

Impartation of Apatite-forming Ability to Chitosan Nanofibers by Using Apatite Nuclei

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Abstract: Chitosan nanofiber-apatite nuclei composites obtained by mixing apatite nuclei which possess high apatite-forming ability with chitosan nanofiber has been expected to be novel bone restorative materials with suitable properties such as light weight, low coefficient of thermal expansion, high mechanical strength, biocompatibility and bioactivity. In this study, we prepared three types of apatite nuclei by changing the reaction time aimed to optimize their crystallinity and fabricated their composites with chitosan nanofiber. In order to evaluate bioactivity in vitro, we tested apatite-forming ability in simulated body fluid. As a result, the materials showed enough apatite-forming ability in a short time by mixing chitosan nanofiber and apatite nuclei with extremely low crystallinity and their high reactivity in SBF.

1. Introduction

In today's super aged society, bone prosthetic materials have been required in various medical situations. Nowadays, the gold standard of bone substitute for bone reconstruction is the autogenous bone graft. However, autograft reconstruction has some problems, such as the need of second surgery at the donor site, the limited quantity and shape of available bone, and the resorption of the graft [1-3]. To reduce the use of autogenous bone graft, various artificial materials have been used, such as metals [4,5] and ceramics like hydroxyapatite [6-9]. Metals have high mechanical strength and toughness but too high elastic modulus. Furthermore, chronic pain is caused by metallic plates, and thinning of the skin also occurs, which leads to extrusion of the plate [10,11]. Hydroxyapatite are good candidates for bone defects because of their osteocompatibility. However, they do not have flexibility, high mechanical strength and toughness, and it is difficult to adjust their shapes to the defects during the operation. Therefore, many new types of biomaterials have been studied for bone regeneration.

Nowadays, polymeric bone restorative materials attract much attention because they have light weight, high mechanical strength and flexibility and are easy to change the shape. Especially, chitosan nanofiber (ChiNF), which is obtained by deacetylation of chitin nanofiber made from crustacean, is expected to be low cost and environmentally friendly material with biocompatibility and biodegradability [12]. However, ChiNF does not have bioactivity by itself. If bioactivity can be imparted to the ChiNF, it will be expected to develop a novel bone restorative material with suitable properties such as high mechanical strength, flexibility, suitable elastic modulus and good osteocompatibility.

Bioactivity is one of the essential properties for materials to achieve a function as osteoconductive orthopedic implants, because materials that do not have bioactivity in vivo are rejected and discharged out of body. Bioactivity can be rephrased apatite-forming ability in vivo.

Hydroxyapatite is a main component of bone. If hydroxyapatite covers the surface of the bone restorative

materials spontaneously in vivo, they can be accepted easily without a body response against a foreign substances and bond directly to the bone [13]. In vitro, bioactivity is often evaluated by simulated body fluid (SBF) immersion test [14-17]. SBF is the solution which contains similar concentration ions to blood plasma. SBF highly reproduces living body environment.

If the chitosan acquires high bioactivity, that is, the above apatite-forming ability, such bioactive chitosan can be applied to bone scaffold materials that regenerate bone defects in many parts of body, because of suitable mechanical properties of chitosan for artificial bone. In a recent 20 years, bioactivity treatment to chitosan was attempted by reinforcing with β -TCP and calcium phosphate invert glass [18], by modifying with silanol groups and calcium ions [19], by phosphorylation and $\text{Ca}(\text{OH})_2$ treatment [20], and so on. However, these materials needed too long time to express apatite-forming ability from a viewpoint of the clinical experience or expressed apatite-forming ability in 1.5SBF (solution with 1.5 times concentration ions as much as SBF) instead of SBF. Also in a recent decade, several types of new bioactivity treatment to chitosan has been proposed. It was reported that the composites of chitosan, gelatin and bioactive glasses needed more than 21 days SBF immersion to observe apatite formation in SBF [21]. In the case of composite of chitosan and nanoclay containing 25 wt% octa-decylamine, apatite-forming ability was observed on the whole surface of the materials within 7 days SBF immersion [22]. In addition, it was reported that a bone scaffold consisted of polysaccharide matrix (chitosan-agarose) reinforced with hydroxyapatite needed 14-21 days to confirm the formation of apatite in SBF [23].

