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Biomimetic deposition of hydroxyapatite on a synthetic polypeptide with β sheet structure in a solution mimicking body fluid

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Abstract Deposition of a hydroxyapatite layer with similar structure to bone mineral is an attractive approach to the fabrication of bioactive coating layers to achieve direct bonding to living bone. To get successful coating of a hydroxyapatite layer on an organic polymer using a biomimetic solution, it is essential to find organic substrates that can effectively induce heterogeneous nucleation of hydroxyapatite after exposure to the body environment. Our previous study showed that sericin, a type of silk protein, has the ability to induce hydroxyapatite nucleation in a biomimetic solution when the sericin has a β sheet structure. To confirm the effectiveness of the β sheet structure in hydroxyapatite nucleation, we focused on investigating hydroxyapatite deposition on a synthetic polypeptide with a β sheet structure in a biomimetic solution. The β sheet forming polypeptides with and without carboxyl groups, poly$(FE)_3FG$, poly$(FQ)_3FG$, poly$(LE)_3LG$ and poly$(LQ)_3LG$, were synthesized in this study. All the polypeptides had mainly β sheet structure. After soaking the polypeptide films in 1.5SBF, which has 1.5 times the inorganic ion concentrations of human blood plasma, hydroxyapatite formed on the surfaces of the polypeptides with carboxyl groups, poly$(FE)_3FG$ and poly$(LE)_3LG$, within 2 days, but not on those without carboxyl groups, poly$(FQ)_3FG$ and poly$(LQ)_3LG$. We confirmed that the β sheet structure was effective for hydroxyapatite nucleation even in the synthetic polypeptide. This finding is useful for the future design of organic polymers that can effectively induce nucleation of hydroxyapatite.

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Introduction

Hydroxyapatite coating onto organic polymers is an attractive method for developing hybrid materials for medical applications. The use of biomimetic processes has focused on fabricating such hybrids, whereby a hydroxyapatite layer can be coated onto organic substrates by using either a simulated body fluid (SBF) with ion concentrations nearly equal to those of human extracellular fluid, or more concentrated fluids, such as 1.5SBF, which has 1.5 times the ion concentrations of SBF [1, 2]. Because SBF is supersaturated with respect to hydroxyapatite even under normal conditions, once hydroxyapatite nuclei form on the surface of the substrate, hydroxyapatite grows spontaneously to give a hydroxyapatite layer. A key to this engineering is to find an organic polymer that can effectively induce hydroxyapatite nucleation, so to achieve successful coating of hydroxyapatite using these solutions.

Previous studies of hydroxyapatite formation in SBF have shown that heterogeneous hydroxyapatite nucleation can be induced by specific functional groups on the surface of a substrate, such as phosphate ($-PO_4H_2$), carboxyl (–COOH) and sulfonic ($-SO_3H$) groups [3, 4]. Because of these observations, in recent years many attempts to coat

hydroxyapatite layers on the surfaces of substrates containing these functional groups have been carried out using this biomimetic process [5–10]. However, the relationship between the arrangement of the functional groups and hydroxyapatite formation has not been well examined.

Formation of hydroxyapatite occurs in the living body to produce bone and tooth. This hydroxyapatite formation, so-called biomineralization, is normally related to many types of proteins. For instance, osteocalcin, the most abundant noncollagenous protein in bone, plays an important role in binding to hydroxyapatite because it contains γ-carboxylated glutamic acids (Gla) residues. The domain in osteocalcin containing these Gla residues has an α helix structure, and it provides precise calcium-binding sites for binding to hydroxyapatite with spatial matching [11]. Dentin matrix protein 1 (DMP1), a protein that is present in bone and tooth, also plays structural or regulatory roles during mineral formation. He et al. [12] examined the role of the domains by using the corresponding peptides with DMP1, and reveled that the self-assembled β sheet acidic domains can be an ideal template for hydroxyapatite nucleation. They proposed the hypothetical model in which self-assembled β sheet acidic domains match (epitaxy) with the hydroxyapatite lattice being nucleated, resulting in lowering the activation energy for hydroxyapatite nucleation and orientation. In both cases, the specific structures of the proteins, such as α helix and β sheet, play important roles in the formation of hydroxyapatite. A similar phenomenon is observed in shell nacre, which is composed of calcium carbonate and an acidic protein matrix containing aspartic acids: the protein has β sheet structure and the directed arrangement of the β sheet plays an important role in mineral formation with crystalline orientation [13]. These observations suggest that both the content of the acidic functional groups and their arrangement must be considered in the effective induction of heterogeneous nucleation of hydroxyapatite.

