The influence of lithium fluoride on *in vitro* biocompatibility and bioactivity of calcium aluminate–PMMA composite cement

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The objective of this study is to assess the influence of lithium fluoride on *in vitro* biocompatibility and bioactivity of calcium aluminate (CA)–polymethylmethacrylate (PMMA) composite cement exhibiting quick setting time (< 15 min), low exothermic temperature ($< 47\,^{\circ}C$), and high compressive strength (> 100 MPa). The biocompatibility was measured by examining cytotoxicity tests such as the agar diffusion test with L929 cell line and the hemolysis test with fresh rabbit blood. To estimate the bioactivity of CA–PMMA composite cement, we determined hydroxyapatite (HAp) formation on the surface of composite cement in the simulated body (SBF) solution by using thin-film XRD, XPS, SEM, EPMA and ICP-AES. The results of biocompatibility tests indicated that all experimental compositions of this study had no cytotoxicity and no hemolysis so that there was no cytotoxicity with regard to non-reacted monomers (MMA and TEGDMA) and lithium fluoride. The results of bioactivity tests revealed that CA–PMMA composite cement without lithium fluoride did not form HAp on its surface after 60 days of soaking in the SBF. On the other hand, $LiAl_2(OH)_7 \cdot 2H_2O$ and HAp were formed on the surface of CA–PMMA composite cement including 1.0% by weight of lithium fluoride after 7 and 15 days of soaking in the SBF, respectively. The 5 μm of $LiAl_2(OH)_7 \cdot 2H_2O$ and HAp mixed layers were formed on the surface of specimen after 60 days of soaking in the SBF.

Introduction

Polymethylmethacrylate (PMMA) based cement was first introduced by Dr John Charnley and has been clinically used for the construction of prosthetic appliances and the fixation of all joint prostheses [1]. Commercial PMMA cement is generally served as two component systems consisting of a powder and a liquid. When the two components of a bone cement system are mixed, the initiator from the powder and the activator from the liquid results in a redox reaction that produces free radicals initiating, in addition, polymerization of the methylmethacrylate (MMA) monomer. As the polymerization reaction continues, the cement paste transits from the liquid state to the solid state and forms a rigid polymer [2].

Several disadvantages such as a weak mechanical property, exotherm character during chemical polymerization and bioinert nature, limit the clinical success of PMMA-based cement currently in use. The weak mechanical property of a PMMA-based system arises from cyclical mechanical loads and over stress beyond endurance, which can result in fracture in the cement structure and the production of wear debris and flaw at the interface between PMMA-based cement and the implant (the bone) [3, 4]. To improve the mechanical property of PMMA-based cement, addition of inorganic fillers such as alumina and silica powders to the powder component have been incorporated with pre-polymerized beads of PMMA.

The high polymerization exotherm of PMMA-based cement plays an important role in the thermal necrosis of bone and surrounding tissue, which can induce early loosening of an implant and has side effects in systemic organs. Lesson [5] reported that the average peak curing

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TABLE I Experimental compositions of CA–PMMA composite cement for biocompatibility and bioactivity test

Name	CA	LiF	PMMA	BPO*
(1) Powder composition (wt %)				
CAC–P	95	0	5	1.87
CAC–LP	94	1		
(2) Liquid composition (vol %)				
MMA: 87.49	DMPT: 3.51	TEGDMA: 9		

*BPO is added separately from total wt %.

temperature of PMMA was 86.9 °C. Thermal necrosis extended to the surgical margins, including cartilage cells and osteocytes. Heat liberation above 47 °C is enough to cause thermal necrosis of bone and denaturalize protein when the temperature is above 56 °C [6]. Therefore, to obtain clinical success in the fixation of the implant and the restoration of defective bone, it is important to lower the exotherm character during chemical polymerization.

The bioinert nature of PMMA-based cement is the most crucial disadvantage of all factors. Its nature results in no chemical bond between artificial biomaterials and bone. Loosening occurs due to polymerization shrinkage between artificial joint prostheses and bone. The clinical success of cemented implants is highly dependent on maintaining a bone–cement–prosthesis construct free of aseptic loosening. The loosening phenomenon involves interfacial failure between the cement and the bone and/or the implant, bone remodeling, and cement failure [7].

On this basis, we developed bioactive calcium aluminate (CA)–PMMA composite cement for cementation of artificial joint prosthesis. CA cement, which is mainly composed of calcium oxide (CaO) and alumina (Al_2O_3) at equivalent molar ratio, was first developed in 1908 by Bied [8]. It features good mechanical properties and an excellent chemical durability. It has also much potential as bone cement for the fixation of total joint prostheses and the restoration of bony defects. In addition, CA cement includes 50% by mole of CaO so that many released calcium ions could increase the degree of the supersaturation of the surrounding simulated body fluid (SBF) with respect to apatite. The byproducts of hydraulic reaction are many kinds of CA hydrates containing hydroxyl (OH) groups. Newly formed OH groups on the surface of CA cement could act as seeds for the formation of hydroxyapatite (HAp) [9–11]. In our previous study, lithium fluoride accelerated the hydration of CA cement and HAp formation on its surface within a short time. We also established the optimum amount of lithium fluoride showing good biocompatibility and bioactivity [12]. Furthermore, we developed CA–PMMA composite cement exhibiting quick setting time (< 15 min), low exothermic temperature (< 47 °C) and high mechanical strength (> 100 MPa) in the pilot study [13].

