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Thermoreversible behavior of κ-carrageenan and its apatite-forming ability in simulated body fluid

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Abstract. Osteoconductive materials with self-setting ability have received much attention because their properties allow developing injectable materials for bone defects. Thermosensitive hydrogel with ability of bone-like apatite formation in a body environment is a candidate of injectable bone fillers with osteoconductivity, because the apatite formation on materials is an essential for materials to show osteoconduction. The present study is focused on development of a thermosensitive hydrogel with the apatite forming ability through suitable modifications of κ-carrageenan that is a polysaccharide with sulfonic groups. Modification of κ-carrageenan was conducted by addition of potassium chloride (KCl) and calcium chloride ($CaCl_2$). Effects of the modification on gelation behavior and the apatite-forming ability were investigated. The gelation temperature of κ-carrageenan solutions increased with increasing the amount of K^+ ions. Apatite formation was observed on the gel after exposing to a simulated body fluid for 0.5 day when the gel was prepared at Ca^{2+} concentration of Ca^{2+}/sulfonic groups=1.5 in molar ratio. These results indicate that thermosensitive κ-carrageenan hydrogel with apatite-forming ability, i.e. potential of osteoconduction, was obtained through addition of appropriate amounts of K^+ and Ca^{2+} ions.

Keyword: κ-carrageenan, Hydroxyapatite, Hydrogel, Simulated body fluid, Injectable material.

1. Introduction

Bioactive ceramics show specific biological affinity to living bone when they are implanted in bony defects to achieve direct bonding to living bone. The bone-bonding property, i.e., osteoconductivity of the bioactive ceramics is generally established by formation of a bone-like apatite layer after exposure to body fluid [1]. Among bioactive ceramics hitherto known, bioactive calcium phosphate paste is one of attractive materials that have a special performance given by its self-setting property. Such material can exhibit not only high biological affinity to living bone but also unique formability in appropriate shapes during operation to fill bone defects. Still, bioactive paste made from calcium phosphates shows brittle and fragile characters in its mechanical property, compared to those of natural bone. Human bone is a composite consisting of hydroxyapatite and collagen. Based on this finding, many researchers attempted to develop bioactive organic-inorganic hybrids to achieve soft materials with bone-bonding property [2,3].

Modification by an organic polymer with silanol (Si-OH) groups and a calcium salt is a typical process to induce apatite forming ability to organic-inorganic hybrids [2]. This design was proposed on the basis of the finding that the silanol group can provide favorable site for nucleation of hydroxyapatite and that the release of Ca^{2+} ion increase

the degree of supersaturation with respect to hydroxyapatite in the surrounding body fluid [4]. It was reported recently that sulfonic groups worked as nucleation sites for the apatite formation [5-6]. This means that the modification by a polymer containing sulfonic group also produces a candidate materials with bone-bonding ability through incorporation of appropriate amount of Ca^{2+} ions. Thus a polymer containing sulfonic groups with thermoreversible gelation around the body temperature would be useful on development of bioactive materials with self-setting capability. It was reported that poly(N-isopropylacrylamide) (PNIPAAm) showed thermogelling behavior [7]. So, it was investigated on its biological applications. However, PNIPAAm showed the limitation as tissue replacement because it has the lack of tissue interface. PNIPAAm is needed modification of organic molecules to improve cell and tissue response. Moreover, the absence of a functional group on PNIPAAm to induce hydroxyapatite nucleation makes the limitation on bone replacement. We have studied carrageenan as a polymer with sulfonic groups to form an apatite in body environment, and found a suitable modification of carrageenan provides potential of apatite formation. Carrageenan is a *Rhodophyta*-derived polysaccharide with side-chains of sulfonic groups. Among the carrageenans, the present study focused κ-carrageenan as a candidate for bioactive material with gelation ability at an appropriate temperature. The

gelation temperature of its solution varies with concentrations of additives such as potassium chloride (KCl) [8-10]. Both the gelation temperature and apatite-forming ability are attributed to the interaction of sulfonic groups in the polysaccharide and cations incorporated in the polymer solution. Previously, our group reported the differences in apatite formation on κ- and λ-carrageenan by incorporation of Ca^{2+} ion [11]. However, the relationship between the thermoreversible gelation behavior and apatite-forming ability is not clear after modification of κ-carrageenan with additives such as KCl and calcium chloride ($CaCl_2$), in spite that this point is quite important for optimization of thermoreversible materials with injectability and osteoconductivity.