Recently, we have focused on high apatite-forming ability of apatite nuclei (AN). By increasing temperature and pH of SBF, nanoparticles of amorphous calcium phosphate, i.e. the above-mentioned AN, were precipitated in SBF. In the previous research, we found that the AN were highly active to induce formation of apatite in SBF [24]. In this research, we aimed to impart apatite-forming ability to ChiNF by mixing with the AN and make materials design

based on the combination of mechanical and biological performance of ChiNF and high bioactivity of AN. We prepared three types of AN with different crystallinity and compare the apatite-forming ability.

2. Materials and Methods

2.1. Preparation of SBF

Firstly, the reagent-grade NaCl, NaHCO₃, KCl, K₂HPO₄·3H₂O, MgCl₂·6H₂O, CaCl₂ and Na₂SO₄ were dissolved in distilled water. By this treatment, SBF with the ion composition as certified in ISO 23317 [17] was prepared and buffered at pH 7.40, 36.5 °C by using tris(hydroxymethyl)aminomethane ((CH₂OH)₃CNH₂) and 1 M HCl.

2.2. Fabrication of AN

An aqueous solution of twice concentration of SBF (2.0SBF) was prepared. This solution was adjusted at pH 8.2, 36.5 °C by dissolving the above tris-buffer, and then held in an incubator at 36.5 °C for 1 day, 4 days and 7 days. By this treatment, AN were precipitated in the 2.0SBF. Thus-obtained AN were filtered by suction, washed with distilled water and air-dried at 36.5 °C for 1 day. We named each type of AN as AN1, AN4 and AN7 depend on the holding time at 36.5. °C. The AN were analyzed by powder X-ray diffraction (XRD; Ultima-IV, Rigaku, Japan), scanning electron microscopy (SEM; SU6600, Hitachi High-Technologies, Japan) and energy dispersive X-ray analysis (EDX; XFlash® 5010, Bruker, USA). Before the SEM and EDX observation, gold sputtering on each sample was carried out. In the XRD measurement, commercially obtained hydroxyapatite powders (Fujifilm Wako Pure Chemical, Japan) were also measured for the reference.

2.3. Fabrication of Composites of ChiNF and AN

ChiNF slurry (BiNF-i-s® 10 wt% chitosan, Sugino Machine, Japan) was mixed with AN1, AN4 and AN7 at a weight ratio of 10 wt%. Then, the mixture was press-molded and dried in an incubator at 50 °C for 4 days. Thus-obtained AN-ChiNF composites were polished with #400 abrasive paper.

2.4. Evaluation of Bioactivity

The bioactivity of each sample was evaluated by the SBF test certified in ISO 23317 [17]. The samples were immersed in SBF under the physical condition (pH 7.4 at 36.5 °C). After the SBF test, the surface of the specimens was analyzed by thin film X-ray diffraction (TF-XRD; Rint 2500, Rigaku, Japan), SEM and EDX.

3. Results and Discussion

Fig. 1, Fig. 2 and Fig. 3 show the SEM images and EDX profiles of AN1, AN4 and AN7. In the SEM images shown in Fig. 1(a) and 2(a), spherical particles with about 100 nm in size, which were features of AN, were observed. In the SEM image shown in Fig. 3(a), however, morphology of AN7 were different from AN1 and AN4 and not only

spherical particles but also flake-like precipitates which is considered to be formed by progress of crystallization of calcium phosphate were observed. These results suggest that the AN were gradually changed to some kinds of crystalline calcium phosphate by 7 days standing of 2.0 SBF in the incubator. In the EDX profiles shown in Fig. 1(b), 2(b) and 3(b), Ca and P were detected. Hence, it is considered that these precipitates were calcium phosphate. In all of the EDX profiles, Au peaks were observed. This derived from gold covered on the samples in samples preparation for SEM observation.

