Bio-Medical Materials and Engineering 13 (2003) 247–259
IOS Press

Evaluation of apatite ceramics containing α-tricalcium phosphate by immersion in simulated body fluid

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Received 1 July 2002

Abstract. The purpose of this study was to estimate the availability of α-tricalcium phosphate (α-TCP) on/in hydroxyapatite (HAP) ceramics for bioactivity as bone-substitute materials by immersion in a simulated body fluid (SBF; Hanks' solution) containing ion concentrations similar to those in human blood plasma. Two α-TCP-surface-modified HAP and α-TCP-HAP composite materials were prepared by orthophosphoric acid treatment of sintered HAP and controlling the crystal phases of calcium phosphate cement, respectively. After immersion in SBF, the sintered HAP modified on the surface in an approximately 0.2 μm α-TCP layer was more effective for the precipitation of carbonated apatites than an approximately 2 μm α-TCP layer and HAP-only layer. In the calcium phosphate cements consisting of HAP and α-TCP phases, after immersion for 1 week, the specimens precipitated large amounts of apatites having α-TCP contents of approximately 25% and 50% in the cement. The results of immersion tests imply the possibility that the α-TCP on/in HAP ceramics may be a bioactive agent for bone-substituting HAP materials.

Keywords: Hydroxyapatite ceramic, α-tricalcium phosphate, simulated body fluid, precipitation, bioactivity

1. Introduction

Hydroxyapatite (HAP; $Ca_{10}(PO_4)_6(OH)_2$) has been widely employed for various biomaterials in the dental and medical fields. The HAP materials are known to bond with bone directly and to possess excellent tissue responses because of their bioactivity and biocompatibility [1–3]. On the other hand, α-tricalcium phosphate (α-TCP; α-$Ca_3(PO_4)_2$) has become popular as a base material for calcium phosphate cements [4–6], though it has never been applied for use as a biomaterial because its solubility is much higher than those of HAP and β-tricalcium phosphate (β-TCP). α-TCP powder sets to form calcium-deficient HAP when mixed with water, and thus is used in dental and medical clinics as a root sealer and bone-filling cement [7–14]. However, the α-TCP crystal phase in the set α-TCP cement takes a long time-several days-for the transformation to HAP. Therefore, α-TCP abundantly exists in the set cement for a long time. We reported that the crystal phase in set α-TCP cement could be changed from α-TCP to HAP within 24 hours by the use of two mixing liquids consisting of calcium chloride and sodium hydrogen phosphate solutions [15]. This occurs because of the better biocompatibility of the α-TCP cement. α-TCP is known to have increased pH when it dissolves in water [4]. This increase of

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pH in the α-TCP cement results in the presence of inflammatory cells in the surrounding set cement until several weeks after implantation [11,12,14,16]. However, α-TCP is a bioactive material because of its high solubility. Therefore, the α-TCP content in the set cement strongly influences the biocompatibility and bioactivity. Many reports have suggested that composite materials consisting of α-TCP and HAP have better bioactivity than HAP-alone material [17–19]. Moreover, these reports indicated that a small quantity of α-TCP in composite calcium phosphate ceramics was better than a large quantity or no α-TCP at all. The effective α-TCP content in the HAP base material is difficult to determine because it differs for each material such as cements or sintered ceramics.

On the other hand, biocompatibility and bioactivity are governed by the character of the surface layer on biomaterials. We tried to develop a functionally gradient ceramic calcium phosphate (FG-CCP) made of sintered HAP material, and having a surface of only α-TCP, the content of which gradually decreased with increasing depth from the outermost surface layer [20,21]. This FG-CCP could be produced by two methods, using a diamond powder and acid treatment, of the HAP ceramic. The FG-CCP with a gradual compositional change from α-TCP (surface) to HAP (inside) may have potential value as a bioactive material (bone replacement and bone substitution), because of the high solubility of the α-TCP on the outermost layer [22]. Although the detailed mechanism of α-TCP bioactivity has not yet been clarified, it seems reasonable that bone formation requires original materials, that is, calcium and phosphate. If this hypothesis is correct, the availability of α-TCP of a biomaterial such FG-CCP will be confirmed by *in vitro* and *in vivo* reactions.

In this investigation, we prepared two calcium phosphate ceramics containing α-TCP. One of the two ceramics was a calcium phosphate cement controlling and/or consisting α-TCP and HAP crystal phases. The other one was a sintered HAP material with a gradual compositional change from α-TCP (outermost surface layer) to HAP (inside). The aim of this investigation was to estimate the availability of α-TCP in HAP materials. The two HAP materials with controlled α-TCP content were immersed in a simulated body fluid (SBF) for a maximum of 8 weeks. After immersion in SBF, the specimens were evaluated by investigating the mass change (precipitation and dissolution), crystal phase and microstructure. The influences of the α-TCP phases in the HAP materials on the precipitation and dissolution with immersion in SBF are discussed.