Our previous study [14] showed that sericin, a type of silk proteins, has the ability to effectively induce hydroxyapatite nucleation in a biomimicking solution. The carboxyl groups contained in the sericin induced the hydroxyapatite nucleation. Furthermore, the ability to induce hydroxyapatite nucleation on sericin with a β sheet structure was higher than that on sericin with a random coil structure [15]. Therefore, sericin may provide an effective surface for hydroxyapatite nucleation because of the existence of carboxyl groups and the formation of β sheet structure. However, it is difficult to investigate these effects on hydroxyapatite nucleation in detail because sericin consists of many types of amino acid such as serine.

The purpose of this study is to investigate fundamentally the effect of β sheet and carboxyl group on hydroxyapatite formation using specifically designed polypeptides without other factors such as hydrophilic amino acids like serine. Synthetic polypeptides were developed based on a polypeptide that contains carboxyl groups and has β sheet structure. The potential for hydroxyapatite formation on the surface of the synthesized polypeptides was examined to assess in detail the effects of carboxyl groups and β sheet structure on the induction of heterogeneous nucleation of hydroxyapatite.

To design the structures of the synthetic polypeptides, previous reports were reviewed. Xiong et al. [16] reported that peptides of alternating nonpolar and polar amino acid residues tend to form β sheet structures. According to this report, peptides consisting of eight amino acid residues alternating polar and nonpolar amino acids were designed. The nonpolar amino acids leucine (L) and phenylalanine (F) were selected. Levitt [17] reported that these amino acids have different preferences for α helix and β sheet, respectively. Phenylalanine favors β sheet formation because of its bulky side chain. Therefore, the peptide containing phenylalanine was expected to form a β sheet structure. Conversely, leucine favors α helix formation. The peptide containing leucine was, therefore, expected to have less β sheet content than the peptide containing phenylalanine. Glutamic acid (E) was selected as the polar amino acid. Glutamic acid contains a carboxyl group in its side chain and it was expected to induce hydroxyapatite nucleation in a solution mimicking the body's environment. As a reference peptide that does not contain carboxyl group, a peptide containing glutamine (Q) instead of glutamic acid was also prepared. Glycine, which is indifferent to racemization occurring during peptide synthesis, was selected as the residue for the C-terminal of the peptides. Thus, four peptides, LELELELG ($(LE)_3LG$), LQLQLQLG ($(LQ)_3LG$), FEFEFEFG ($(FE)_3FG$) and FQFQFQFG ($(FQ)_3FG$), were synthesized. The hydroxyapatite formation on polymerized films of the resultant synthesized polypeptides was examined after exposure to 1.5SBF, a solution that has an enhanced degree of supersaturation with respect to hydroxyapatite.

Experimental procedure

Preparations of peptides

Peptides were prepared using an ABI-433A Peptide Synthesizer (Applied Biosystems, USA) using solid-phase Fmoc chemistry. They were cleaved from the resin with either trifluoroacetic acid (TFA, Peptide Institute, Inc., Japan) containing 5% water or 2.5% ethanedithiol and 2.5% water. The peptides were used without purification because they were poorly soluble in water.

Polymerization of peptides

The peptides were polymerized in dimethylsulfoxide (DMSO, MERCK, Germany) using diisopropylethylamine (DIPEA, Peptide Institute, Inc., Japan), 1-hydroxybenzotriazole (HOBt, Peptide Institute, Inc., Japan) and 1-ethyl 3-(3-dimethylaminopropyl)-carbodiimide (EDC·HCl, Peptide Institute, Inc., Japan). Ten milligram of the peptides were dissolved at a concentration of 10 mmol/L in DMSO in a glass test tubes at room temperature, and an equivalent molar amount of DIPEA, HOBt, and three equivalent molar amounts of EDC·HCl were added. After adding all of the chemicals into the peptide solutions, the test tubes were kept at 20 °C and stirred. After stirring at 20 °C for 48 h, approximately 5 mL of phosphate buffered saline (PBS) was added into the tube to completely stop the polymerization process. They were dialyzed in ultrapure water using a cellulose membrane tube (Viskase. Co.,USA, molecular weight cut-off (MWCO) = 14,000). to obtain aqueous solutions of the polypeptides.