The aim of this study is to evaluate the conditions for preparation of injectable material made from κ-carrageenan hydrogels under the presence of cations. We investigated the effects of K^+ and Ca^{2+} ions on rheological properties of κ-carrageenan hydrogels to optimize the gelation temperature. Apatite formation on the κ-carrageenan gels modified with the cations was also investigated in a simulated body fluid, and discussed in terms of ionic interaction of the κ-carrageenan.

2. Experimental procedure

2.1 Preparation of κ-carrageenan hydrogels

The κ-carrageenan was purchased from Tokyo chemical industry Co. Ltd., Japan, and used without further purification. Chemical structure of κ-carrageenan was shown in Figure 1. Aqueous solution of 1 mass% κ-carrageenan was prepared by dissolving κ-carrageenan into ultra-pure water at 50 °C. KCl (Nacalai Tesque Inc., Kyoto, Japan) and $CaCl_2$ (Nacalai Tesque Inc.) were then added into the κ-carrageenan solution at a concentration ranging from 0.0 to 1.5 in molar ratio against sulfonic groups in the κ-carrageenan. The examined compositions were given in Table 1. After the dissolution of the κ-carrageenan, KCl and $CaCl_2$, the gelation behavior of the solution were observed during cooled to room temperature. Viscosity of the solution was measured from 30 °C to 55 °C by a viscometer (Visconic EHD, Tokyo Keiki Inc., Tokyo, Japan). The gelation temperatures of the κ-carrageenan solutions were determined as a temperature where the viscosity of the solution increased.

2.2 *In vitro* evaluation of apatite formation

Apatite formation of the prepared hydrogels was examined by exposure to a simulated body fluid proposed by Kokubo and his colleagues. The apatite-forming ability on the surface of the materials is well correlated to show the potential of osteoconductivity in bony defects [12,13]. The simulated body fluid (SBF; Na^+ 142.0,

K^+ 5.0, Mg^{2+} 1.5, Ca^{2+} 2.5, Cl^- 147.8, HCO_3^- 4.2, HPO_4^{2-} 1.0, SO_4^{2-} 0.5 mol $\cdot m^{-3}$) has nearly equal ion concentrations to those of human blood plasma with pH 7.25 at 36.5 °C. 5 cm^3 of the κ-carrageenan solutions added with KCl and $CaCl_2$ were transferred to polystyrene bottles (40 cm^3), followed by natural cooling to room temperature, in order to obtain the hydrogels. 30 cm^3 of SBF was then added to the bottles containing the hydrogel, to be kept at 36.5 °C for various periods. After the predetermined periods, the hydrogels were removed from the SBF, and washed with the mixed solvent of ultra-pure water and *tert*-butanol at water:*tert*-butanol=50:50 in volume ratio using shaking apparatus for 2 days. The hydrogels were then applied to freeze-drying. The hydrogels were characterized by X-ray diffraction (XRD; RINT2100, Rigaku Co., Japan). The surfaces of the hydrogels coated by sputtering gold were observed under a scanning electron microscope (SEM; JSM-5600, JEOL Ltd., Japan). The concentrations of calcium (Ca), phosphorus (P) and sulfur (S) in SBF after exposure of the hydrogels were measured by induced coupled plasma atomic emission spectroscopy (ICP-AES; Optima 2000DV, PerkinElmer Japan Co. Ltd., Japan). The pH changes of the SBF were also measured by a pH electrode (HM-25R, DKK-TOA Corporation, Japan).

3. Results

Figure 2 shows the relationship between temperature and viscosity of the κ-carrageenan solutions with various contents of KCl and with a constant concentration of $CaCl_2$. In the case of K00Ca15 solution, viscosity of the solution distinctly decreased in the temperature range from 32 to 36 °C. K05Ca15 solution shows higher temperature regions, i.e. from 40 to 42 °C, on the decreasing the viscosity. K10Ca15 solution showed relatively small increase in the viscosity in temperature region from 46 to 52 °C. Since the measurement of the viscosity of the solution was measured during the cooling the solution from 55 to 30 °C, the increase in the viscosity relates the gelation during the cooling. Addition of KCl in the κ-carrageenan solutions gave heightening the temperature on gelation when the solution contained same amounts of $CaCl_2$. After the gelation during the cooling, the gels were broken to decrease viscosity of the hydrogels at lower temperature than gelation. Figure 3 shows appearance of the K05Ca15 kept at about 55 °C, followed by cooling to 30 °C, and then heated to 55 °C. Reversible sol-gel transition was observed according to change in temperature. These results indicate that thermoreveresible hydrogel which can gel at around 40 °C was successfully prepared by addition of KCl as well as $CaCl_2$.