2. Materials and methods

2.1. Preparation of HAP ceramics containing α-TCP

For preparation of sintered HAP ceramics containing α-TCP, the precipitated HAP powder used for sintering was calcined at 800°C for 5 h. After crushing and controlling the calcined HAP, the average particle size measured by a sedimentation method based on Stokes' law with the use of an automatic centrifugal particle analyzer (CAPA 300, Horiba, Kyoto, Japan) was 2 μm. A powder compact 6 mm in diameter and 3 mm thick was prepared by dry-pressing the HAP powder. The powder compact was sintered by heating at 1280°C for 3 h. The bulk density of sintered HAP was 2.95 g/cm^3 (HAP density; 3.16 g/cm^3). For preparation of sintered calcium phosphates with a compositional gradient change from α-TCP (surface) to HAP (inside), the sintered HAP was immersed in an orthophosphoric acid (H_3PO_4) solution of 1.0 or 5.0 mol/l for 1 h [21]. After immersion, the specimens were first rinsed in distilled water, and then ultrasonically washed in distilled water for 15 min. The two types of sintered HAP treated with the acid solutions were heated to 1250°C at a heating rate of 5°C/min, and kept at 1250°C

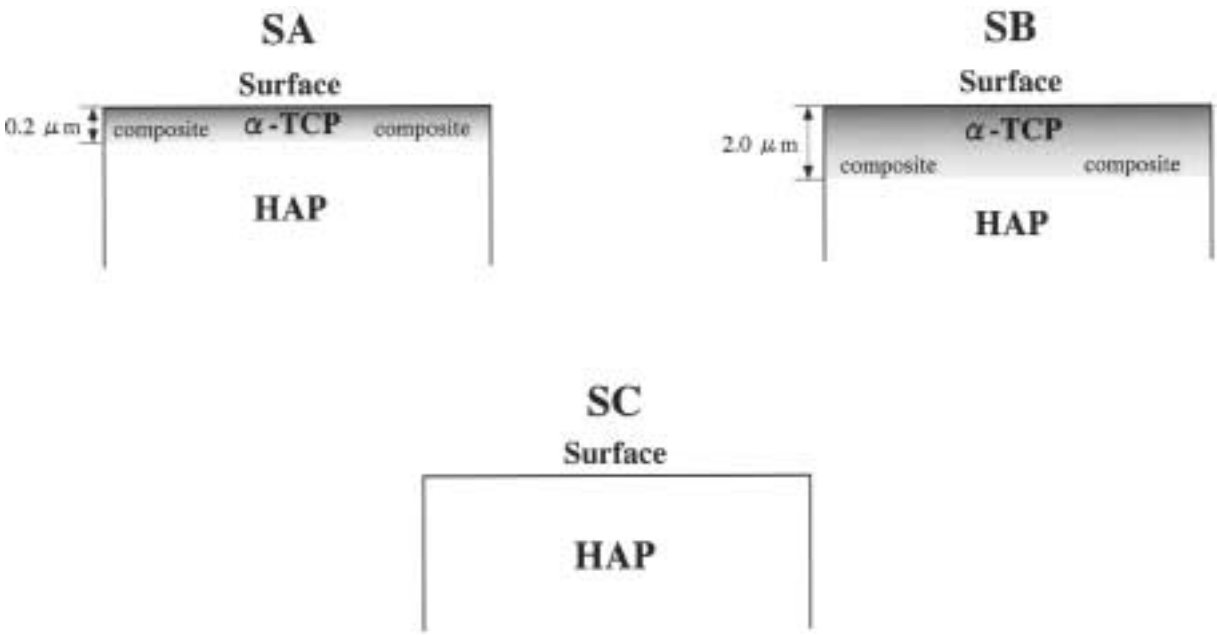

Fig. 1. Schematic illustration of sintered HAP specimens surface-modified with 1.0 mol/l or 5.0 mol/l orthophosphoric acid (H_3PO_4) solution and heat (at 1250°C) treatments. SA: 1.0 mol/l H_3PO_4 solution, SB: 5.0 mol/l H_3PO_4 solution, SC: untreated specimen used as a control. HAP: hydroxyapatite, α-TCP: α-tricalcium phosphate.

for 1 h in a furnace, because of the α-TCP formation on the surface layer. The sintered specimens were classified into the following three groups: SA, 1.0 mol/l H_3PO_4 solution and heat treatments; SB, 5.0 mol/l H_3PO_4 solution and heat treatments; and SC, untreated used as a control, as shown in Fig. 1. The α-TCP on the surface layers of specimens SA and SB with acid and heat treatments extended to the depths of approximately 0.2 and 2.0 μm from the outermost surface layers, respectively, measured by X-ray photoelectron spectroscopy (XPS; Quantum 2000, Perkin-Elmer Co., Wellesley, MA, USA) in a previous study [21]. However, on the outermost layer of specimen SA to a depth of around 0.05 μm there was calcium phosphate with a Ca/P ratio of approximately 1.0 [21].

For calcium phosphate cements containing α-TCP, the cement powder, α-TCP, was prepared from a mixed powder consisting of calcium carbonate ($CaCO_3$) and dicalcium phosphate dihydrate (DCPD: $CaHPO_4 \cdot 2H_2O$) with a Ca/P ratio of 1.5. The mixed powder was heat-treated at 1400°C for 5 h, and quenched in air to form α-TCP. After heat treatment, X-ray diffraction of the resultant α-TCP revealed no other crystals. The α-TCP was crushed to a fine powder in an alumina ceramic cell by a ball mill (P-7 Planetary Micro Pulverizer, Fritsch Co., Idar Obertein, Germany). As liquid phases for mixing the α-TCP powder, distilled water, calcium chloride ($CaCl_2$) and sodium hydrogen phosphate (NaH_2PO_4) were applied for different crystal phases in set cements [15]. To combine the powder and liquid phases, the α-TCP powder was first mixed with $CaCl_2$ solution, and then mixed with the same volume of NaH_2PO_4 solution as for the calcium solution at a total powder/liquid ratio of 2.0. The total time for mixing of cement was 1.0 min. After mixing, the cement paste was placed in an acrylic mold 6 mm in diameter $\times$ 3 mm thick. After setting, the specimens were taken out of the molds and kept in an incubator at temperature of 37°C and a relative humidity of approximately 100% for 24 h. The cement specimens were classified into the following three groups; CA) mixed with 1.0 mol/l $CaCl_2$ and 0.6 mol/l NaH_2PO_4 solutions, CB) mixed with 0.2 mol/l $CaCl_2$ and 0.12 mol/l NaH_2PO_4 solutions, and CC) mixed only