Characterization of polypeptides

The molecular weights and secondary structures of the polypeptides were determined using gel permeation chromatography (GPC) and circular dichroism (CD) spectroscopy, respectively. Aqueous solutions containing the polypeptides were frozen at –30 °C and freeze-dried using a freeze dryer (FRD-82M, IWAKI (ASAHI Techno Glass), Tokyo, Japan). For GPC measurement, the freeze-dried polypeptides were dissolved at the concentration of 2 mg/mL in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, Wako Pure Chemicals, Japan). The measurements were performed at the Tosoh Analysis and Research Center (Japan) and the molecular weights were calculated from a standard curve obtained using polymethylmethacrylate (PMMA). For CD measurement, the freeze-dried polypeptides were dissolved at a concentration of 0.6 mg/mL in HFIP, and then an equal volume of ultrapure water or HFIP was added to give 0.3 mg/mL polypeptide solutions in H_2O/HFIP = 1/1 or in HFIP. Spectra were acquired at 25 °C in a 1 mm cuvette using Spectra Manager Ver. 1.53E equipped to a circular chroism spectropolarimeter (J-820, JASCO, Japan). The content of secondary structure were calculated from the spectra using the secondary structure analysis software JWSSE-480(32) Ver. 1.00A.

The secondary structures of the polypeptides in forms of films were determined using CD and Fourier transform–infrared (FT-IR) spectroscopy. For CD measurements, the solutions of polypeptides in HFIP/H_2O = 1/1 or HFIP were cast onto silica glass plates and dried under ambient conditions at 40 °C. Spectra were acquired at 25 °C. For FT-IR measurements, the polypeptide solutions obtained by dialysis after polymerization were cast onto a polyethylene film, and then dried under ambient conditions at 40 °C. Spectra were acquired using an FT-IR spectrometer (Spectrum one, PerkinElmer Ltd., UK).

Soaking in 1.5SBF

Polypeptide films were prepared by casting the solution as prepared after dialysis, containing about 1 mg/mL of polypeptide in H_2O, on the bottom of a Petri dish to form a polypeptide film. The cast films were exposed to 15 mL of 1.5SBF (Na^+ 213.0, K^+ 7.5, Mg^{2+} 2.3, Ca^{2+} 3.8, Cl^- 221.7, HCO_3^- 6.3, HPO_4^{2-} 1.5 and SO_4^{2-} 0.8 mM). The solution was buffered at pH 7.25 using 75 mmol/L tris(hydroxymethyl)aminomethane and appropriate amounts of hydrochloric acid, according to the methods reported by Kokubo et al. [2]. The solution was poured into the Petri dish on which bottom polypeptide film was formed and then it was stored at 36.5 °C without renewing the solution.

After storage at 36.5 °C for various periods, the surface of the film was characterized using an electron scanning microscope (FE-SEM: S-4800 Hitachi Ltd., Japan) and thin-film X-ray diffraction (TF-XRD; RINT2200V/PC-LR, Rigaku Co., Japan).

Results

The weight-average molecular weights (M_w) and molecular weight distributions (M_w/M_n) of the polypeptides were summarized on Table 1. This result showed that polydisperse polymer with M_w in the range of 6,000–12,000 was obtained. Figure 1 shows the CD spectra of the polypeptides. The spectra of the polypeptides were measured in HFIP and in H_2O containing HFIP, as the polypeptides were not soluble in H_2O. The spectra acquired in H_2O containing HFIP showed a negative Cotton band at 205 nm and a negative shoulder at 220 nm for poly(LE)$_3$LG and poly(LQ)$_3$LG. These spectra provide clear supporting evidence that the polypeptides containing leucine residues form α helices containing some amount of random coil structure [18–21]. For poly(FE)$_3$FG in H_2O containing HFIP, a positive Cotton band at 190 nm and a negative

Table 1 Average molecular weights (M_w) and distributions of molecular weight (M_w/M_n) of polypeptides

Polypeptide	M_w	M_w/M_n
poly(LE)$_3$LG	6000	1.5
poly(LQ)$_3$LG	8600	1.2
poly(FE)$_3$FG	7500	1.3
poly(FQ)$_3$FG	12000	1.3

