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In vitro apatite formation on organic–inorganic hybrids in the $CaO–SiO_2–PO_{5/2}$–poly(tetramethylene oxide) system

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Abstract The osteoconduction potential of artificial materials is usually evaluated in vitro by apatite formation in a simulated body fluid (SBF) proposed by Kokubo and his colleagues. This paper reports the compositional dependence of apatite formation on organic–inorganic hybrids in the $CaO–SiO_2–PO_{5/2}$–poly(tetramethylene oxide) system, initiated from tetraethoxysilane (TEOS), triethyl phosphate ($OP(OEt)_3$), calcium chloride ($CaCl_2$) and poly(tetramethylene oxide)(PTMO) modified with alkoxysilane. Formation of an apatite layer was observed on the surface of the organic–inorganic hybrids with molar ratios of TEOS/$OP(OEt)_3$ ranging from 100/0 to 20/80. The rate of apatite formation remarkably decreased when the hybrids were synthesized with TEOS/$OP(OEt)_3$ ratios of 40/60 or less. Hybrids without TEOS showed no apatite formation in SBF for up to 14 days. Addition of small amounts of $OP(OEt)_3$ to TEOS in the hybrids led to the high dissolution of calcium and silicate, while addition of large amounts of $OP(OEt)_3$ decreased the dissolution of calcium and silicate ions and resulted in reduced apatite formation regardless of the dissolution of phosphate ions from the hybrids.

1 Introduction

Bioactive glasses and glass–ceramics have been widely investigated because they exhibit specific biological activities that imply bone-bonding properties [1, 2]. The ability of glasses, glass–ceramics and sintered polycrystalline ceramics to make direct contact with bone is sometimes simply referred to as bioactivity in researches for bone substitutes [3]. The phenomenon of new bone formation on the surface of bioactive ceramics is used to identify osteoconductive materials. The main benefit of using osteoconductive ceramics is their potential for tight bonding to living bone after long-term implantation. Some bioactive ceramics have been used in clinical applications as bone substitutes [4]. The application range for bioactive ceramics is still narrow for less load-bearing parts owing to the mechanical properties, such as brittleness, of the bioactive ceramics known to date. To develop bioactive materials with high flexibility, attempts to design organic–inorganic hybrids through organic modification of calcium silicates have been reported, since calcium silicate can act as an essential component for apatite deposition in the body environment [5]. The fundamental idea behind this design comes from previous research on the compositional dependence of the hydroxyapatite (abbreviated to apatite hereafter) formation ability on glasses in the $CaO–SiO_2–P_2O_5$ system [6] in a simulated body fluid (SBF) proposed by Kokubo and his colleagues [7, 8]. Apatite formation is basically observed on glasses in the $CaO–SiO_2$ binary system because release of calcium ions from the glasses increases the degree of supersaturation with respect to apatite, and the formation of silanol (Si–OH) groups can induce heterogeneous nucleation of apatite on the surface of the glasses. Glasses mainly consisting of $CaO–P_2O_5$ compositions did not show any ability for the apatite formation. However, the conventional melt-quenching techniques only allow divided regions in the compositions between $CaO–SiO_2$-based and $CaO–P_2O_5$-based areas, because the melting temperature is too high to obtain a homogeneous liquid state. Hench and his colleagues

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reported that sol–gel processing was able to produce glasses with wider compositional regions in the ternary CaO–SiO_2–P_2O_5 system [9] compared with conventional melt-quenching techniques. However, there have been no reports on the compositional dependence of apatite formation on organic–inorganic hybrids based on the CaO–SiO_2–P_2O_5 ternary system. In the present study, we attempted to synthesize hybrids by combining organic components of poly(tetramethyleneoxide)(PTMO) with inorganic components in the CaO–SiO_2–P_2O_5 system and examined the in vitro apatite formation in SBF. A previous study has already indicated that organic–inorganic hybrids are able to show in vitro apatite formation through modification of the CaO–SiO_2 system with PTMO [10]. During synthesis through sol–gel processing, a starting compounds of triethyl phosphate ($OP(OEt)_3$) was reported to be available, leading to conversion of the inorganic composition into CaO–SiO_2–$PO_{5/2}$ system, and is shown in Fig. 1 with recalculation from the previously reported data [6]. In Fig. 1, the nominal compositions of the inorganic constituents are also shown. A modified protocol based on the previous report regarding synthesis of CaO–SiO_2–PTMO was applied to produce CaO–SiO_2–$PO_{5/2}$–PTMO hybrids. In vitro apatite formation was examined in SBF to estimate the osteoconduction potentials of the hybrids, i.e. their bioactivities. Based on the previous study, we found that in vitro apatite formation was observed within 1 day after exposure to SBF when a CaO–SiO_2–PTMO hybrid was prepared at a nominal molar ratio of CaO/SiO_2 of 0.15/1 with starting materials of $CaCl_2$ and tetraethoxysilane (TEOS) incorporated with PTMO with a mass ratio of PTMO/TEOS of 30/70. This composition was modified in the present study to prepare other hybrids, because of its high potential for apatite formation.