with distilled water. At 24 h after mixing, there were three types of HAP and α-TCP crystals in the set cements. The α-TCP contents in set cement specimens CA, CB and CC were approximately 25%, 50% and 90%, respectively, measured by the X-ray diffraction method.

2.2. Immersion in SBF (Hanks' solution)

As a simulated body fluid, Hanks' solution was prepared without organic species, and pH was adjusted to 7.4 with 7.5% $NaHCO_3$ solution [23–25]. The final inorganic ion concentrations (mol/l) of the solution were 1.42×10^{-1} Na^+, 5.81×10^{-3} K^{2+}, 8.11×10^{-4} Mg^{2+}, 1.26×10^{-3} Ca^{2+}, 1.45×10^{-1} Cl^-, 7.78×10^{-4} HPO_4^{2-}, 8.11×10^{-4} SO_4^{2-}, 4.17×10^{-3} CO_3^{2-}. All sintered and cement specimens containing α-TCP were immersed in Hanks' solution at 37°C in a Teflon-sealed bottle (capacity in 150 ml) for 1, 2, 4 or 8 weeks. The volume of Hanks' solution per unit of surface area for the specimens was 20 ml/cm^2. Hanks' solution for the immersion was renewed every 2 days because the ion concentrations in the solution were changed by a precipitation and dissolution on the specimen. After immersion, the specimens were washed, dried, and weighed by using a balance accurate to 0.01 mg (AEG-45SM, Shimadzu, Kyoto, Japan). Mass changes per unit of surface area before and after immersion were determined. Ten specimens from each condition were prepared for the measurement of mass changes. The data of mass changes were statistically analyzed to determine the influence of immersion time by one-factor analysis of variance (ANOVA) and Fisher's PLSD post-hoc multiple comparison procedure. The pH change of Hanks' solution was measured until 2 weeks by storing each specimen without renewing the solution. Then, the changes of calcium and phosphorus concentrations in Hanks' solution were measured using a sequential plasma spectrometer (ICP; ICPS-7500, Shimadzu, Kyoto, Japan).

2.3. Analysis of specimens

The crystal phases in the specimens were analyzed with a powder X-ray diffractometer system (XRD; ADG-301, Toshiba Co., Tokyo, Japan). The XRD conditions were Ni monochromatized Cu K_α radiation ($\lambda = 0.1540$ nm) generated at 30 kV and 16 mA. The specimens for XRD analysis were prepared by crushing with an agate mortar. A scanning electron microscope (SEM: JSM-5300, JEOL, Tokyo, Japan) was used to observe the microstructures on the outermost surface layers of the specimens before and after immersion in Hanks' solution. For SEM observation, the specimen was sputtered with gold. Moreover, the specimens after immersion in Hanks' solution were analyzed with a Fourier transform infrared spectrometer (FTIR; FTS-40, Bio-Rad, Hercules, CA, USA). The specimen for FTIR was prepared by scraping the surface layer on the specimen after immersion in the solution.

3. Results

Figure 2 shows the mass changes of sintered calcium phosphates and calcium phosphate cements, immersed in Hanks' solution for 1, 2, 4 and 8 weeks. The mass of sintered specimen SA with 1.0 mol/l phosphoric acid and heat treatments was increased with the increase of immersion time in Hanks' solution (ANOVA, $p < 0.05$). Sintered specimen SB with 5.0 mol/l phosphoric acid and heat treatments slightly decreased in mass at 1 week after immersion. However, the mass was remarkably increased with the immersion for 2 weeks. In the immersion from 2 weeks to 8 weeks, the mass of specimen SB was not increased. In the case of untreated specimen SC not containing α-TCP, the mass was slightly increased