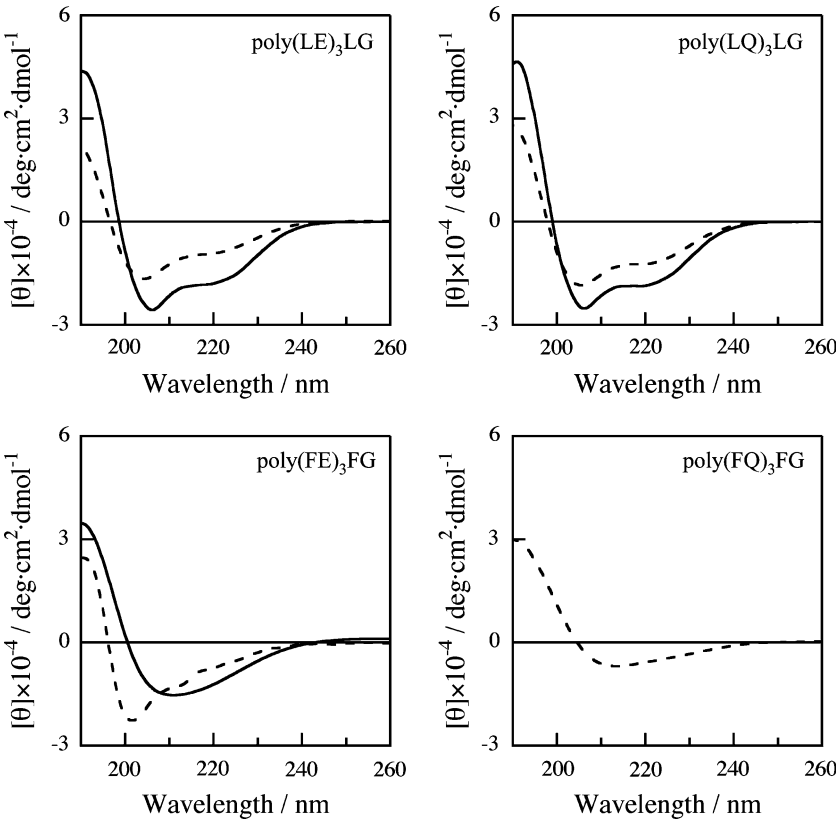

Fig. 1 CD spectra of polypeptides, ————————:in H_2O/HFIP, - - - - - - - : in HFIP

Cotton band at 217 nm, assigned to a β sheet, were detected. For poly(FQ)$_3$FG, the spectrum could not be measured in H_2O containing HFIP, because it did not dissolve. The spectra acquired in HFIP indicated a decrease in the α helix or β sheet structure content of the polypeptides other than poly(FQ)$_3$FG. It was obvious that the HFIP had prevented these peptides from forming specific secondary structures such as α helix and β sheet. For poly(FQ)$_3$FG, a positive Cotton band at 190 nm and a negative Cotton band at 217 nm, assigned to a β sheet, were detected.

As the secondary structures of the polypeptides shown above are their structures in solution, the structures in the polypeptide films were investigated using FT-IR and CD spectroscopy. Figure 2 shows the FT-IR spectra of the polypeptide films. The two absorption bands located at 1,626 and 1,550 cm^{-1} were assigned to the amide I (υC=O) and amide II (δN–H) stretches, respectively. The strong peak at 1626 cm^{-1} in the amide I band was attributed to the β sheet structure [22]. This result implies that all the polypeptides form a β sheet structure in the films. Figure 3 shows the CD spectra of polypeptide films. All polypeptide films contained mainly β sheet structure characterized by a positive Cotton band around 197 nm and a negative Cotton band around 217 nm. The content of secondary structures in the polypeptide films was calculated using the secondary structure analysis software accompanying the CD spectrometer (Fig. 4). Polypeptides with leucine residues contained about 60% β sheet and polypeptides with phenylalanine residues contained about 75% β sheet. Both results of CD and FT-IR spectra of polypeptide films surely indicated that the polypeptides formed mainly β sheet.

Figure 5 shows SEM images of polypeptide films after soaking in 1.5SBF for various periods. Deposition of fine particles was observed on the surfaces of poly(LE)$_3$LG and poly(FE)$_3$FG films after soaking in 1.5SBF for more than 2 days. The morphology of the particles was similar to that of hydroxyapatite crystals observed on bioactive glasses soaked in SBF [23, 24]. In contrast, no changes were observed on the surfaces of the poly(LQ)$_3$LG and poly(FQ)$_3$FG films.