From the results of gelation behavior of the solution, the samples K05Ca10 and K05Ca15 were examined on its apatite-forming ability in SBF. Figure 4 show the SEM

photographs of the surfaces of □□κ-carrageenan hydrogels soaked in SBF for various periods. Spherical particles were observed on the surfaces of the K05Ca15 hydrogel, whereas no formation of precipitates suggesting formation of calcium phosphate was observed on the surface of K05Ca10 hydrogel soaked in SBF even for 7 days. Figure 5 shows X-ray diffraction patterns of K05Ca10 and K05Ca15 hydrogels soaked in the SBF for various periods. The “0 day” indicates hydrogel samples without soaking in SBF. Broad diffraction was detected on the patterns on the K05Ca10 hydrogel without soaking in SBF, as well as K05Ca15. The samples of K05Ca10 soaking in SBF showed broad diffraction even after soaking for 7 days. Whilst K05Ca15 samples showed peaks at 2θ = 26° and 32°after soaking in SBF for 0.5 day and more. The peaks at 2θ = 26° and 32° were assigned to (002) plane and to envelope of (211), (112) and (300) planes of hydroxyapatite (PDF Card #09-0432). These results indicate that the K05Ca15 formed bone-like hydroxyapatite on its surface as a particle within 0.5 days after exposure to SBF, but not K05Ca10.

Figure 6 shows the changes in calcium (Ca), phosphorous (P) and sulfur (S) concentrations and pH of SBF due to the immersion of the κ-carrageenan hydrogels K05Ca10 and K05Ca15. The Ca concentrations of the SBF after exposure of the hydrogels for 0.5 day were rapidly increased both for the K05Ca10 and K05Ca15. The

increase in Ca concentration of the SBF was attributed to dissolution of Ca^{2+} ions from the hydrogels. K05Ca15 showed larger amounts of released Ca^{2+} ions than K05Ca10. P concentration of SBF was decreased remarkably after immersion of K05Ca15 compared to that after immersion of K05Ca10. The large decrease in P concentration was attributed to consumption of phosphate ions from SBF to form the apatite on the surface of the hydrogels. Slight decrease in P was detected on the changes in P concentration of SBF after immersion of K05Ca10. This means a probable formation of amorphous calcium phosphate on the hydrogel but not hydroxyapatite on K05Ca10 hydrogels. Slight decrease in pH of SBF after exposure of K05Ca15 was also observed. The decrease in pH of SBF was also caused by formation of apatite precipitates in SBF. Increase in S concentrations of the SBF was attributed to dissolution of κ-carrageenan from the hydrogel. Larger amounts of dissolved sulfur was observed for K05Ca10 that for K05Ca15.

4. Discussion

It is apparent from the results of the changes in temperature dependence of viscosity due to the amounts of additives that the gelation temperatures of the κ-carrageenan hydrogels can be controlled by addition of K^+ ions. Previous reports

showed that the gelation of κ-carrageenan solution depends on the addition of cations, especially K^+ ion [14-16]. The gelation is explained by temperature-dependent transition from random coil to helix, followed by formation of the helices aggregate. The aggregation forms a physical crosslinkage, leading to a macroscopic three-dimensional network. Consequently, the κ-carrageenan hydrogel is obtained by the transition of the random coil to helix. During the conformational change of κ-carrageenan, the addition of K^+ ions leads interaction between two coils or two helices. Namely the addition of K^+ ions enhances the formation of aggregation and subsequent gelation, since mobility of the κ-carrageenan molecules is decreased. In the present study, the gelation temperature by addition of K^+ ions into κ-carrageenan system, under the presence of Ca^{2+} ions was distinctly increased by increasing the molar ratio of K^+ ions/sulfonic groups (K^+/SO_3H) at constant amounts of calcium chloride. Among the examined compositions, the K05Ca15 hydrogel seems appropriate for the injectable material because its gelation occurred at the temperature ranging 40-42 °C. This result shows that the K05Ca15 hydrogel can be useful as a candidate of injectable bone fillers, because the phase transition of κ-carrageenan will occur in the bone defects after injection.