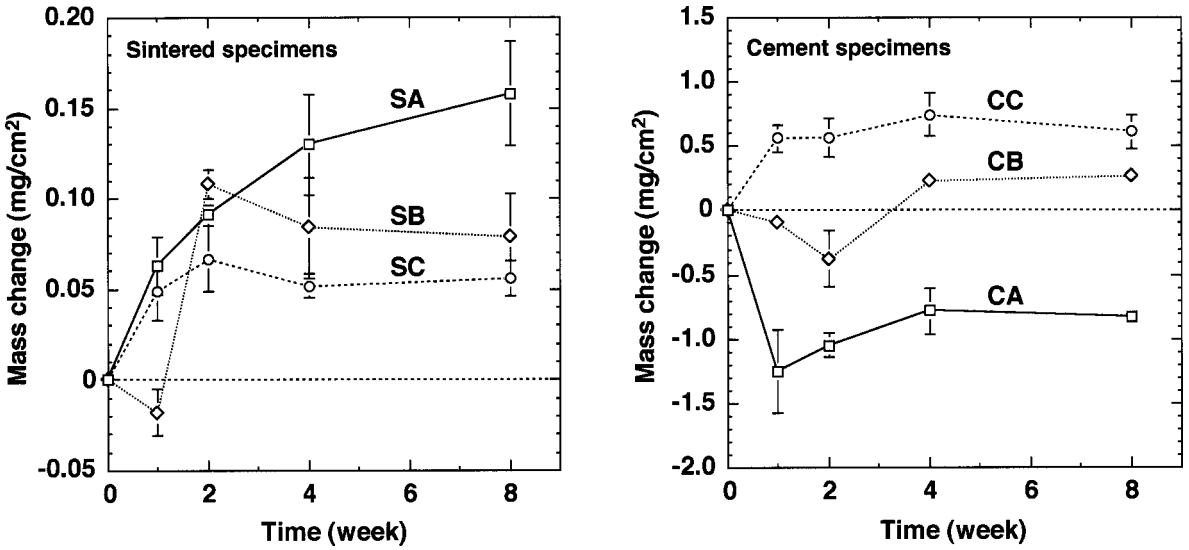

Fig. 2. Mass changes of sintered HAP specimens (SA, SB, SC) and calcium phosphate cement specimens (CA, CB, CC) containing α-TCP before and after immersion in Hanks' solution. Error bars show standard deviations.

Table 1

Calcium (Ca) and Phosphorus (P) concentrations in Hanks' solution at 14 days after immersion of calcium phosphate cements, measured by ICP

Specimens	α-TCP content at 24 h after setting (mass%)	Ca (mg/l)	P (mg/l)
CA	25	78.3	28.1
CB	50	66.4	33.9
CC	90	73.8	28.9
Hanks' solution (initial concentration)		46.2	24.5

with the immersion until 2 weeks ($p < 0.05$). However, no mass changes of specimen SC were confirmed by the immersion from 2 weeks to 8 weeks ($p > 0.05$). On the other hand, the mass changes of calcium phosphate cements (CA, CB and CC) containing α-TCP had remarkable variations. The mass of specimen CC containing approximately 90% α-TCP before immersion was increased with the immersion for 1 week. No increases of mass were confirmed with the immersion from 1 week to 8 weeks ($p > 0.05$). Specimen CA remarkably decreased in mass with immersion for 1 week. However, the mass of specimen CA was increased with the immersion time of 2 weeks, compared with the mass change at 1 week. In the case of specimen CB, the mass was also decreased with the increase of immersion time until 2 weeks ($p < 0.05$). An increase of mass was shown with immersion for 4 weeks ($p < 0.05$).

Table 1 shows calcium (Ca) and phosphorus (P) concentrations in Hanks' solution when the calcium phosphate cements, CA, CB and CC, were immersed for 14 days. The Ca concentration in Hanks' solution was remarkably increased by the immersion of all cement specimens, compared with the initial Ca concentration in Hanks' solution. Though the P concentrations of all specimens were also increased, the increases were smaller than those of Ca concentrations. Ca and P concentrations in Hanks' solution for specimens CA and CC were almost the same. However, Ca and P concentrations in the case of specimen CB were smaller and larger, respectively, compared with those of specimens CA and CC. In the case of

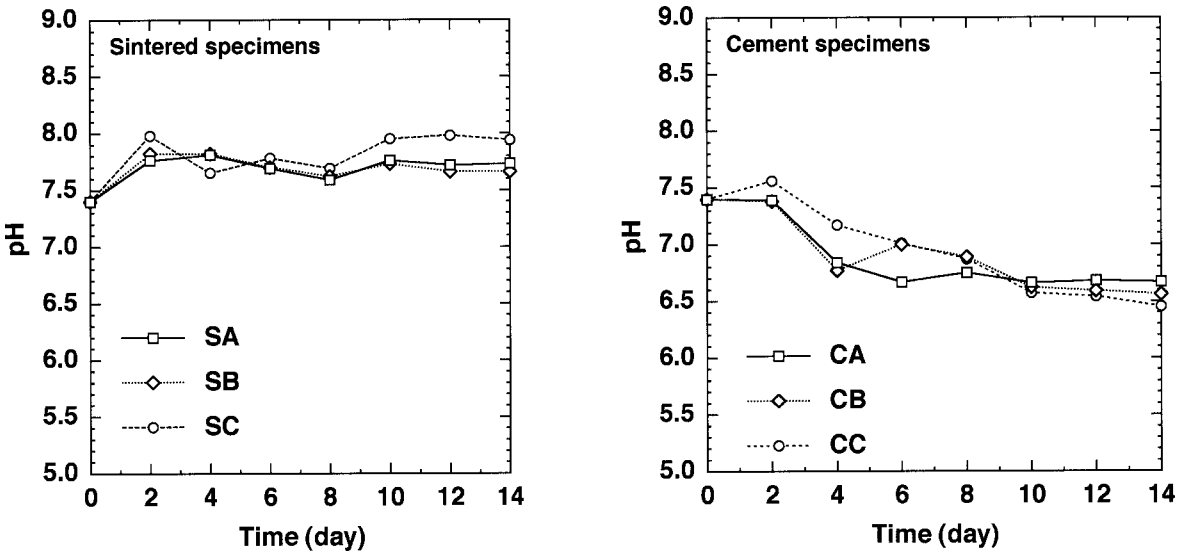

Fig. 3. The pH changes in Hanks' solution for 14 days with the immersion of sintered HAP specimens (SA, SB, SC) and calcium phosphate cement specimens (CA, CB, CC) containing α-TCP.

sintered specimens SA, SB and SC, Ca and P concentrations in the solution were decreased by immersion for all specimens. The pH changes in Hanks' solution in which sintered and cement specimens were immersed until 14 days are shown in Fig. 3. The pH values in Hanks' solution for sintered specimens SA, SB and SC were slightly increased at the very beginning of the immersions. However, the pH for the immersion of all sintered specimens was almost unchanged from 4 days to 14 days, having an approximately neutral value. On the other hand, the pH values in Hanks' solution for cement specimens CA, CB and CC were decreased with the increase of the immersion time. After 14 days, the solutions had pH values of approximately 6.5. Specimen CC slightly increased at the immersion time of 2 days.