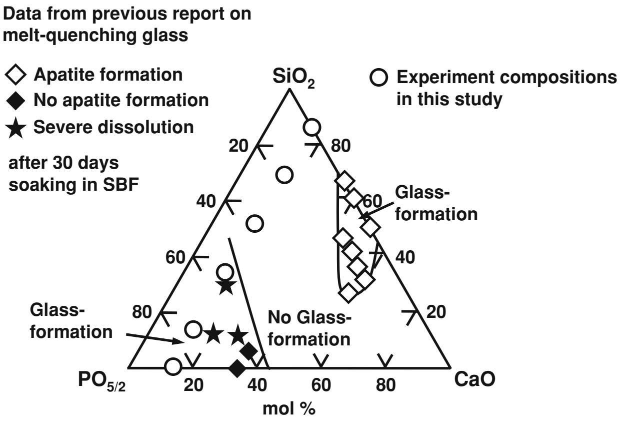


Fig. 1 Compositional dependence of apatite formation on glasses in the CaO–SiO_2–P_2O_5 system in SBF (Kokubo solution). Duplicated from a previous report (J Ceram Soc 1991;99:1–6) with a modification after obtaining permission from the journal

2 Experimental procedures

2.1 Synthesis of hybrids

Table 1 summarizes the nominal compositions for the preparation of hybrids in the CaO–SiO_2–$PO_{5/2}$–PTMO system. The protocol for the synthesis basically followed that in a previous report [11], which involved synthesis of hybrids in the ternary CaO–SiO_2–PTMO system. The starting materials were commercially available reagents without further purification. PTMO (HO–$(CH_2CH_2CH_2CH_2O–)_n$–H, $n =$ 1,000; Aldrich Chemical Co., USA) was modified with 3-isocyanatopropyltriethoxysilane (IPTS; $(C_2H_5O)_3SiCH_2CH_2CH_2NCO$; Aldrich Chemical Co.) to obtain the triethoxysilane-functionalized poly(tetramethylene oxide) (Si–PTMO) according to a previously reported method [12]. A mixture of PTMO and IPTS with a molar ratio of PTMO/IPTS of 1/2 was kept at 70°C for 5 days under a nitrogen atmosphere.

TEOS ($Si(OC_2H_5)_4$; Nacalai Tesque Inc., Japan) and Si–PTMO were dissolved in isopropyl alcohol (IPA; Nacalai Tesque Inc.) and tetrahydrofuran (THF; Nacalai Tesque Inc.), and ion-exchanged water as the solvents. Next, $OP(OEt)_3$ (Nacalai Tesque Inc.) and calcium chloride ($CaCl_2$; Nacalai Tesque Inc.) plus a 35% hydrochloric acid solution (Nacalai Tesque Inc.) as a catalyst were added in the initial solution.

The molar ratios of TEOS/$OP(OEt)_3$ in the synthesized hybrids varied from 100/0 to 0/100, abbreviated as sample names of P3T10P00 and P3T00P10 in Table 1, respectively. The mass ratios of PTMO/(TEOS + $OP(OEt)_3$) of the synthesized hybrids ranged from 40/60 to 20/80 (shown as P4T08P02 and P2T08P02 in Table 1, respectively). The molar ratio of $CaCl_2$/(TEOS + $OP(OEt)_3$) was constant at 0.15.

The obtained solutions were poured into a cylindrical Teflon® container, which was then covered with a paraffin film containing several pinholes. The solutions were sequentially dried in the container at 40°C for 3 weeks and 60°C for 1 week. Gel formation was visually observed. A Fourier-transformed infrared (FT-IR) spectroscope (FT/IR-610; Jasco, Japan) was used to analyze the structures of the obtained hybrids. The samples were pulverized and then mixed with powered KBr before the FT-IR measurements. Energy dispersive X-ray analysis (EDX; JEOL Ltd., Japan) was conducted to characterize the chemical constituents of the hybrids before soaking in SBF.