The specific purposes of this study are to estimate the influence of lithium fluoride on *in vitro* biocompatibility of CA–PMMA composite cement and the feasibility of HAp formation on the surface of the cement with the diversity of induction periods in a SBF solution.

Materials and methods

Preparation of CA–PMMA composite cement

Calcium aluminate powder was prepared as follows. $CaCO_3$ and Al_2O_3 were wet-mixed at the same molar ratio of CaO to Al_2O_3 by using alumina ball mill for 24 h. The blended slurry was dried at 150 °C for 12 h and sintered at 1350 °C for 6 h. The sintered product was pulverized with processing attrition milling at 600 rpm for 2 h. The average particle size of CA powder was approximately 3.5 μm and main composition was $CaO \cdot Al_2O_3$ including a little $12CaO \cdot 7Al_2O_3$. CA–PMMA composite cement was prepared for two component systems composed of a powder component and a liquid component. A powder component consisted of CA, PMMA (Sigma-Aldrich, Mw = 120 000, USA) and benzoyl peroxide (BPO; Lancaster, 75% in water, England). A liquid component was blended with MMA (Junsei, GR, Japan), *N*,*N*-dimethyl-para-toluidine (DMPT; Junsei, GR, Japan) and TEGDMA (Sigma-Aldrich, USA). Experimental compositions in this study are listed in Table I. The mixing ratio of liquid to powder was 0.35 ml/g. A cement paste was prepared by hand-mixing powder and liquid, filled in syringe, and then injected into cylindrical Teflon split mold ($\phi = 10$ mm, $h = 2$ mm). Glass plates on each end of the molds were firmly fastened using a C-clamp. Experimental compositions of CA–PMMA composite cement including 1.0% by weight of lithium fluoride and including no lithium fluoride are designated as CAC–LP and CAC–P, respectively. From the pilot tests of CAC–LP and CAC–P, setting time ranged 12–15 min, exothermic temperature did not exceed 47 °C and compressive strength ranged 115–130 MPa [13].

Agar diffusion test [14]

We used established L-929 cell (normal cell; fibroblast connective tissue of 100 days old male mouse) lines from American Type Culture Collection (Rockville, MD, USA). Each 1 mL of cell line was mixed up with 20 mL of Eagle's minimum essential medium (MEM) contained 5% fetal bovine serum (FBS). It was plated onto petri dish ($\phi = 90$ mm) and incubated at 37 °C, 5% CO_2 for 1 week. When the concentrations of cell exceeded 3×10^5/mL, cells with culture media were subcultured and repeated incubation. The agar media were made from 50% agar and 50% Eagle's MEM with

TABLE II Description of decolorization index, lysis index and interpretation of response index

Decolorization index	Description of decolorization
0	No detectable zone around or under sample
1	Zone limited to area under sample
2	Zone not greater 5 mm in extension from sample
3	Zone not greater 10 mm in extension from sample
4	Zone greater than 10 mm in extension form sample, but not involving entire plate
5	Zone involving entire plate
Lysis index	**Description of zone**
0	No observable lysis
1	Up to 20% of zone lysed
2	Over 20–40% of the zone lysed
3	Over 40–60% of the zone lysed
4	Over 60–80% of the zone lysed
5	Over 80% lysed within the zone
Cytotoxicity	**Response index (Zone index/Lysis index)**
None	0/0
Mild	1/1–1/5, 2/1
Moderate	2/2–2/5, 3/1–3/5, 4/1–4/3
Severe	4/4–4/5, 5/1–5/5

5% FBS. Agar media were replaced each Eagle's MEM with FBS in a same volume and allowed to gel in a room temperature for 30 min. Each agar medium was stained with neutral red vital stain solution for 30 min.

Preparation of extracts from specimens

Ethylene-oxide-sterilized specimens were extracted from the culture medium (0.2 g/ml) at 37 °C for 72 h. The sterilized papers sized $10 \times 10\,mm^2$ were immersed in extract for 30 min. Two immersed papers per one extract overlaid onto each petri dish filled up with the agar medium along with a negative (polyethylene) and positive (polyvinylchloride) controls after removed the stain solution. Each petri dish was subsequently incubated at 37 °C, 5% CO_2 for 24 h. Cytotoxicity estimation was followed by response index that is equal to the ratio of decolorization index to lysis index. Descriptions of decolorization index, lysis index and criteria of cytotoxicity are listed in Table II.