Figure 4 shows the powder XRD patterns for the crystal phases in cement specimens CA and CC before and after immersion in Hanks' solution. In all cement specimens, including specimen CB, it was confirmed that the α-TCP in the specimen was mostly transformed to HAP by the immersion in Hanks' solution for 1 and 4 weeks. Moreover, the immersion for 8 weeks did not change the crystallinity of HAP in the specimens. The XRD patterns for the sintered specimens showed that the crystal phases were not different before and after immersion. Figure 5 shows the FTIR spectra for the scraped surface layers of cement specimens CA, CB and CC after the immersion in Hanks' solution for 1 week. The FTIR spectra for specimens CA and CC suggested that CO_3 radicals existed on the surface layer with approximately 873 cm^{-1}, 1410 cm^{-1} and 1470 cm^{-1} bands. A small amount of CO_3 radicals was confirmed by the FTIR spectrum for specimen CB. FTIR spectra for all sintered specimens revealed CO_3 radicals after immersion for 2 weeks. The FTIR measurements suggested that the surface layers of all cement and sintered specimens precipitated carbonated apatite after immersion in Hanks' solution.

SEM micrographs of the surfaces of sintered specimens SA and SC, before and after immersion in Hanks' solution for 1, 2 and 8 weeks are shown in Fig. 6. The precipitates on the surfaces of all sintered specimens (SA, SB and SC) were confirmed at immersion for 1 week by the SEM observations. The behavior of precipitatants on specimens SA and SC was different and related to the mass changes (see Fig. 2). A large number of island-like deposits were observed on specimen SA at 1 and 2 weeks after immersion. The precipitating behavior for specimen SB was similar to that for specimen SA. Figure 7

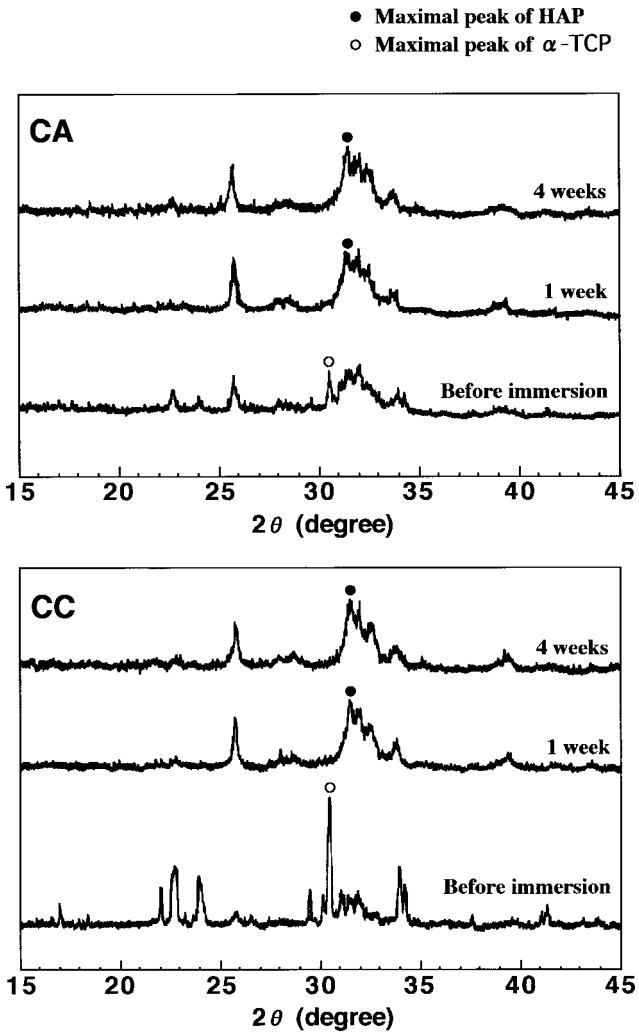

Fig. 4. X-ray diffraction patterns of calcium phosphate cement specimens (CA and CC) containing α-TCP before and after immersion in Hanks' solution for 1 and 4 weeks.

shows micrographs of cement specimens CA, CB and CC, before and after the immersion for 1 week. The precipitates and their behavior for each specimen were remarkably different. In cement specimen CA, the precipitates filled up spaces or gaps between plate-like crystals after immersion for 1 week. For specimen CB after immersion for 1 week there were precipitates of plate-like and needle-like crystals. On the surface of specimen CC there were crystallized new plate-like crystals. Moreover, small quantities of other precipitates were observed on the surfaces of its plate-like crystals.