Osteoconductive materials able to bond to living human bone generally show ability on formation of bone-like apatite in an acellular simulated body fluid (SBF)

proposed by Kokubo and his colleagues. The direct contact of the surface of materials to living bone is achieved through the bone-like apatite layer formed on the surface of the materials after implantation [13]. Previous studies reported that the apatite formation is induced by the presence of the functional groups that play a role in heterogeneous nucleation sites for hydroxyapatite [17-19], while Ca^{2+} ions released from a material increase the degree of supersaturation with respect to hydroxyapatite of the surrounding fluid. Sulfonic groups existed in κ-carrageenan are reported to have a potential to induce heterogeneous nucleation of hydroxyapatite, while incorporated $CaCl_2$ released Ca^{2+} ions in the body fluid. The present study indicated that K05Ca15 hydrogel showed potential of bone-like apatite formation on its surface after exposure to SBF for 0.5 day, while Ca05Ca10 did not. The increase in Ca concentration of SBF after soaking of K05Ca15 was larger than that of K05Ca10, so that the increased degree of the supersaturation of the surrounding body fluid with respect to hydroxyapatite mainly determines the potential of bone-like apatite formation, that is osteoconductivity, of the κ-carrageenan hydrogel. Molar ratio of Ca^{2+}/sulfonic groups in K05Ca15 gel is 1.5. Because the complexes, such as $-SO_3Ca^+$ or $(-SO_3)_2Ca$, formed in κ-carrageenan may little contribute to release Ca^{2+} ions, the excess amounts of Ca^{2+} that hardly interact with sulfonic groups in κ-carrageenan may be released from the hydrogel. Actually, the

dissolution of κ-carrageenan hydrogel is more apparent on K05Ca10 than K05Ca15 from the results of increased sulfur concentrations due to immersion of the hydrogels. This means that the K05Ca15 is more stable in SBF than K05Ca10. Lager amounts of Ca^{2+} may contribute to crosslinkage between molecules of κ-carrageenan. K05Ca15 has an ability of formation of bone-like hydroxyapatite on the surface to cover the hydrogels. Thus it is expected that the formed apatite-layer also prevent the further dissolution of the κ-carrageenan and K^{+} and Ca^{2+} ions to the surrounding body fluid. All the finding suggested the optimization of amounts of additives not only of Ca^{2+} but also of K^{+} is essential to develop thermoreversible osteoconductive hydrogel derived from for the modification of κ-carrageenan.

5. Conclusions

We have succeeded to obtain thermoreversible κ-carrageenan hydrogel with apatite-forming ability by addition of K^{+} ions as well as Ca^{2+} ions. It was found that the gelation temperature of κ-carrageenan can be controlled by the amounts of potassium contents added. The apatite-forming ability of the hydrogel was controlled by addition of Ca^{2+} ions. The κ-carrageenan hydrogel modified with K^{+} and Ca^{2+} ions allows to produce injectable osteoconductive materials for reconstruction of damaged bone.

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Table 1 Sample notations and molar ratio of K^+ and Ca^{2+} ions against sulfonic groups (S) in 1mass% solution of κ-carrageenan

Notation	KCl		$CaCl_2$	
	Concentration / mol m^{-3}	K/S	Concentration / mol m^{-3}	Ca/S
K00Ca15	0.0	0.0	39.0	1.5
K05Ca15	13.0	0.5	39.0	1.5
K05Ca10	13.0	0.5	26.0	1.0
K10Ca15	26.0	1.0	39.0	1.5

Figure captions

Figure 1 Chemical structure of κ-carrageenan.

Figure 2 Relationship between temperature and viscosities of the κ-carrageenan solutions.

