effective for deposit morphology. Magnified images of the deposits after 10 and 20 min incubation showed that there was no remarkable difference in their microstructures (Figures 1f and 1g). We were able to confirm formation of the sheet-like deposits; however, it seemed that control of length or width of the sheet structures was hardly achieved under the present experimental conditions. Energy-dispersive X-ray spectroscopy (EDX, Sigma; Kevex) revealed that they contained calcium and phosphorous, in addition to small amounts of sodium and magnesium, which is characteristic for bone-like HAp obtained from SBF,[3,4] and elements originating from peptides (Figure 2). These results support formation of peptide–CaP hybrids. We also evaluated CaP mineralization induced by B-HABP, the results of which showed that there is no observable effect by introducing biotin moiety into the HABP sequence on mineralization ability (Figure 2).[9] The values of Ca/P elemental ratios obtained from the EDX spectra for several samples were distributed and most of them appeared lower than that for stoichiometric HAp (1.67). Such lower Ca/P ratios indicate that these CaP were not HAp, or they were bone-like HAp where some calcium ions were substituted with other ions such as magnesium ions. EDX spectra supported the latter case. However, surface morphology of these deposits seems to indicate that these CaP crystalline phases were amorphous rather than HAp.[10] Gungormus et al. reported CaP mineralization by HABP, where aggregates of plate-like objects, mainly consisted of octacalcium phosphates, were obtained.[6] One of the reasons to make such a difference in morphologies between their study and the present study probably comes from the difference in reaction solutions. They used calcium ion-containing Tris-HCl buffer solutions to which were added phosphate anions continuously using enzymatic reaction, whereas we used SBF, that was already supersaturated with respect to HAp.

For the case of B-HABP immobilized on solid substrates, hemispherical deposits consisting of plate-like objects, which were characteristic morphologies of HAp deposited from SBFs,[3,4] were found in most parts of the substrates after one-week incubation in 1.5SBF (Figure 3A). Such deposition was not observed for the case of surfaces without B-HABP (data not shown). EDX spectra (Figure 3B) showed similar elemental appearances to those for the case of the dispersed peptides (Figure 2B). FT-IR spectra of the sample surfaces obtained using a single-reflection attenuation total-reflection (ATR) apparatus (Nicolet 380 combined with Smart-Orbit module; Thermo Fisher Scientific Inc.) exhibited two intense absorption peaks in the region of about 1150–900 and 650–500 cm^{-1}, which were respectively assignable to the stretching vibration (ν_3) of the phosphate (PO_4^{3-}) groups and the bending vibration (ν_4) of the phosphate (PO_4^{3-}) groups (Figure 3C).[11] These peaks are frequently observed for HAp-deposited substrates.[3,4] These results strongly support that B-HABP immobilized on solid substrates can act as heterogeneous nucleation sites for HAp. We could not observe deposition formation when the substrates were incubated in SBF for 1 week. This might indicate limitation of HAp nucleation ability of B-HABP.

The detailed mechanism for HAp binding by HABP has not been clarified. In the reference study, Gungormus et al. proposed a mechanisms for their CaP mineralization systems, which initiate from the preformed CaP nuclei.[6] We referred to their study and proposed the presumable mechanisms for CaP mineralization in the present study in Figure 4. In the case of the dispersed peptides, at first interaction between spontaneously formed nanosized CaP aggregates (we call them CaP nuclei) and HABP might occur, which results in formation of peptide–CaP nanohybrids. Such nanosized hybrids then assemble into sheet-like structures. HAp binding property of HABP should contrib-

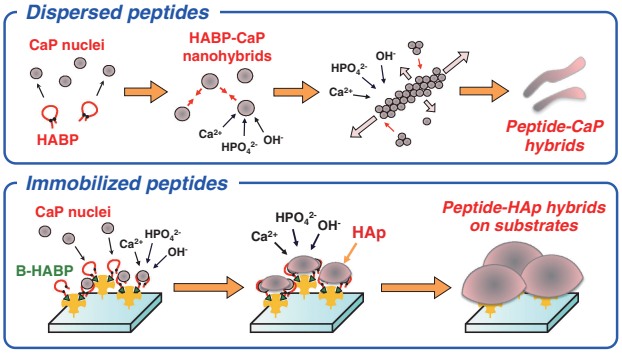

Figure 4. Presumable mineralization mechanisms for dispersed peptides (upper illustration) and immobilized peptides (lower illustration).

ute such assembly processes. During this process, growth of CaP components can also occur simultaneously. On the other hand, first preformed CaP nuclei can adsorb on the substrate surfaces for the case of peptides (B-HABP) immobilized on solid substrates. Further nucleation and growth of HAp from these adsorbed CaP components at the substrate–solution interfaces then occur, which results in formation of HAp layers on the surfaces. Nanosized CaP components or peptide–CaP hybrids are a key factor for such mechanisms, therefore their isolation is one of our next research targets.

We demonstrated that even small peptides dispersed in SBFs have the ability to induce CaP mineralization, which results in formation of peptide–CaP hybrids. In addition, we also revealed that distribution of the peptides affects morphology of the mineral phase. The latter point should be considered when fabricating organic–inorganic hybrids using analogous approaches. We believe that further modification of peptide sequences and experimental conditions for the present system will lead control of morphology and CaP crystallinity of the resulting hybrids more precisely.

References and Notes

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8 For B-HABP immobilization, excess amounts of B-HABP were treated in order to bind to all biotin-binding sites displayed by avidin that densely coated on glass substrates.

9 Identification of the peptides using a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer supported cyclization of HABP via disulfide bond formation ($[M + H]^+$, m/z 966.33 (calcd), 966.64 (found)), whereas B-HABP was not cyclized just after purification ($[M + H]^+$, m/z 1320.55 (cyclized, calcd), 1322.57 (not cyclized, calcd), 1323.38 (found)). This is probably due to steric hindrance by introducing a biotinyl lysine residue. We think that spontaneous cyclization during the sample preparation for mineralization experimens can occur, but we have not proven it. We confirmed that noncyclized HABP sequences still induced CaP mineralization in separate experiments.

10 At this moment we could not determine CaP crystalline phase for these samples using X-ray diffraction (XRD) studies because we have not obtained sufficient amounts of the deposits to conduct XRD measurements accurately, even for the case we used a special sample holder for small amounts of samples. We are now continuing experiments to obtain enough amounts of samples. Crystallinity of the CaP components and further investigation for characteristics of the present deposition systems will be described in our future reports.

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