4. Discussion

Simulated body fluid (SBF) has been utilized for the first step in evaluating biomaterials such as a bone-substituting material [24–27]. The Hanks' solution used in this study is one of several SBFs. It is generally known that carbonated apatite is precipitated on the surface of biomaterial by immersion in a

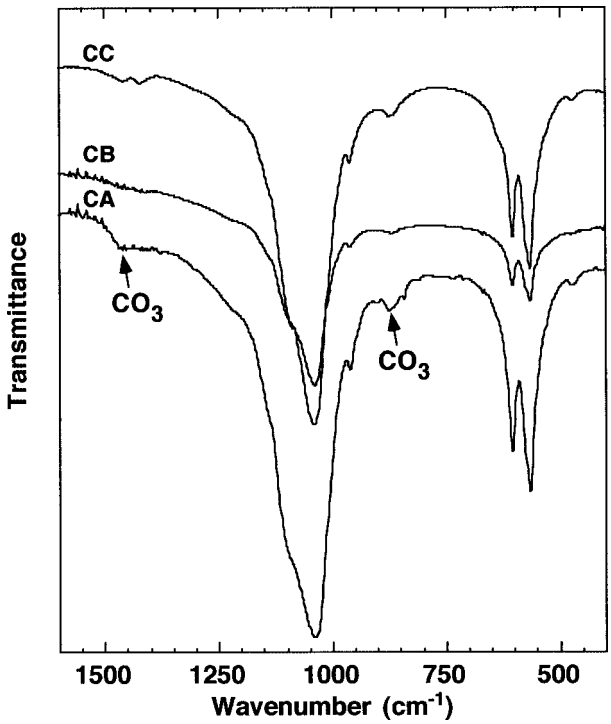

Fig. 5. FTIR spectra of calcium phosphate cement specimens (CA, CB, CC) containing α-TCP at 1 week after immersion in Hanks' solution.

SBF because of its biocompatibility and bioactivity, that is, osteointegration or osteobonding. The precipitates are grown by consuming the calcium, phosphate, carbonate, and hydroxide ions in the SBF [28]. Its evaluation is performed by observing the precipitation time and volume of carbonated apatites. However, the estimation of the biocompatibility and bioactivity with SBF must be considered very carefully as it may differ from that of a practical *in vivo* reaction. We have been trying to adopt weight measurement for the estimation of biomaterials with immersion in SBF since ten years ago [22,24,25]. Such weight measurement can elucidate the dissolution and precipitation on the surfaces of biomaterials, though the dissolution of biomaterials is difficult to observe.

The calcium phosphate ceramics prepared in this study were sintered or cement materials consisting HAP and α-TCP phases. HAP is known to be chemically stable in neutral pH and the body environment compared with other calcium phosphates. However, α-TCP is not a stable compound under environmental conditions around neutral pH, compared with HAP [29]. In immersion in SBF, it is expected that the α-TCP in the specimens will be dissolved in SBF or transformed to calcium-deficient HAP. The dissolution of α-TCP occurs because of its high solubility. In the transformation, the α-TCP is one of the raw materials used for the precipitation of HAP [4,8–10]. Graham and Brown reported that α-TCP is transformed to HAP through precipitation of octacalcium phosphate (OCP: $Ca_8H_2(PO_4)_6 \cdot 5H_2O$) under environmental conditions at basic pH [30,31]. Therefore, HAP transformed from α-TCP is generally a plate-like crystal because the plate-like crystal originates from a crystal form of OCP.

The surface layers of sintered specimens SA and SB prepared in this study were composed of α-TCP, because the bioactivity and biocompatibility for a biomaterial are governed by the character of the surface layer. After immersion in SBF, the weights of all sintered specimens were increased compared with