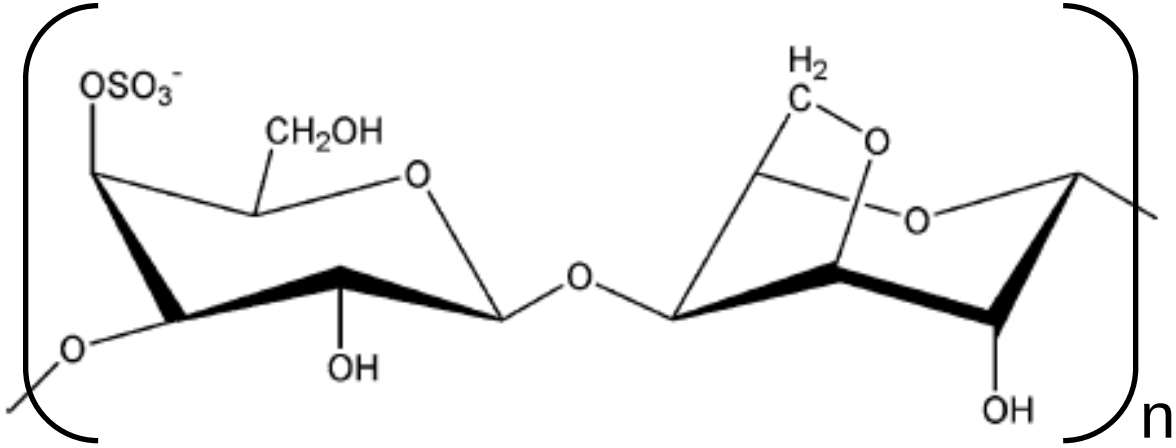
Figure 3 Appearance of K05Ca15 solution, in the conditions, (a) heating at 50 °C after preparation, (b) cooling at 30 °C, and (c) re-heating at 50 °C.

Figure 4 SEM photographs of the surfaces of K5-Ca10 and K5-Ca15 hydrogels exposed to the simulated body fluid (SBF) for various periods.

Figure 5 X-ray diffraction patterns of K5-Ca10 and K5-Ca15 hydrogels exposed to the simulated body fluid (SBF) for various periods.

Figure 6 Changes in Ca, P and S concentrations and in pH of the simulated body fluid (SBF) due to immersion of the hydrogels.