Figure 6 shows the TF-XRD patterns of the surfaces of poly(LE)$_3$LG and poly(FE)$_3$FG films before and after soaking in 1.5SBF for various periods. Broad peaks that could be assigned to hydroxyapatite were detected around 2θ = 26° and 32° for the samples after soaking in 1.5SBF for more than 2 days.

Discussion

To obtain peptides with a β sheet structure, four peptides alternating polar and nonpolar amino acid residues were synthesized. Polypeptides containing phenylalanine formed β sheet structure even in conditions with HFIP, whereas

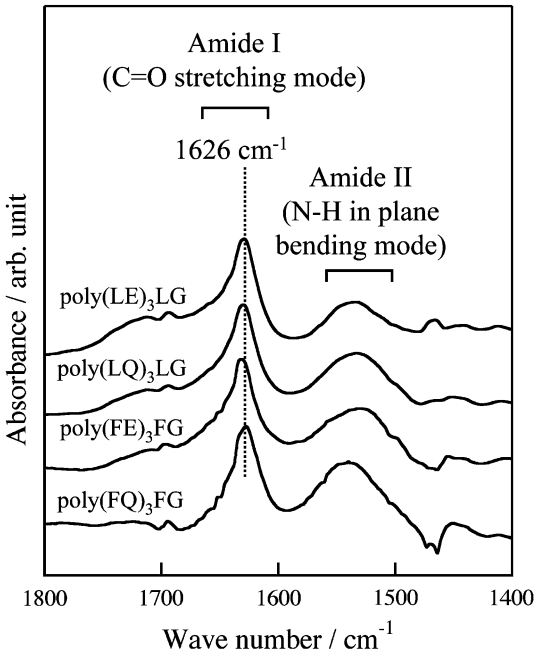

Fig. 2 FT-IR spectra of polypeptide films

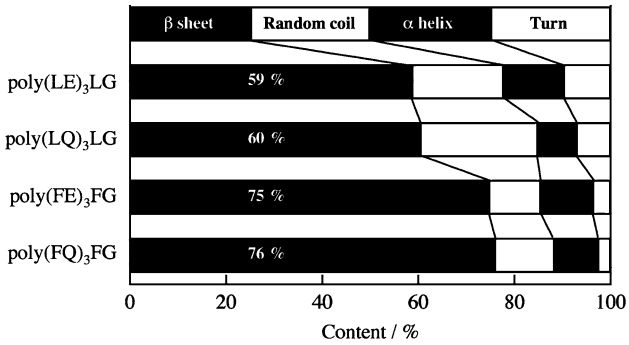

Fig. 4 Contents of secondary structures in polypeptide films

polypeptides containing leucine formed an α helix structure. These results relate to the preferences of leucine and phenylalanine for α helix and β sheet structures, respectively.

The FT-IR and CD measurements of the polypeptide films indicated that all the polypeptides in the films predominantly contained β sheet. The solution structures of the polypeptides containing phenylalanine remained even after film formation, whereas the structures of the polypeptides containing leucine residues were changed from random coils or α helices to β sheet. These phenomena can be explained by the alternating sequence that favors the formation of β sheet. The alternating sequence of the peptide matches the structural repeat of the β strand, with successive side chains pointing up then down. Therefore, the sequences of the polypeptides are suitable for formation of β sheet [16]. In the process of film formation, the con-