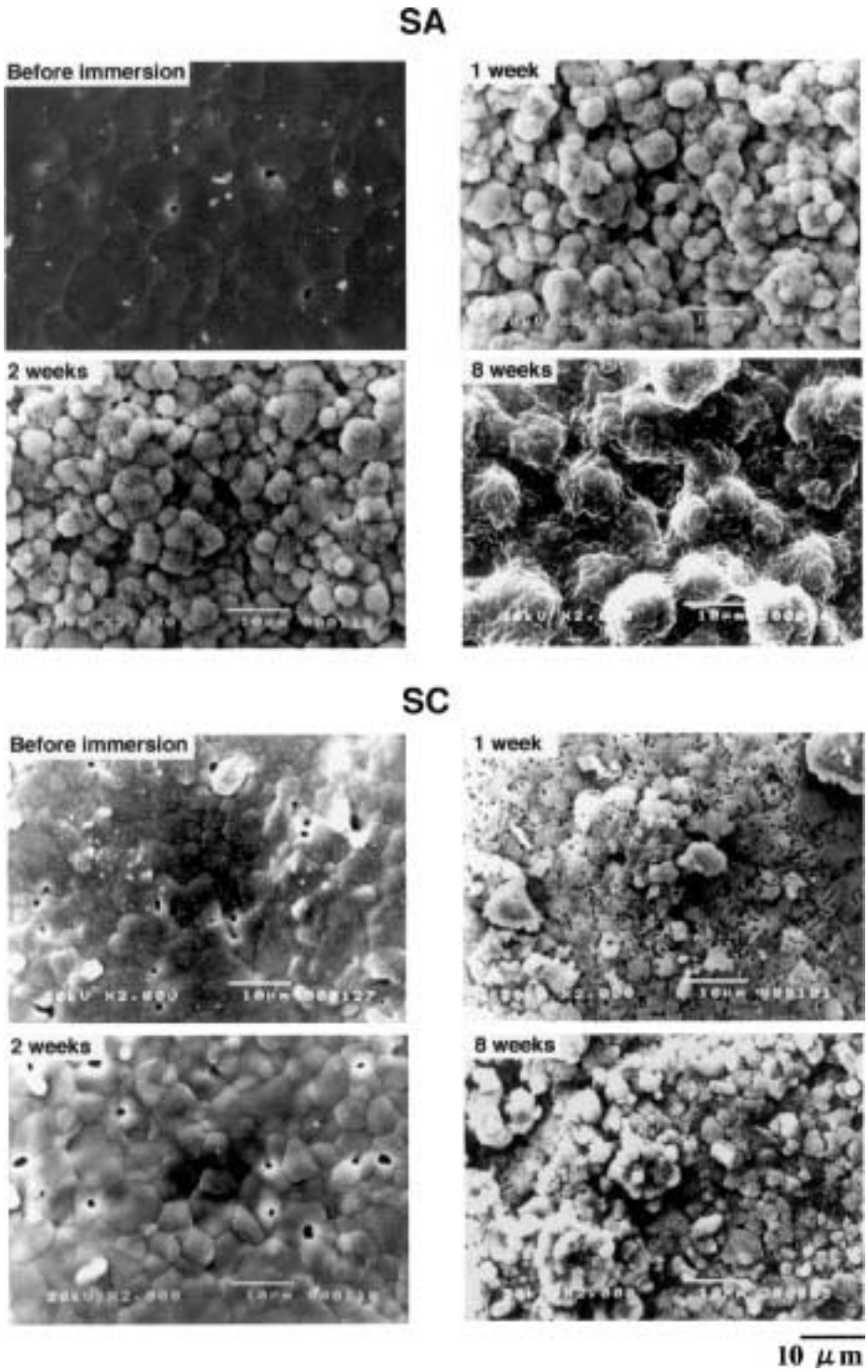

Fig. 6. Scanning electron micrographs showing surface microstructures of sintered HAP specimens (SA and SC) before and after immersion in Hanks' solution for 1, 2 and 8 weeks.

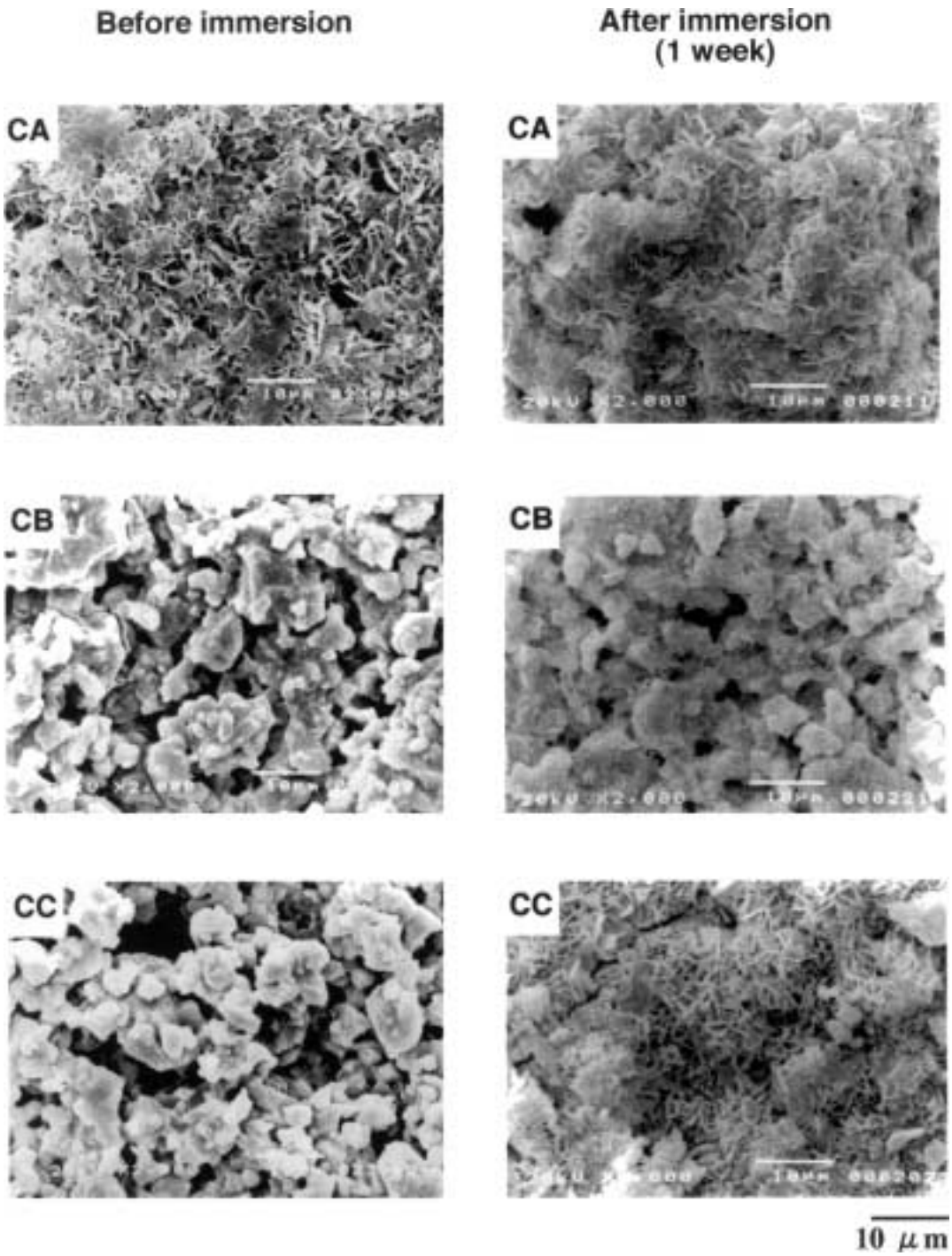

Fig. 7. Scanning electron micrographs showing surface microstructures of calcium phosphate cement specimens (CA, CB, CC) before and after immersion in Hanks' solution for 1 week.