Figures



α(1–3)-D-galactose **β**(1–4)-3,6-anhydro-D-galactose

Figure 1 Kim *et al.*

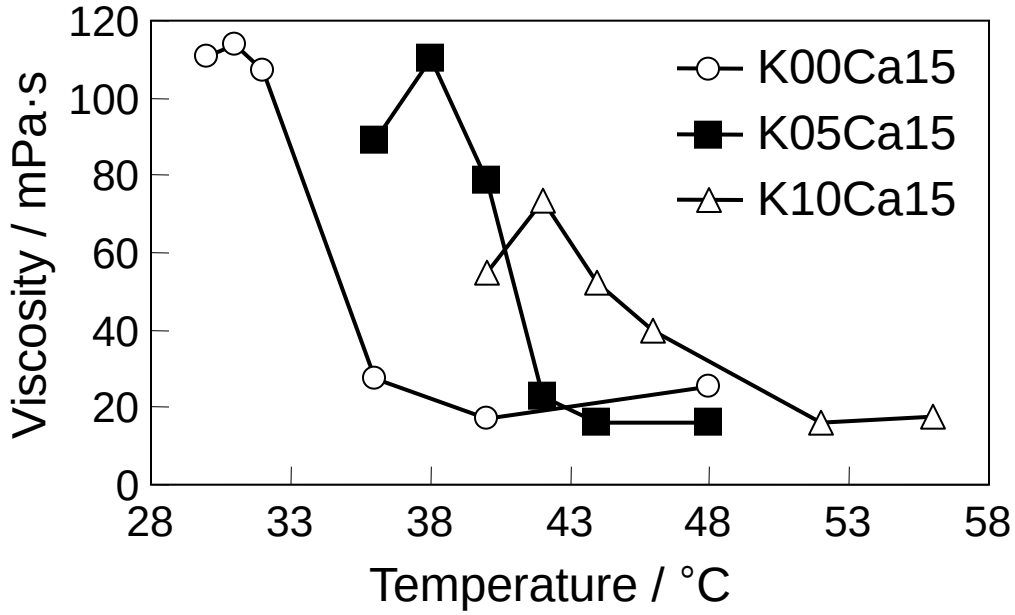


Figure 2 Kim *et al.*

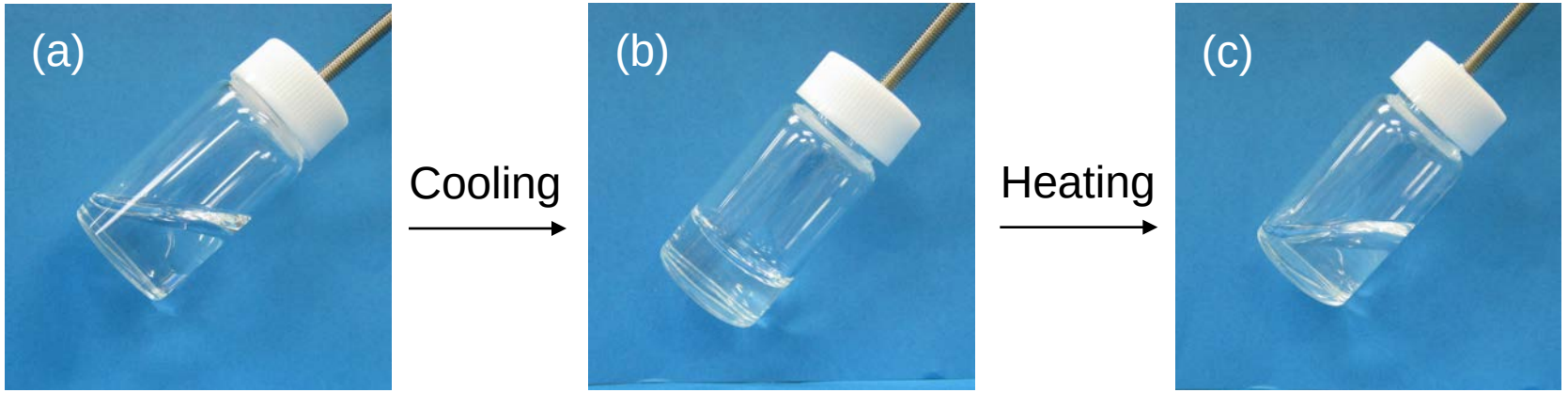
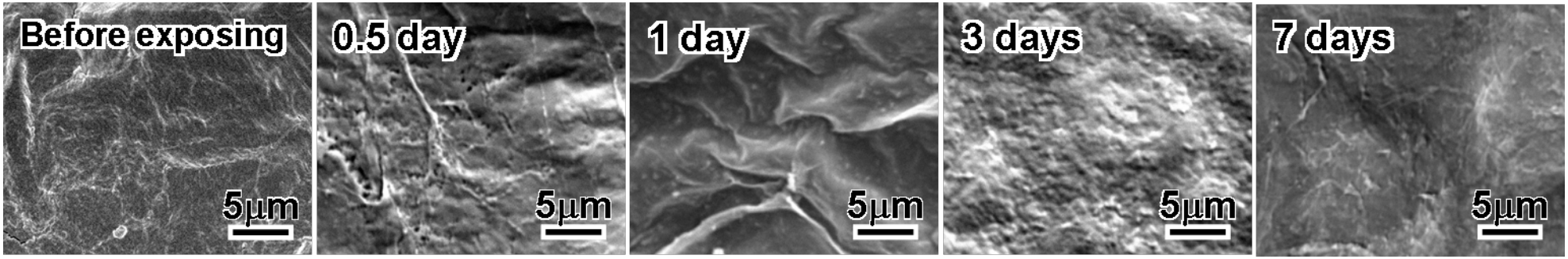