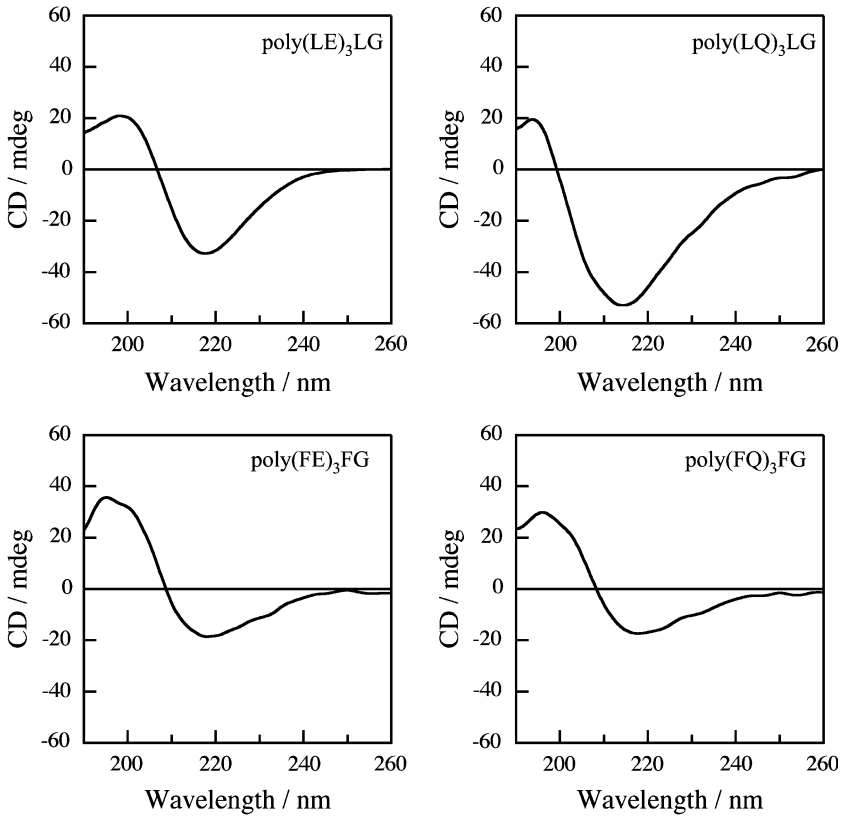

Fig. 3 CD spectra of polypeptide films

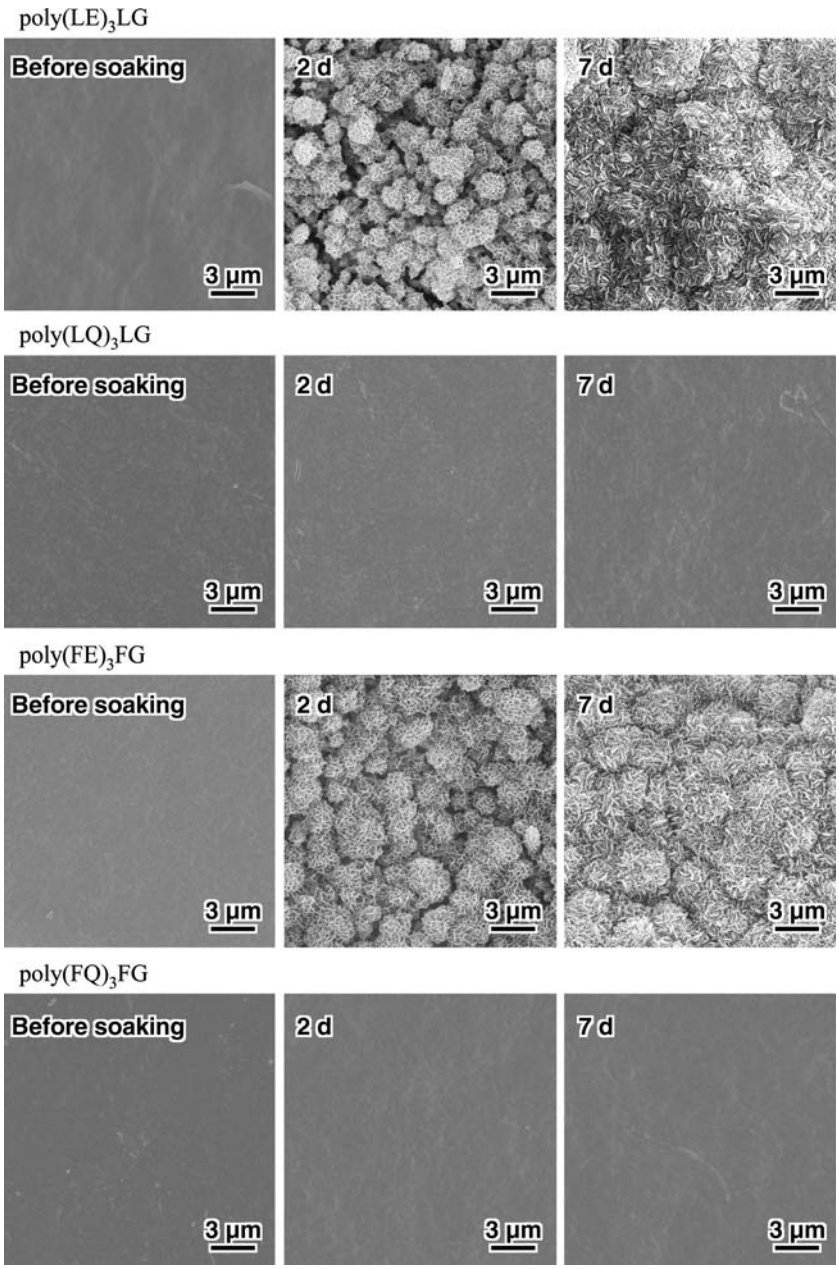

Fig. 5 SEM images of the surfaces of polypeptide films before and after soaking in 1.5SBF for various periods