Hemolysis test [15, 16]

The extract was prepared by 5 g of set cement soaking in 10 ml of physiological saline at 37 °C for 30 min. After removing set cement, 0.2 ml anticoagulated rabbit blood was gradually added to the extract and suspension was stored at 37 °C for 1 h. Suspension was centrifuged at 1000 rpm for 10 min and optical density of supernatant was measured at 545 nm by ELISA plate reader (EL308, Bio-Tek, USA). For the positive control and negative control, rabbit blood was added to distilled water and physiological saline respectively. Rate of hemolysis was calculated according to the following equation:

$$\text{Rate of hemolysis } (\%) = [(OD_{specimen} - OD_{negative})/(OD_{positive} - OD_{negative})] \times 100$$

Soaking in a SBF solution

To evaluate the bioactivity of calcium aluminate cement, we examined HAp formation on the surface of specimen in a SBF solution after each induction time. Concentrations of various ions in a SBF solution were adjusted to be similar to those in human blood plasma as shown in Table III according to Kokubo *et al*. [17] by dissolving reagent grade NaCl, $NaHCO_3$, KCl, K_2HPO_4–$t3H_2O$, $MgCl_2$–$6H_2O$, $CaCl_2$ and Na_2SO_4 into distilled water in this order and a SBF solution was adjusted to pH 7.4 with 5 mM tris(hydroxymethyl)-aminomethane and 1 M hydrochloric acid. After stored in incubator at 36.5 °C and 100% of relative humidity, cylindrical specimen was cleansed with distilled water and immersed in 20 ml SBF solutions per one piece of specimen at 36.5 °C for 1, 2, 7, 15, 30 and 60 days. After the predetermined soaking time, specimen was removed from the SBF solution to be gently rinsed with distilled water, and then dried at 60 °C for 24 h.

Analysis of HAp formation

Surface analysis and morphological observation of specimen were performed by thin film X-ray diffractometer (thin film XRD: DMAX 2500, Rikaku, Japan), X-ray photoelectron spectroscope (XPS: Escalab 220i. VG Scientific, UK), and scanning electron microscope (SEM: JSM-840A, Jeol, Japan). In the thin film XRD measurements, Cu-Kα radiation ($\lambda = 1.5405$ Å) was used as the X-ray source. The glancing angle of the specimen was fixed at 1° against the incident beam, which enabled the detection of XRD patterns to be about a depth of 1 μm from the top source of the substrate. The XPS analysis was performed by using unmonochromated Mg-Kα radiation at an instrument pressure of about 133.3 nPa. The binding energy was referenced to the C_{1s} of adventitious hydrocarbon at 284.6 eV. For SEM observation, the section was mounted on metal holders for gold coating, followed by SEM observations. The depth of

TABLE III Ion concentrations of simulated body fluid and human blood plasma

	Concentration (mM)							
	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	HCO_3^-	HPO_4^{2-}	SO_4^{2-}
Simulated body fluid	142.0	5.0	2.5	1.5	147.8	4.2	1.0	0.5
Human blood plasma	142.0	5.0	2.5	1.5	103.0	27.0	1.0	0.5

TABLE IV Results of agar diffusion test and hemolysis test of CAC–P and CAC–LP

(1) Agar overlay test

Name	Zone index	Lysis index	Response index	Cytotoxicity
CAC–P	1	1	1/1	Mild*
CAC–LP	2	1	2/1	Mild*
PVC (Positive control)	5	3	5/3	Severe
PE (Negative control)	0	0	0/0	None*

(2) Hemolysis test

Name	Optical density (mean)	Hemolysis rate (%)
CAC–P	0.268	1.65**
CAC–LP	0.251	0.80**
Positive control	2.231	100.00
Negative control	0.235	0.00**

*These specimens have no cytotoxicity from the criterion.
**These specimens have no hemolysis (criterion $< 5\%$).

accumulated layers on the surface of cement was measured by using electron probe micro analyzer (EPMA: JXA-8900R, Jeol, Japan). The measurements of Ca, Al and P concentrations in the SBF solution were used by inductively coupled plasma atomic emission spectroscopy (ICP-AES: ICPS1000IV, Shimadzu, Japan).

Results

Biocompatibility of CA–PMMA composite cement

The results of agar diffusion test and hemolysis test are listed in Table IV. The results of agar diffusion test indicated that CAC–LP and CAC–P ranked mild state and these compositions had no cytotoxicity from the criterion. The results of hemolysis test with rabbit blood revealed that hemolysis rates of CAC–LP and CAC–P were 1.65 and 0.80, respectively. The upper limited value of hemolysis rate is 5, thus these compositions had no hemolysis from the criterion of ISO 10993 [15].

Surface analysis of CA–PMMA composite cement with various induction times

Fig. 1 shows the thin film XRD patterns of the surfaces of CAC–LP and CAC–P soaked in a SBF solution with the diversity of induction periods. After 7 days of soaking,