Figure 3 Kim *et al.*

K05Ca10



K05Ca15

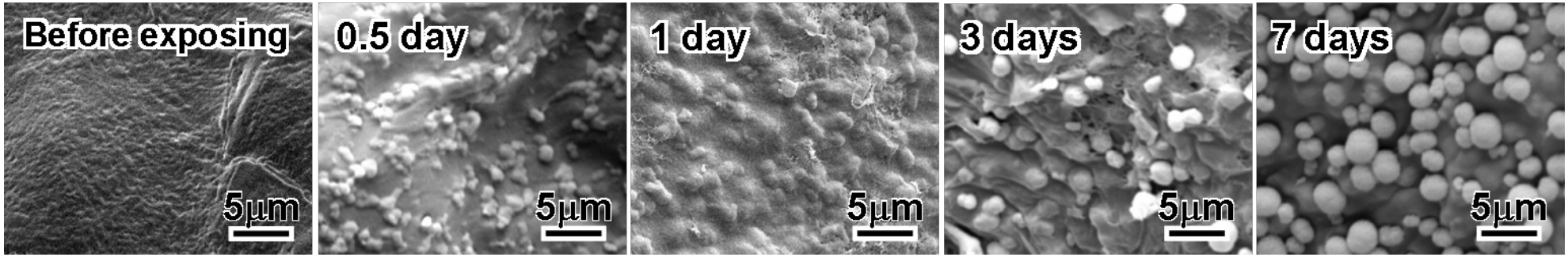
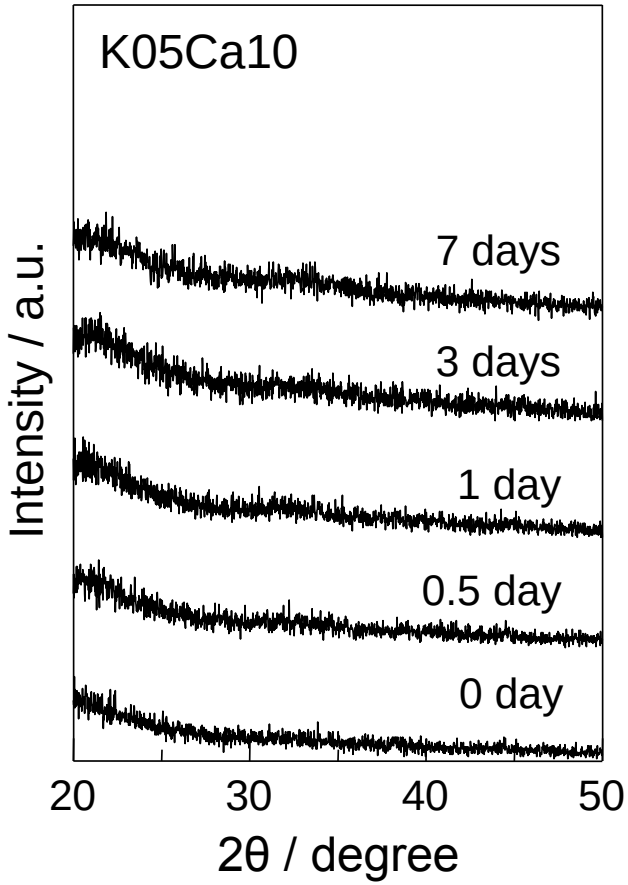