centration of polypeptide solution increases with evaporation of the solvent. This increase in the concentration would enhance the interaction between the polypeptide chains and increase the intermolecular hydrogen bonds because of sequence matching, and thus result in the formation of β sheet structure, even in the films of polypeptides containing leucine residues.

From the SEM and XRD analysis results of the polypeptide films before and after soaking in 1.5SBF, it was obvious that poly$(LE)_3$LG and poly$(FE)_3$FG had the ability to induce hydroxyapatite nucleation in 1.5SBF. Hydroxyapatite nucleation must have been induced by the carboxyl groups contained in glutamic acids in the polypeptides [3]. In the XRD patterns, the intensity of hydroxyapatite formation on poly$(FE)_3$FG after soaking in 1.5SBF for 7 days seemed larger than that of poly$(LE)_3$LG. This may indicate the possibility that poly$(FE)_3$FG, containing a higher content of β sheet, has a little higher ability to induce hydroxyapatite formation than does poly$(LE)_3$LG, although the difference is very small.

Peptides containing alternating polar and nonpolar amino acid residues allow the resultant β strands to have a successive arrangement of side chains pointing above and below the peptide chain; thus, peptides $(LE)_3$LG and $(FE)_3$FG have carboxyl groups arranged on one side of the peptide chain. In the aggregation of β strands into β sheet, two different β sheet structures can be built. In the first structure, the polar side chains are equally distributed on

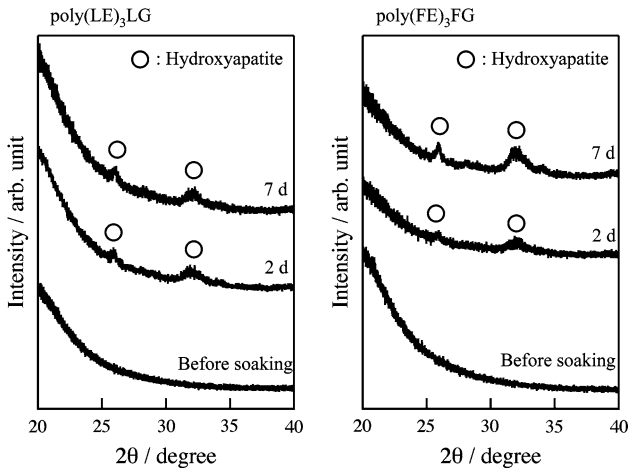

Fig. 6 TF-XRD patterns of the surfaces of polypeptide films before and after soaking in 1.5SBF for various periods

both sides of the sheet: the polar side chains are directed towards one side, those of another chain towards the other. In the second structure, all polar side chains point to the same side of the β sheet. Although the true structures of poly(LE)$_3$LG and poly(FE)$_3$FG have not been identified in this study, these polypeptides with β sheet structure must have a surface on which the carboxyl groups in glutamic acid are arranged. The ordered and concentrated carboxyl groups due to β sheet formation can thus be successful templates for hydroxyapatite nucleation.

This finding brings much information on the coating of biomimetic hydroxyapatite on organic polymer substrates under physiological conditions, and provides an attractive method for fabrication of hydroxyapatite–organic polymer hybrids through biomimetic processing, as well as for understanding the mechanism of biomineralization.

Conclusion

Polypeptides that contain β sheet structure—poly(LE)$_3$LG and poly(FE)$_3$FG—can effectively induce hydroxyapatite deposition on their surface in a solution mimicking body fluid. The induction of hydroxyapatite nucleation can be attributed to the highly concentrated or highly ordered carboxyl groups on the surfaces of poly(LE)$_3$LG and poly(FE)$_3$FG. These polypeptides with a high content of β sheet structure may be suitable substrates for the induction of hydroxyapatite deposition. The findings in this study provide important information for developing guidelines for the design of organic polymer substrates for the preparation of a novel hydroxyapatite–organic polymer hybrids, as well for the understanding of the mechanisms of biomineralization.

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