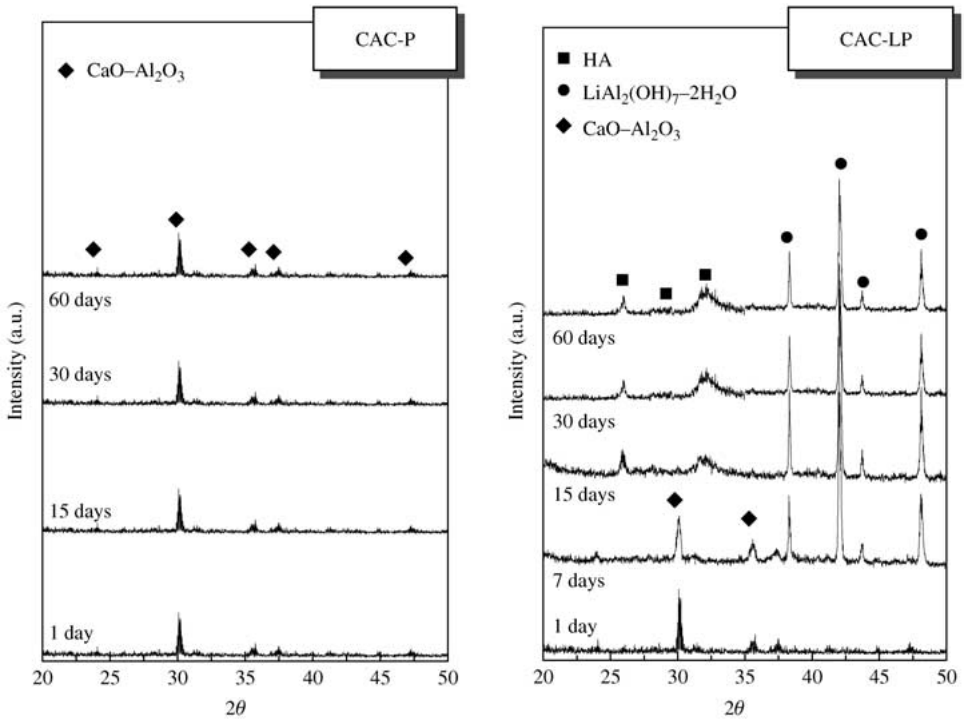

Figure 1 Thin film XRD patterns of CAC–P and CAC–LP soaked in SBF with variance of induction periods.

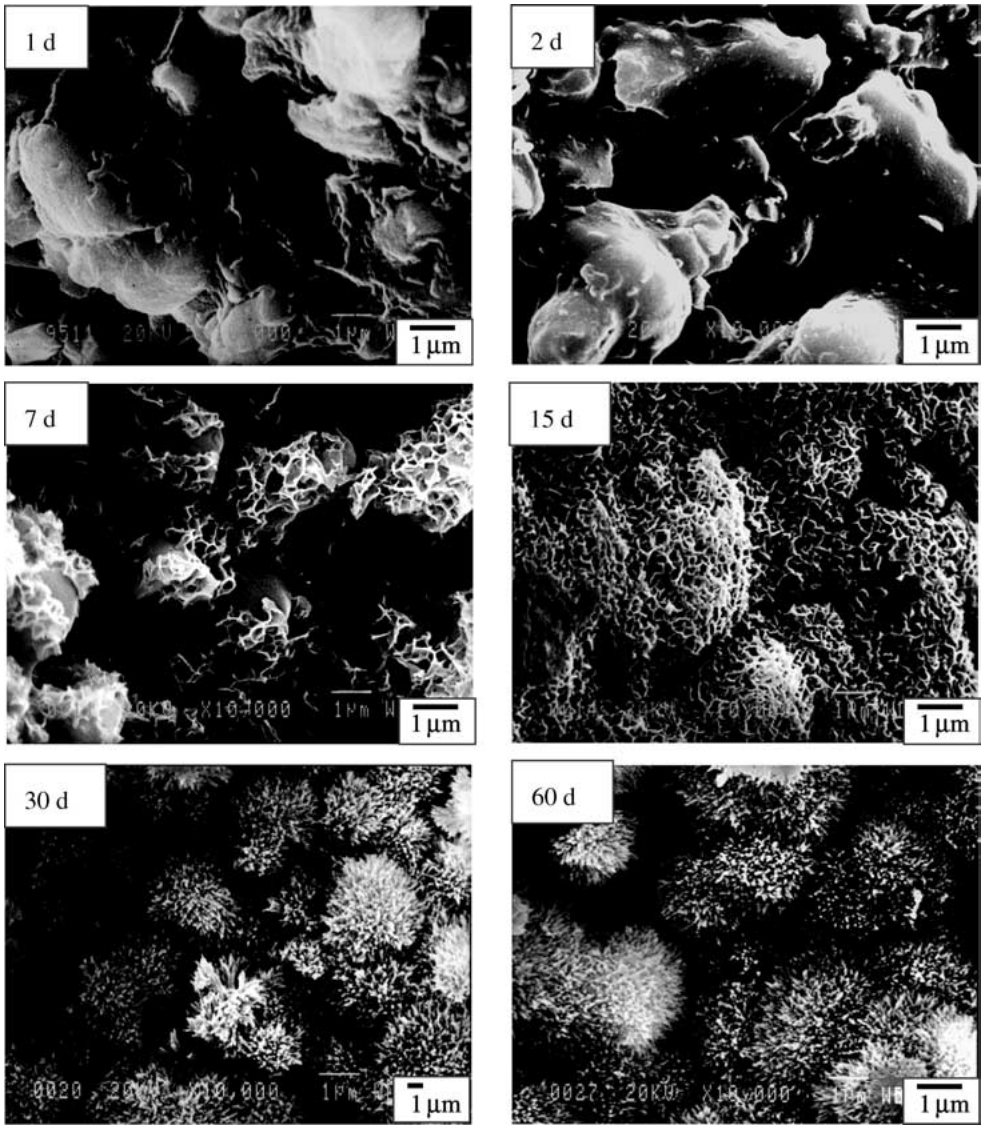

Figure 2 SEM surface views of CAC–LP soaked in SBF with the diversity of induction periods.