2.2 Evaluation of in vitro apatite formation

The obtained hybrids were cut to a size of 10 × 10 × 2 mm^3 and soaked in SBF (30 cm^3; pH 7.25) in the container at 36.5°C for various time periods. The SBF had a

Table 1 Nominal compositions of the organic–inorganic hybrids synthesized in this study

Name	Composition						
	PTMO/(T + P[a]) (mass ratio)	TEOS/$OP(OEt)_3$ (molar ratio)	$CaCl_2$/(T + P) (molar ratio)	H_2O/(T + P) (molar ratio)	HCl/(T + P) (molar ratio)	IPA (cm^{-3})	THF (cm^{-3})
P3T10P00	30/70	100/00	0.15	3	0.03	3	2
P3T08P02	30/70	80/20	0.15	3	0.03	3	2
P3T06P04	30/70	60/40	0.15	3	0.03	3	2
P3T04P06	30/70	40/60	0.15	3	0.03	3	2
P3T02P08	30/70	20/80	0.15	3	0.03	3	2
P3T00P10	30/70	00/100	0.15	3	0.03	3	2
P2T08P02	20/80	80/20	0.15	3	0.03	3	2
P4T08P02	40/60	80/20	0.15	3	0.03	3	2

[a] T + P: TEOS + $OP(OEt)_3$

similar ion concentration to that of human blood plasma [7]. The surfaces of the hybrids were analyzed by X-ray diffraction (XRD; RINT PC 2100; Rigaku Co., Japan) before and after soaking in SBF and observed with a scanning electron microscope (SEM; JSM-5600, JEOL Ltd). Changes in the element concentrations of calcium (Ca), phosphorus (P) and silicon (Si) in the SBF before and after soaking for 1, 3, 7 and 14 days were measured by inductively coupled plasma emission spectroscopy (ICP; Optima 2000DV; PerkinElmer Inc., USA). The pH of the SBF was also measured at this time points using a glass-electrode pH meter (D-51; Horiba Ltd., Japan).

3 Results

Figure 2 shows a typical example (P3T08P02) of the appearance and flexibility of the obtained hybrids. Among all the compositions, hybrids were obtained as transparent and pore-free bulk gels in the macro-scale. The size of each sample was approximately 50 × 35 × 2 mm^3. Although all of the synthesized specimens showed similar flexibilities, the samples seemed to be softened with increasing amounts of $OP(OEt)_3$. Figure 3 shows the FT-IR spectra of the hybrids before soaking in SBF. The absorption peak at 800 cm^{-1} was assigned to a vibration of Si–O–Si bending, while the peaks around 420 and 1,150 cm^{-1} were assigned to vibrations of Si–O bending and the longitudinal optical mode of Si–O, respectively [13]. The absorption peak of the Si–OH group was detected around 950 cm^{-1} [14]. The peaks at 1,375 and 1,450 cm^{-1} were assigned to C–H bonding of PTMO. The intensity of the peak around 1,120 cm^{-1} increased with increasing amounts of $OP(OEt)_3$ [14], and was distinct for the P3T00P10 hybrid, which did not contain TEOS. The peak around 1,120 cm^{-1} on the spectra was assigned to P–O bonding. Figure 4 shows the results of EDX analysis of the obtained hybrid surfaces before soaking in SBF. The intensity of P ions increased with

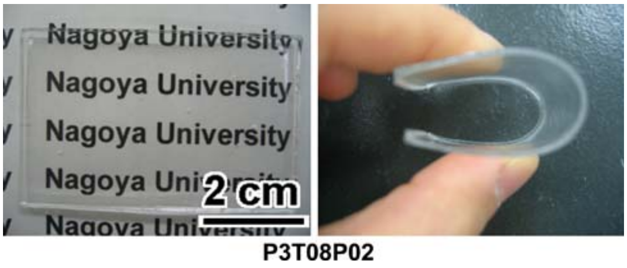


Fig. 2 An example (P3T08P02) of the appearance and flexibility of the obtained hybrids