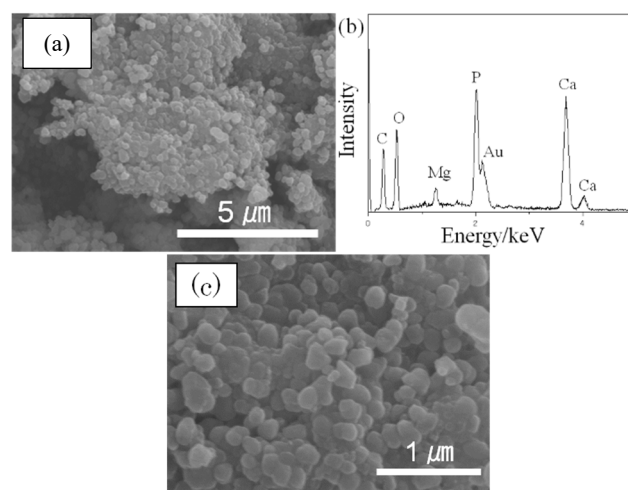


Fig. 1. (a) SEM image, (b) EDX profile and (c) SEM image in higher magnification of AN1

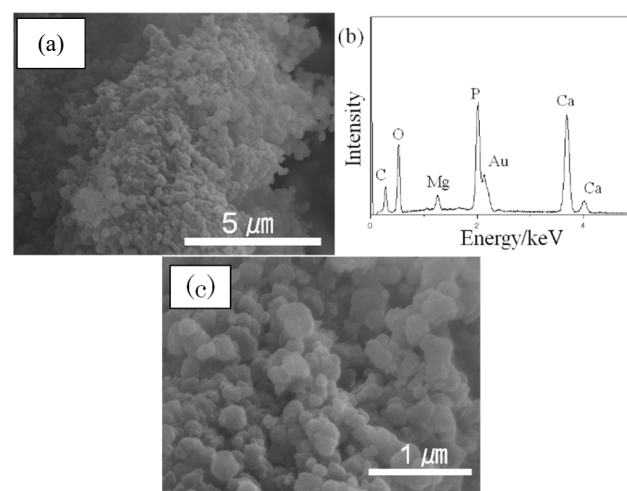


Fig. 2. (a) SEM image, (b) EDX profile and (c) SEM image in higher magnification of AN4

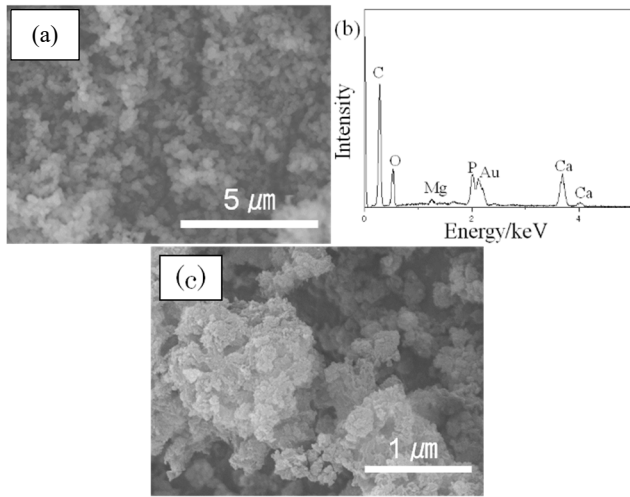


Fig. 3. (a) SEM image, (b) EDX profile and (c) SEM image in higher magnification of AN7

Fig. 4 shows powder XRD profiles of AN1, AN4, AN7 and commercially obtained hydroxyapatite powders. Diffraction peaks of commercially obtained hydroxyapatite were corresponded to JCPDS Powder Diffraction File [25]. In the case of AN obtained in this study, AN7 clearly showed diffraction peaks of hydroxyapatite with broad shape. For the AN1 and AN4, in contrast, no diffraction peaks of hydroxyapatite were observed. These results suggest that AN1 and AN4 were consisted of amorphous calcium phosphate (ACP) and AN7 contains not only ACP but also crystalline hydroxyapatite formed by progress of crystallization of ACP in the 2.0SBF.