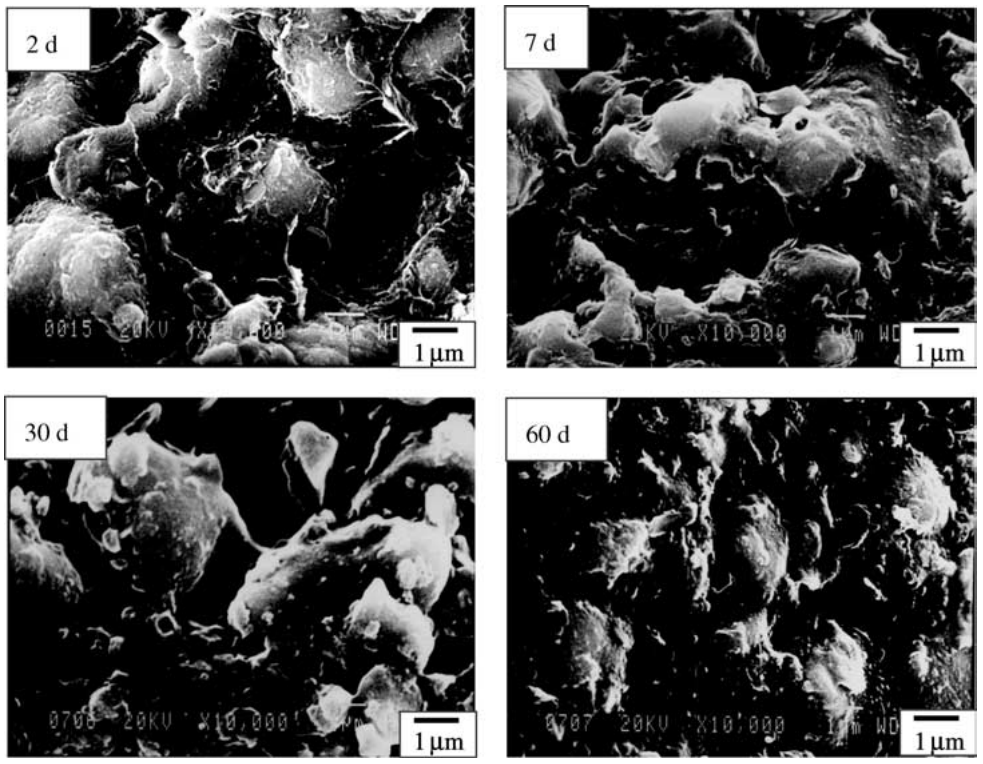

Figure 3 SEM surface views of CAC–P soaked in SBF with the diversity of induction periods.