that of each specimen before immersion. The weight of the sintered HAP-only specimen (SC) was also increased by the immersion, because HAP is a bioactive material and can bond directly with bone. The weight of specimen SA, which possessed a thin surface layer (approximately 0.2 μm) of α-TCP was most increased with the immersion in SBF until 8 weeks (see Fig. 2). This phenomenon may have occurred because a small quantity of α-TCP on the outermost layer of a specimen has a soluble or precipitate action. Moreover, it can be assumed that the surface area of the specimen increases with the dissolution of α-TCP on the outermost surface layer. SEM observation revealed that the precipitate behaviors of carbonated apatite on the specimen surfaces (SA and SC) were remarkably different with immersion for 1 week (see Fig. 6). The difference of precipitates between SA and SC was observed by the volume of island-like deposits on the substrates at 1 week after immersion. Furthermore, the precipitate on specimen SA at 1 week was composed of fine plate-like crystals, as shown in Fig. 6. This result suggested that the α-TCP contributed to the precipitation of apatite because of its plate-like crystals. On the other hand, specimen SB containing α-TCP from the outermost layer to approximately 2 μm depth was decreased in weight after immersion for 1 week because of the dissolution of its α-TCP. The mass change provided evidence that the dissolution of α-TCP on the surface markedly precipitated apatite at 2 weeks after immersion (see Fig. 2). Therefore, these results of sintered specimens proved that the α-TCP on the surface of sintered HAP materials was available for bioactivity. This implies that even the α-TCP on the surface 200 nm layer is more effective for bioactivity than HAP-only material.

On the other hand, immersion with calcium phosphate cements was performed to estimate the contribution of α-TCP to the composite phases consisting HAP and α-TCP. However, estimation for cement specimens is difficult with the immersion in SBF because the specimens are dissolved and precipitated by the large surface area or high reactivity itself. For all cement specimens large amounts of Ca and P ions were dissolved by immersion in the SBF compared with those concentrations in SBF itself (see Table 1). Therefore, the mass change of cement specimens after immersion in SBF varied remarkably with α-TCP contents in the cement. Before immersion, cement specimens CA, CB and CC contained approximately 25, 50 and 90% α-TCP, respectively. However, the α-TCP phase in all cement specimens including the inside layer was transformed to HAP by the immersion in SBF for 1 week (see Fig. 4). Moreover, surface areas of cement specimens were remarkably different, as shown in Fig. 7. The behavior of cement specimens after 1 week of immersion, therefore, is very important for the estimation of α-TCP in cement. Although the crystal phase in specimen CA was composed of a small amount of α-TCP before immersion, the weight was strongly decreased at 1 week after immersion. However, SEM observation indicated that the spaces between plate-like crystals were filled up by the precipitates at 1 week after immersion though the weight was decreased (see Fig. 7). These results suggested that the dissolution of α-TCP and the precipitation of carbonated apatite occurred simultaneously with immersion for 1 week. Specimen CC prepared with a maximal α-TCP phase showed a mass increase at 1 week, contrary to the expectation of a mass decrease. Although the weight was increased, the quantity of precipitates was very small (see Fig. 7). The α-TCP on the surface of set cement transformed to HAP such as plate-like crystals passed through a crystal form of OCP. The microstructure of specimen CC at 1 week after immersion was similar to that of set cement CA before immersion. It seemed that the α-TCP of specimen CC reacted with the SBF, like the setting reaction of specimen CA. In the case of cement specimens, an immersion test with a short term within 1 week may be necessary for the estimation of α-TCP-containing cements because pH values in SBF for each cement varied with immersion for the short term within 8 days (see Fig. 3). These results for cement specimens imply that a small quantity of α-TCP in calcium phosphate cements may be more effective for bioactivity and biocompatibility than a large quantity of α-TCP.

Our results indicate the possibility that the α-TCP on/in HAP ceramics could be a bioactive agent for a bone-substituting HAP material. More rapid dissolution of calcium and phosphate with α-TCP from HAP composite ceramics can be expected than in the case of HAP alone when implanted in the body [22]. This is thought to induce rapid precipitation of carbonated apatite, and thus rapid osteobonding can be expected if this is used as a bone-substituting material. In future work, the availability of α-TCP in HAP ceramics should be investigated by estimating the osteoconductivity in animal experiments (*in vivo*) and the expression of mRNA in type I collagen, alkaline phosphatase activity and osteocalcine in cell culture. The application of α-TCP is not limited to HAP ceramics because it can also be applied to several biomaterials coated with or containing calcium phosphates.

Acknowledgement

This study was supported in part by a Grant-in-Aid for Scientific Research (13672040) from the Ministry of Education, Science, Sports and Culture, Japan.

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