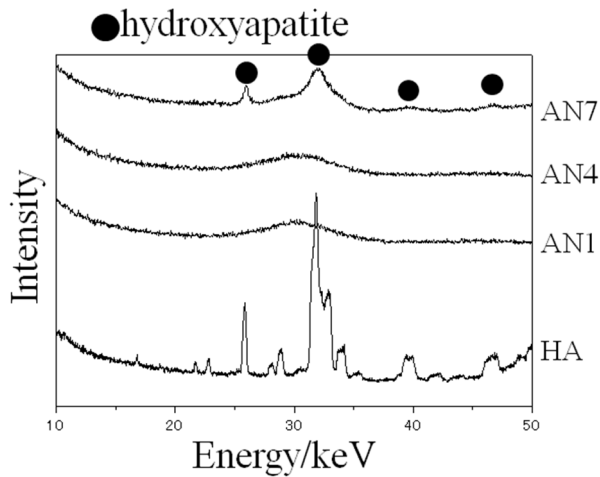


Fig. 4. Powder XRD profiles of AN1, AN4, AN7 and commercially obtained hydroxyapatite

Fig. 5 shows the SEM images and EDX profiles of the surfaces of 10 wt% AN1-ChiNF composite before and after soaking in SBF for various periods. Before soaking in SBF, smooth surface was observed. After 1 day immersion, flake-like crystallites characteristic to apatite formed on various bioactive ceramics in SBF [14-16, 26] were

observed on only a part of the surface. After 4 days and 7 days immersion, flake-like crystallites covered the whole surface of the samples. As the immersion time was increased, the intensity of peaks of calcium and phosphorous became higher compared with other peaks in the EDX.

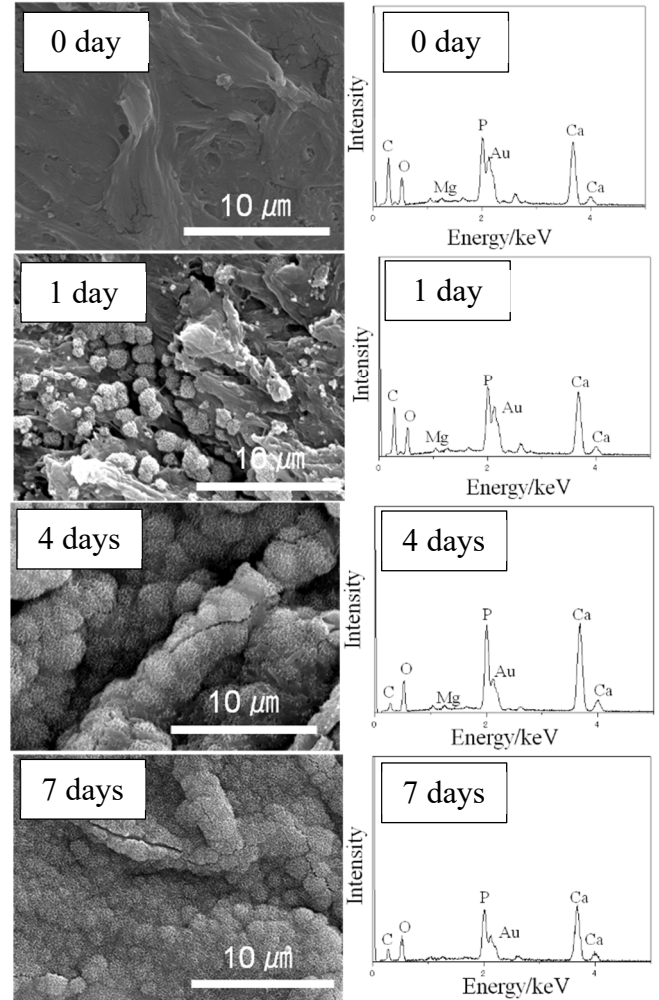


Fig. 5. SEM images and EDX profiles of the surfaces of 10 wt% AN1-ChiNF composites after soaking in SBF for various periods