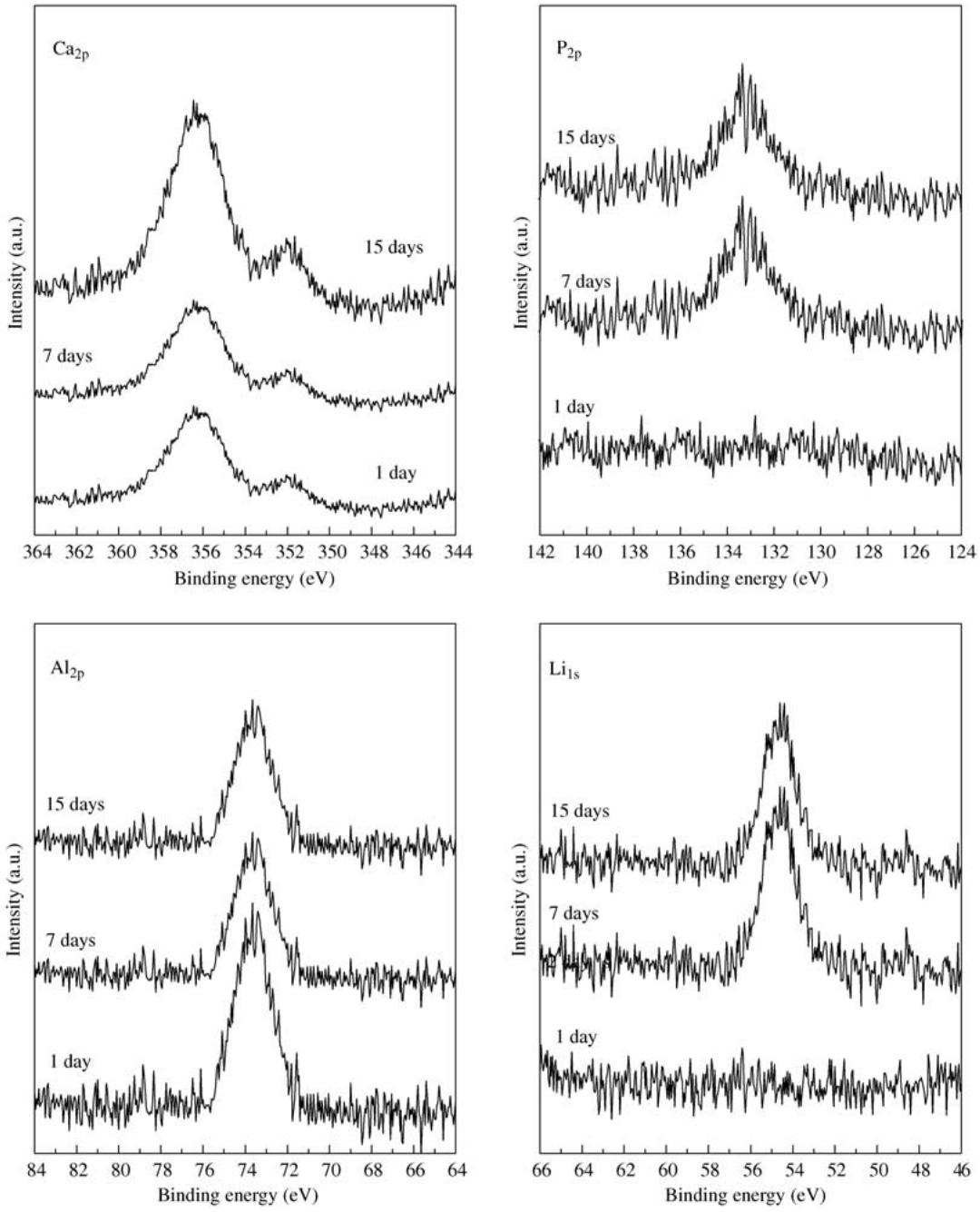

Figure 4 XPS spectra of CAC–LP immersed in SBF with the diversity of induction periods.

$LiAl_2(OH)_7 \cdot 2H_2O$ peaks were detected on the surfaces of CAC–LP. After 15 days of soaking, a new XRD peak along with the peak of $LiAl_2(OH)_7 \cdot 2H_2O$ were found at $2\theta = 25.9°$, 29° and 31.8° (broad peak) in the two days soaked sample, which is assigned to the *002* reflection, *210* reflection and the overlap of the *211*, *112*, and *300* reflection of HAp respectively [18]. On the contrary, only $CaO \cdot Al_2O_3$ was found on the surface of CAC–P within all soaking times.

Figs. 2 and 3 illustrate SEM photographs of the surfaces of CAC–LP and CAC–P immersed in a SBF solution. It can be seen from Fig. 2 that leaflike particles were detected on the surfaces of CAC–LP after 7 days of immersion and needlelike particles were formed on all surfaces of CAC–LP after 15 days of soaking in the SBF. On the contrary, for CAC–P, no particle was observed on its surface after 60 days of soaking (Fig. 3).

Fig. 4 shows the result of XPS analysis of CAC–LP soaked in a SBF solution for various induction periods. Al_{2p} and Ca_{2p} peaks were detected after 1 day of soaking and Li_{1s} peak appeared at 7 days after immersion. After 15 days of soaking, P_{2p} peak was detected on the surface of CAC–LP. These results indicated that $LiAl_2(OH)_7 \cdot 2H_2O$ layer was formed on the surface of CAC–LP after 7 days and $LiAl_2(OH)_7 \cdot H_2O$ and HAp coexisted on the surface of CAC–LP after 15 days of soaking with regard to the results of thin film XRD.

Fig. 5 shows the cross-sectional views and depth profile of CAC–LP and CAC–P after 60 days of soaking in a SBF solution. It can be seen from SEM photograph of CAC–LP that one layer was formed on its surface and the thickness of this layer was approximately 5 μm. From the result of EPMA, the layer on the surface of CAC–LP was composed of Al, Ca and P. On the contrary, no layer was