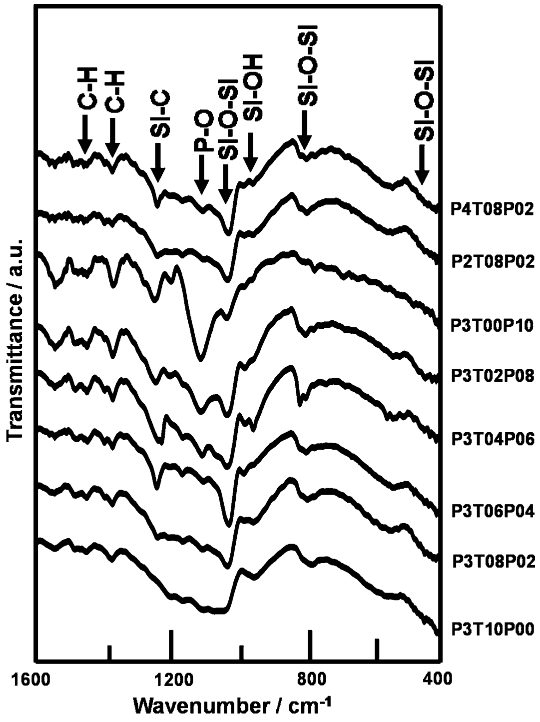


Fig. 3 FT-IR spectra of the hybrids before soaking in SBF

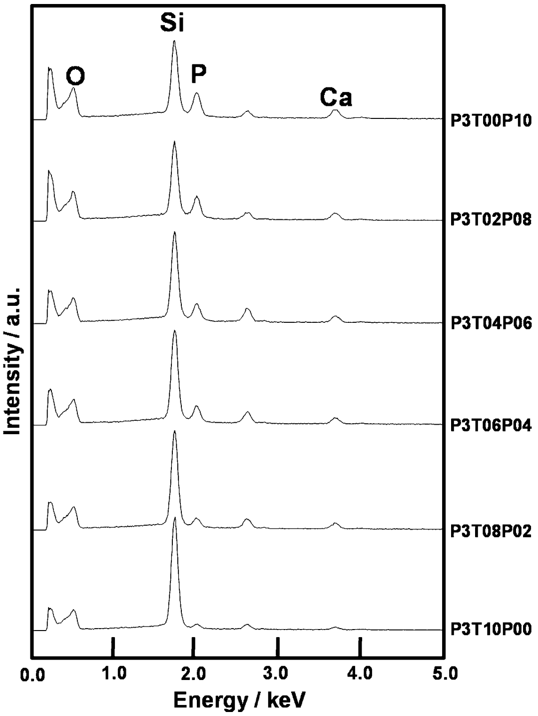


Fig. 4 EDX spectra of the peaks of Si, O, P and Ca for the hybrids before soaking in SBF

increasing amounts of $OP(OEt)_3$, whereas Si ions decreased with increasing amounts of $OP(OEt)_3$.

SEM images of the surfaces of the hybrids before and after exposure to SBF are shown on Fig. 5. The soaking periods were 1, 3, 7 and 14 days. The surfaces of the hybrids were clean and had no cracks or pores at the microscale before soaking. After soaking in SBF, the hybrids formed ball-shaped precipitates that resembled apatite, based on their morphological similarity to apatite described in a previous report [11]. The precipitates were observed to form on the surfaces of the hybrids starting from a compositions containing TEOS, and the time periods required for apatite formation lengthened as the amounts of $OP(OEt)_3$ increased to molar ratios of TEOS/$OP(OEt)_3$ of 20/80 and more. The P3T00P10 hybrid, which was prepared as a composition without TEOS, showed no apatite formation, even after soaking in SBF for 14 days. Apatite formation on the hybrids was confirmed by the observed thin-film X-ray diffraction patterns (Fig. 6). The broad peaks at $2\theta = 26°$ and $32°$ were assigned to apatite with low crystallinity. The X-ray diffraction patterns of the surfaces were consistent with the apatite-forming tendencies in the SEM images. The P2T08P02, P3T08P02 and P4T08P02 hybrids exhibited similar potentials for apatite formation.

Figure 7 shows the changes in the element concentrations and pH values of SBF before and after soaking the hybrids in SBF for 1, 3, 7 and 14 days. The increases in the Ca, P and Si concentrations in SBF after soaking were related to the release of Ca, P and Si from the samples, while the decreases in the Ca and P concentrations were related to the formation of apatite on the surface of the hybrids. An increase in the Ca concentration was observed within 1 day for all samples. The degree of increase in the Ca concentration promoted the apatite-forming ability of the hybrids in SBF. In contrast, the P concentrations in SBF increased with increasing amounts of $OP(OEt)_3$. In the case of the P3T10P00 hybrid without $OP(OEt)_3$, the P concentrations in SBF after soaking decreased within 14 days. The interval of the peak was larger for samples with high apatite-forming abilities. The Si concentrations increased with increasing time in all of the samples. The pH of the SBF decreased with increasing amounts of the $OP(OEt)_3$ in the hybrids.