Fig. 6 shows the TF-XRD profiles of the surface of 10 wt% AN1-ChiNF composite. In this result, diffraction peaks of hydroxyapatite appeared after 1 day immersion test. After longer than 4 days immersion, the intensity of the diffraction peaks of hydroxyapatite became higher. These results suggest that 10 wt% AN1-ChiNF expressed enough apatite-forming ability by SBF immersion for longer than 4 days immersion. In the case of chitosan scaffolds reinforced by β -TCP and calcium phosphate invert glass, it has been reported that they need 4 weeks immersion to confirm enough apatite-forming ability in SBF [18]. In the case of chitosan treated with phosphorylation and $\text{Ca}(\text{OH})_2$, it has been reported that they needs longer than 7 days immersion in 1.5SBF to express apatite-forming ability [20]. It has been also reported that the whole surface of the chitosan

membrane modified with silanol groups and calcium ions were covered with hydroxyapatite by 7 days SBF immersion [19]. Even in the recent reports, for example, the composites of chitosan, gelatine and bioactive glasses, chitosan and nanoclay containing 25 wt% octa-decylamine, and the bone scaffold composed of polysaccharide matrix (chitosan-agarose) reinforced with nanohydroxyapatite need at least 7 days to be observed enough apatite-forming ability in SBF [21-23]. In comparison with previously reported bioactive chitosan composites, it is considered that the method mixing ChiNF with AN1 with low crystallinity prosed in this study showed advantage in significance of higher apatite-forming ability as well as simplicity of the methodology and in SBF.

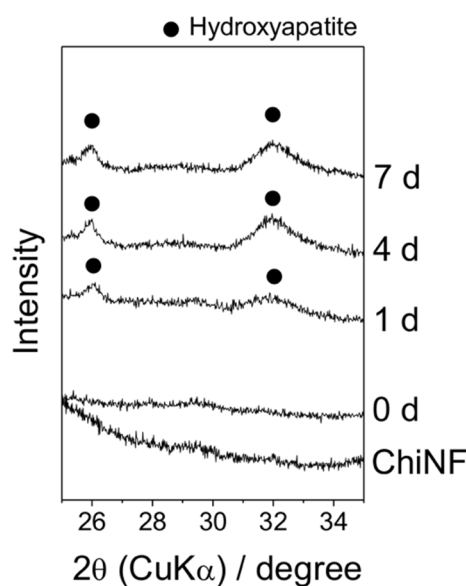


Fig. 6. TF-XRD profiles of the surfaces of 10 wt% AN1-ChiNF composites after soaking in SBF for various periods

Fig. 7 and Fig. 8 show the SEM images, EDX profiles and TF-XRD profiles of the surfaces of 10 wt% AN4-ChiNF composites before and after soaking in SBF. It was indicated that these results by applying AN4 were similar to the results in 10 wt% AN1-ChiNF composites. After 4 days immersion, the SEM and EDX observations clarified that the whole surface of the sample was covered with flake-like crystallites and the TF-XRD measurements clarified that diffraction peaks of hydroxyapatite appeared by 1 day immersion and they were intensified after 4 days immersion.

In contrast, 10 wt% AN7-ChiNF composites showed different behavior from former two composites. Fig. 9 and Fig. 10 show the SEM images, the EDX profiles and the TF-XRD profiles of the surface of 10 wt% AN7-ChiNF composites before and after soaking in SBF for various periods. In the results of the SEM even after 7 days SBF immersion, flake-like crystallites were observed not on the whole surface but only on a part of the surface of the samples. In the TF-XRD results, the intensity of diffraction peaks of hydroxyapatite was not so changed depending on the immersion period in SBF. These results mean that

apatite-forming ability of 10 wt% AN7-ChiNF composites was relatively low in comparison with AN1- or AN4-ChiNF composites. The reason why diffraction peaks of hydroxyapatite were detected on the samples even before SBF immersion is considered that AN7 consisted of hydroxyapatite and this result corresponds to the result of powder XRD shown in Fig. 4.