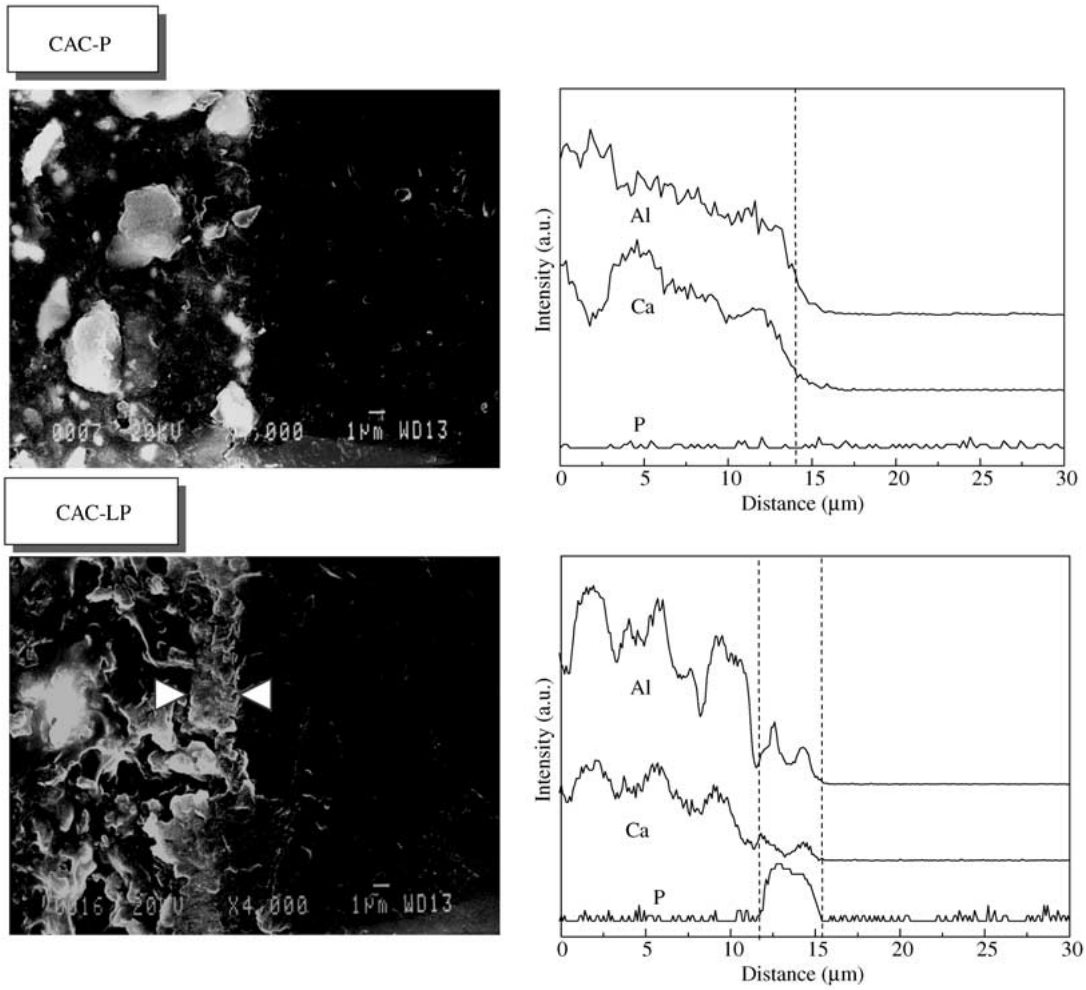

Figure 5 SEM cross-sectional views and EPMA profiles of CAC–P and CAC–LP after 60 days of soaking in SBF.

formed on the surface of CAC–P and only Ca and Al were detected at the interface between cement and resin block from the results of EPMA.

Fig. 6 shows changes in pH and element concentrations of SBF solutions soaked CAC–LP and CAC–P. There was no difference of pH between CAC–LP and CAC–P. The Ca and Al concentration of each SBF solution soaked CAC–LP increased as soaking time increased and P concentration of them decreased. In contrast, the Ca, Al and P concentration of the SBF solutions soaked CAC–P had little change with the diversity of induction times.

Discussion

The addition of calcium aluminate to PMMA-based cement is considered one of the possible ways to improve the mechanical and biological properties of PMMA-based cement. Adding ceramic filler is made to decrease the relative amount of MMA in the composite, which would decrease the exotherm, polymerization shrinkage, and the release of toxic MMA into the tissue, thus improving the biocompatibility and bioactivity of PMMA-based cement [19–21].

In the testing of CA–PMMA composite cement with bioactivity potential, estimating the biocompatibility of CA–PMMA cements is of particular importance because cytotoxicity in a PMMA-based system is mainly dependent on the non-reacted MMA and TEGDMA [22, 23]. The leached monomers from PMMA-based cement were attributed to adverse effects on internal organ and normal tissue [24]. In addition to non-reacted monomers, we should be concerned about the influence of lithium fluoride on the biocompatibility, because lithium fluoride, which was added as the setting accelerator of calcium aluminate cement, displayed cytotoxicity at more than 0.5% by weight in a previous study [12]. It can be seen from the results of the biocompatibility test (Table IV) that both CAC–LP and CAC–P had no cytotoxicity and hemolysis, respectively. In the pilot study, the degrees of polymerization of CAC–LP and CAC–P were more than 99% (data not shown), so we supposed that all specimens of two experimental groups had no influence on the cytotoxicity and hemolysis. The CA-PMMA composite cement including 1.0% by weight of lithium fluoride also showed no cytotoxicity. Accordingly, it seemed reasonable that there were no side effects of biocompatibility regarding the non-reacted monomers (MMA and TEGDMA) and lithium fluoride in this system.