4 Discussion

Organic–inorganic hybrids in the $CaO–SiO_2–PO_{5/2}$–PTMO system were successfully prepared with transparent and monolithic forms from TEOS, $OP(OEt)_3$, $CaCl_2$ and Si–PTMO by the sol–gel process, at a mass ratio of PTMO/(TEOS + $OP(OEt)_3$) of 30/70. Organic–inorganic hybrids could have an advantage for examination of their bioactivities depending on their compositions. This advantage allows not only examination of the potentials of hybrids with various reactivities, but also examination of the compositional dependence of the bioactivities, i.e. the bone-bonding properties. The results of the present study reveal that the wide ranges of compositions were difficult to examine in glasses synthesized using a conventional melt-quenching technique.

In a previous study, we discussed the synthesis and apatite-forming abilities of hybrids using starting compositions of PTMO/TEOS with mass ratios ranging from 20/80 to 90/10 in the $CaO–SiO_2$–PTMO system [11]. These hybrids formed a bone-like apatite layer from starting compositions of PTMO/TEOS with mass ratios ranging from 20/80 to 50/50. In the present study, we designed hybrids with similar compositions to the previous hybrids but with added $OP(OEt)_3$. The mass ratios of the organic component/inorganic component, PTMO/(TEOS + $OP(OEt)_3$), were varied from 40/60 to 20/80, and the molar ratios of TEOS/$OP(OEt)_3$ were varied from 100/0 to 0/100. Some of the obtained hybrids with molar ratios of TEOS/$OP(OEt)_3$ ranging from 100/0 to 20/80 showed apatite-forming abilities in SBF. The apatite-forming abilities of these hybrids were significantly governed by Si–OH groups on their surfaces. The FT-IR spectra of the hybrids showed that the amount of Si–OH groups increased as the TEOS content increased. This means that the apatite-forming abilities of