In our previous study, we clarified that commercially available hydroxyapatite with higher crystallinity expressed extremely lower apatite-forming ability than AN mainly consisted of ACP [27]. In addition, we proposed that such difference of apatite-forming ability was derived from the difference of dissolution speed in an aqueous solution because of their difference of crystallinity [28]. Based on our previous findings, the predicted mechanism of apatite-forming ability in this study is thought as follows. First of all, Ca^{2+} and PO_4^{3-} in AN were released to around the surface of the ChiNF. Then, the concentration of Ca^{2+} and PO_4^{3-} locally became higher. Such supersaturation of Ca^{2+} and PO_4^{3-} resulted in promotion of apatite formation on the surface of ChiNF in a short time. Hence, it is considered that lower crystallinity of AN supported effective impartation of high apatite-forming ability from a viewpoint of relatively higher solubility than crystalline hydroxyapatite.

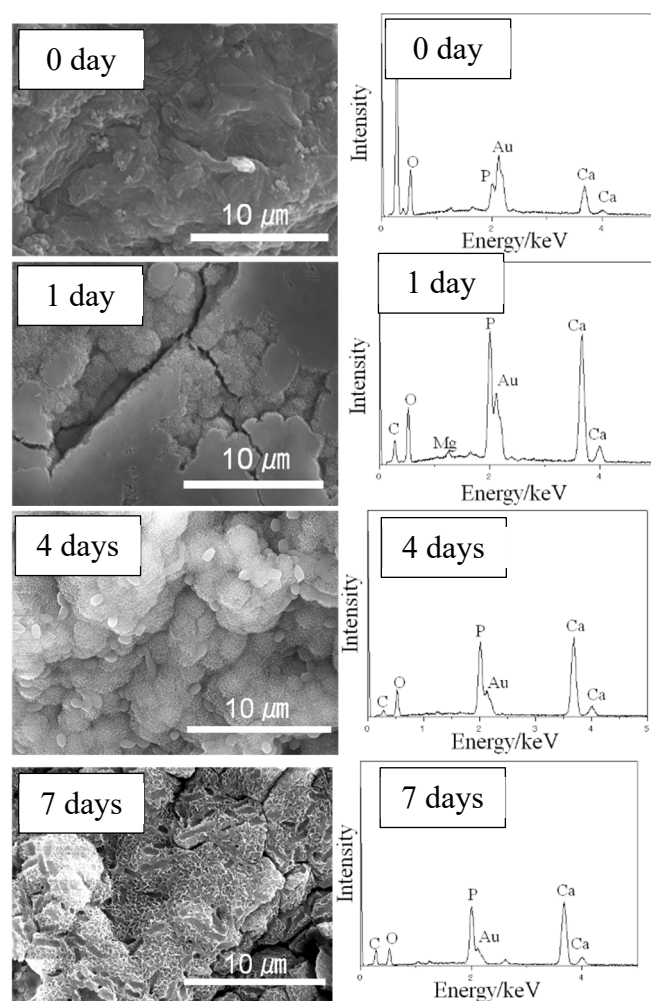


Fig. 7. SEM images and EDX profiles of the surfaces of 10 wt% AN4-ChiNF composites after soaking in SBF for various periods

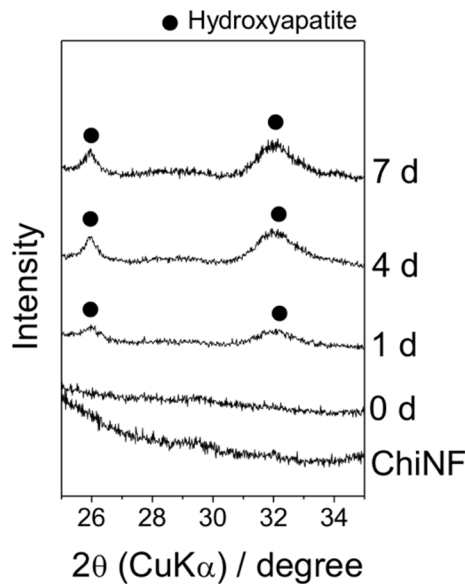


Fig. 8. TF-XRD profiles of the surfaces of 10 wt% AN4-ChiNF composites after soaking in SBF for various periods