Figure 4 Kim *et al.*



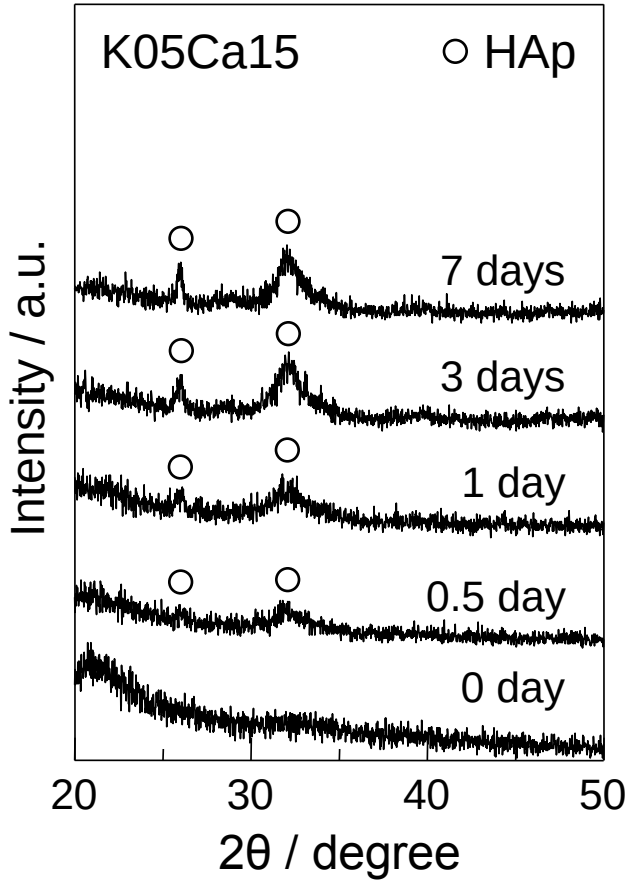


Figure 5 Kim *et al.*

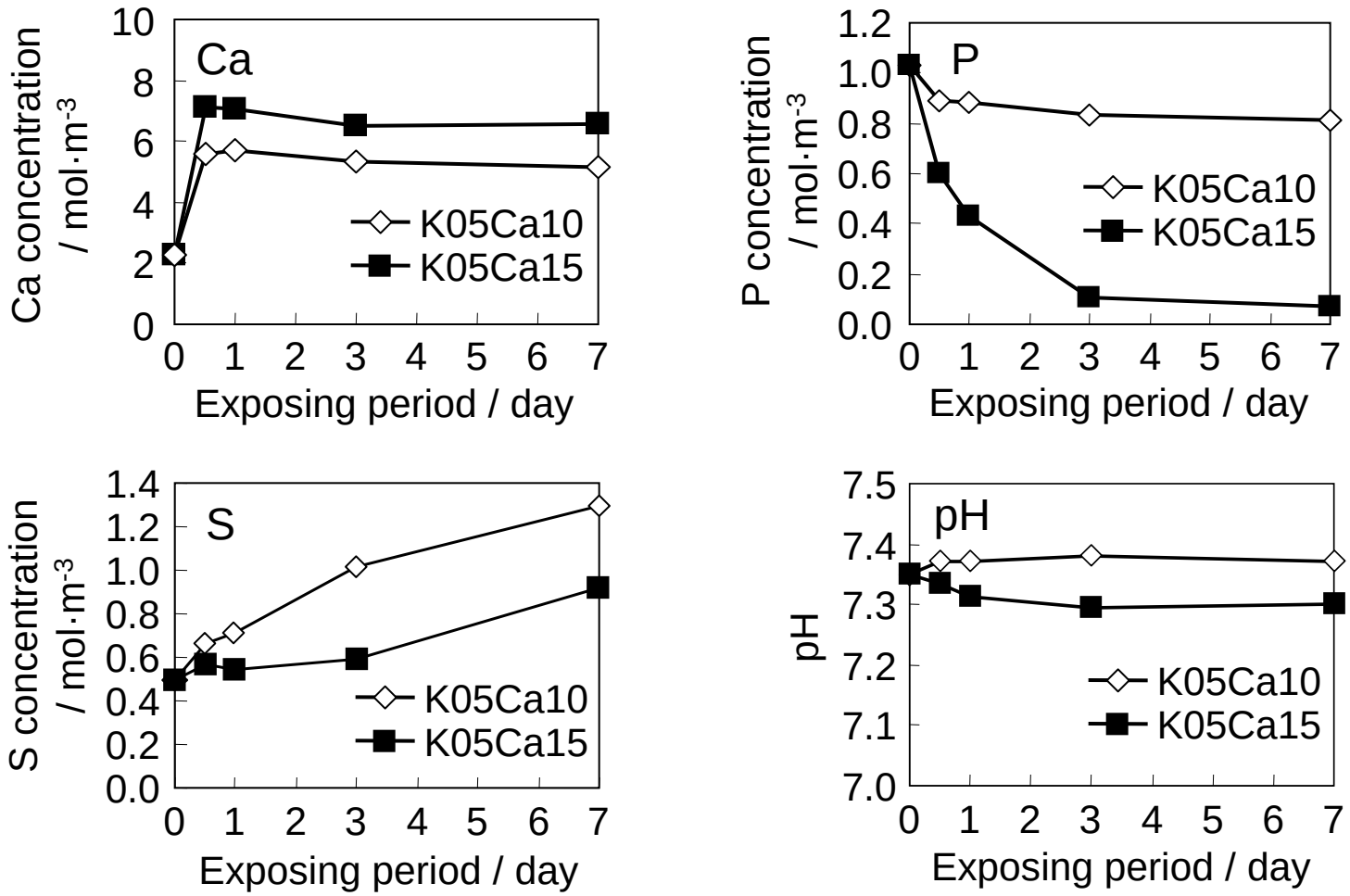


Figure 6 Kim *et al.*