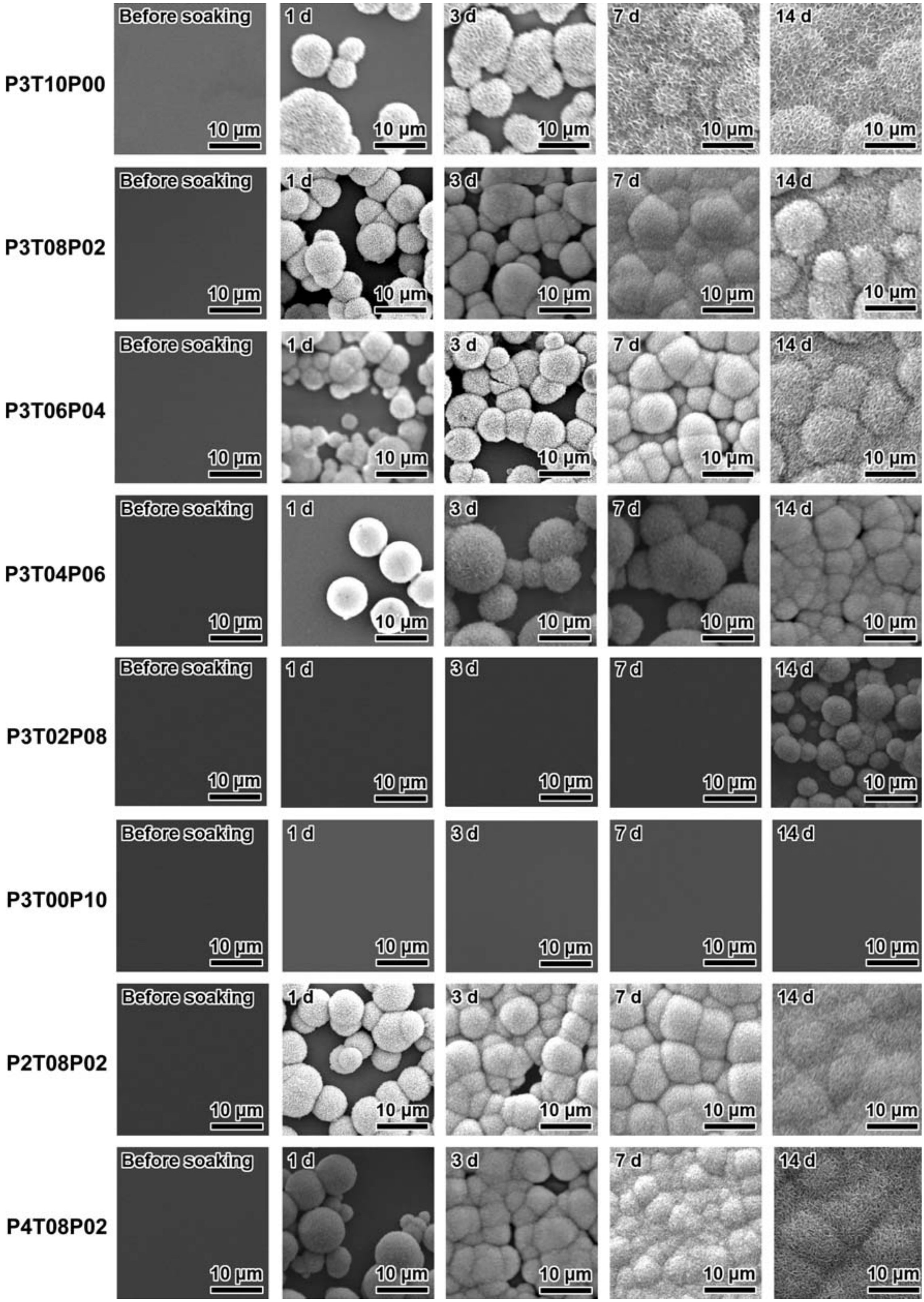
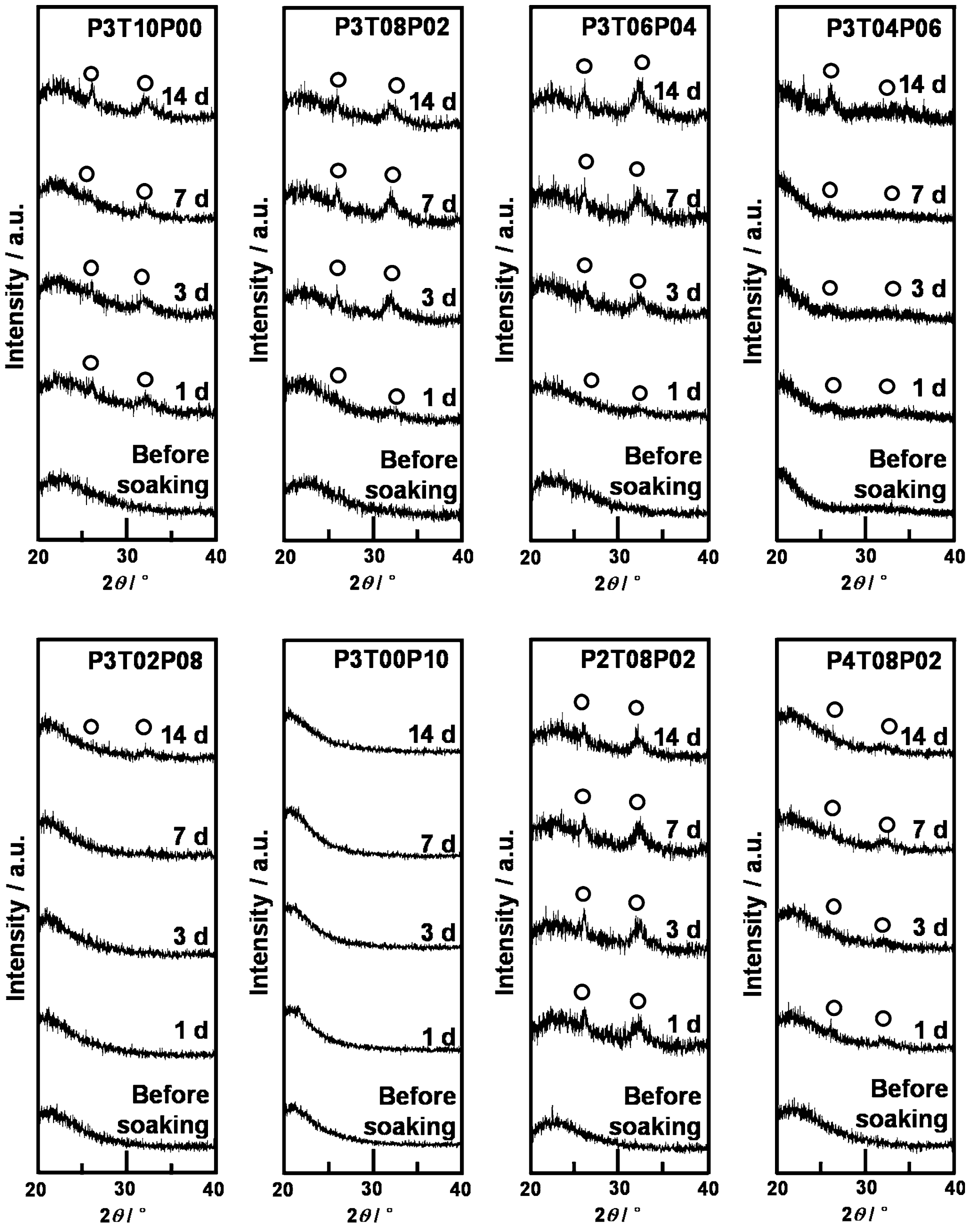