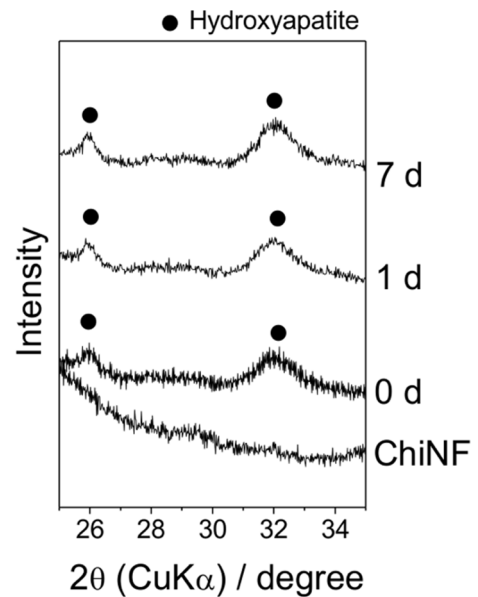


Fig. 10. TF-XRD profiles of the surfaces of 10 wt% AN7-ChiNF composites after soaking in SBF for various periods

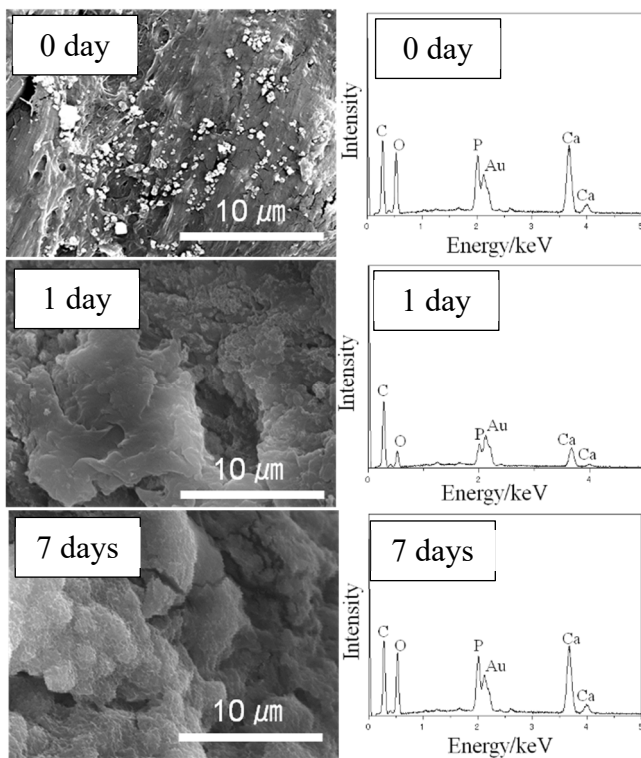


Fig. 9. SEM images and EDX profiles of the surfaces of 10 wt% AN7-ChiNF composites after soaking in SBF for various periods

4. Conclusions

We aimed to impart high apatite-forming ability to ChiNF by with three types of AN focusing on their particle properties. In the results, 10 wt% AN1-ChiNF composites and 10 wt% AN4-ChiNF composites showed good apatite-forming ability by SBF immersion test. In contrast, 10 wt% apatite-forming ability of AN7-ChiNF was relatively lower. This is because AN7 contains crystalline hydroxyapatite instead of ACP mainly. In the conclusion, mixing of ChiNF with AN1 or AN4 with extremely low crystallinity was remarkable method in the point of simplicity of material processing and its high apatite-forming ability supported by their low crystallinity. This material is expected as one of the promising biomaterials with unique mechanical and biological properties as well as bioactivity.

5. References

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