Fig. 5 SEM images of the surfaces of the hybrids before and after exposure to SBF

Fig. 6 X-ray diffraction patterns of the hybrids before and after exposure to SBF. Open circle: peaks assigned to apatite

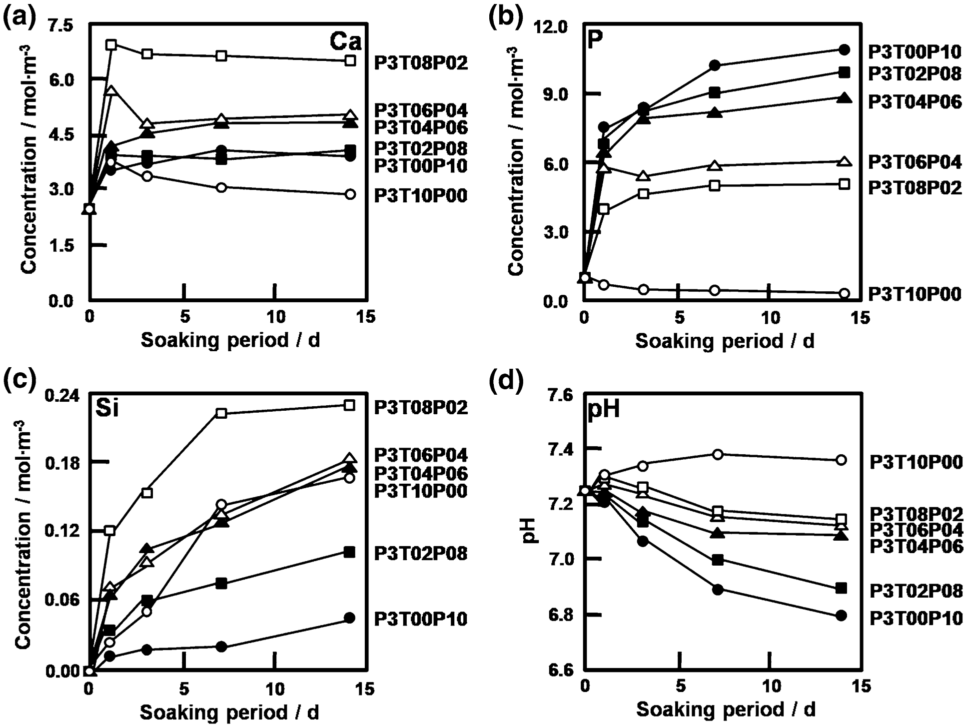


the hybrids with higher TEOS contents were increased. It has also been reported that phosphate ions have an effect on the apatite-forming ability [13]. The amount of phosphate ions exposed the surface of the hybrids increased with increasing amounts of $OP(OEt)_3$ based on the EDX results, and the amount of phosphate ions released from the hybrids increased with increasing amounts of $OP(OEt)_3$. The apatite-forming ability depended on the amount of phosphate ions included in the hybrids. This finding means that the amount of phosphate ions and a high TEOS content are important factors for the apatite-forming ability of the hybrids. Moreover, the pH of the SBF is an essential factor for apatite formation because the ionic activity product (*IP*) for apatite could reflect the calcium and phosphate ion concentrations and the pH of the SBF before and after soaking. The changes in the ionic activity product *IP* of apatite were estimated according to previous reports, using:

$$IP = \left[Ca^{2+}\right]^{10}\left[PO_4{}^{3-}\right]^{6}[OH^{-}]^{2}(\gamma_{Ca^{2+}})^{10}\left(\gamma_{PO_4{}^{3-}}\right)^{6}(\gamma_{OH^{-}})^{2}$$

where $\gamma_{Ca^{2+}}$, $\gamma_{PO_4{}^{3-}}$ and γ_{OH^-} are supposed to be 0.36, 0.06 and 0.72 at physiological ionic strength ($\mu = 0.16$), respectively [15]. The calculated values of *IP* are shown in Fig. 8. Increases in *IP* contributed to increased rates of nucleation and crystal growth of apatite. The degree of supersaturation of the SBF increased at 1 day after immersion for all of the hybrids. After 1 day, the *IP* for apatite in the SBF decreased for the P3T10P00, P3T08P02,

Fig. 7 Changes in the element concentrations and pH of SBF before and after soaking of the hybrids for 1, 3, 7 and 14 days. Open circle: P3T10P04; open square: P3T08P02; open triangle: P3T06P04; filled triangle: P3T04P06; filled square: P3T02P08; filled circle: P3T00P10



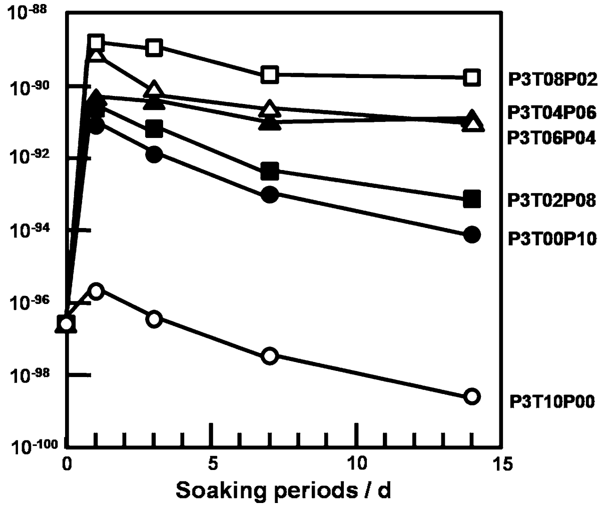


Fig. 8 Changes in the ionic activity products of SBF caused by immersion of the hybrids. Open circle: P3T10P04; open square: P3T08P02; open triangle: P3T06P04; filled triangle: P3T04P06; filled square: P3T02P08; filled circle: P3T00P10

P3T06P04, and P3T08P02 hybrids, which formed apatite on their surfaces within 1 day, followed by decreases in *IP* that were related to the consumption of calcium and phosphate as well as the decrease in pH. It should be noted that the P3T10P00 hybrid formed apatite on its surface at the lowest degree of increased *IP* among the samples. It is interesting that P3T10P00 showed a lower degree of *IP*, reflecting a lower degree of supersaturation of the SBF, than the P3T00P10 hybrid that showed no apatite formation in SBF for up to 14 days. Previous reports support the finding that Si–OH groups on the surface of the hybrids can induce heterogeneous nucleation of apatite in SBF. Even on the hybrids with PTMO combined with phosphate in their structure, Si–OH groups were obviously important for apatite deposition. There is still an assumption of inhibition of the induction of apatite formation owing to local decreases in pH around the surface after dissolution of phosphate from the hybrids, because of the difficulty in detecting the local pH near the surface. However, it is clear that simple modification with calcium phosphate hardly produces osteoconductive materials owing to the lower potential for apatite deposition even on organic–inorganic hybrids. The addition of appropriate amounts of phosphate to the CaO–SiO_2–PTMO system can enhance the dissolution of the hybrids in SBF as observed in the data for the released calcium and silicon (Fig. 7). These results support the potential of controlled degradability of organic–inorganic hybrids with addition of phosphate. In other words, controlled degradability can be achieved by addition of phosphate to organically modified ceramics of calcium silicate.

5 Conclusions

Transparent organic–inorganic hybrids were successfully synthesized by a sol–gel process in the CaO–SiO_2–$PO_{5/2}$–

PTMO system. The obtained hybrids formed apatite on their surfaces in SBF when the molar ratio of $OP(OEt)_3$/TEOS ranged from 20/80 to 0/100. The apatite-forming ability of the hybrids was dependent on the calcium and phosphate ion concentrations dissolved from the hybrids, the pH of the solution and the Si–OH groups existing on the hybrid surfaces. Biodegradable and osteoconductive materials can be developed by organic modification of calcium silicate ceramics with phosphate.

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