As shown in the results of the *in vitro* bioactivity test, the addition of 1.0% by weight of lithium fluoride showed good bioactivity in comparison to the CA–PMMA composite cement without lithium fluoride. The CA–PMMA composite cement prepared via polymerization yields cement with the calcium aluminate particles coated with the polymer, thus prohibiting any interaction with the SBF. Rapid dissolution of low molecular weight

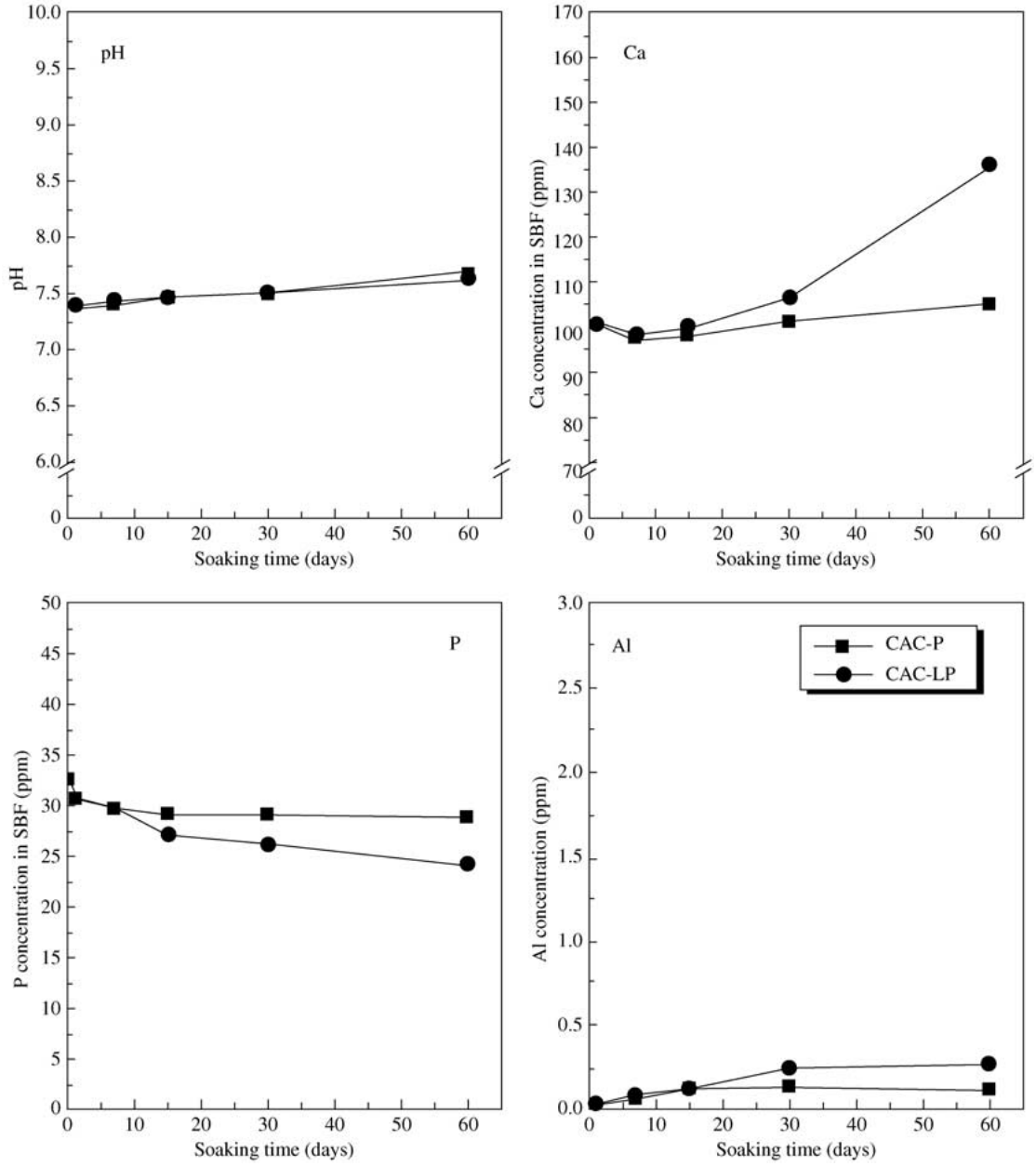

Figure 6 Changes in pH and element concentrations of a SBF solution storing CAC–P and CAC–LP.

PMMA particles increases the amount of MMA in the polymerizing composite and allows the formation of a uniform resin layer on the surface that completely covers the calcium aluminate particles [25]. Calcium aluminate showed good bioactivity in the SBF through the release of many calcium ions into the surrounding body fluids in our previous study [26]. In this system, we found it difficult for CA–PMMA composite cement to form HAp on its surface within all experimental periods because the polymerized PMMA layers prohibit ceramic particles from being exposed to the surrounding body fluids. To overcome this shortcoming of PMMA-based cement, much research has been tried with regard to the change of PMMA's molecular weight [27] and silane coupling method [28, 29] as well as the addition of various kinds of ceramic filler [30–32]. In this research, we focused on the addition effect of highly reactive ceramic filler such as lithium fluoride on reducing the drawbacks of conventional PMMA based system (see Table I).

Lithium has a high ionization potential (5.39 eV) so that the ease of ionization makes lithium highly reactive and electropositive in chemical reactions [33]. Lithium fluoride has been used as the conventional accelerator of setting reaction of cement [34]. It reacts easily with H_2O and precipitates by-products within a few minutes. Accordingly, we suggested that this high reactivity of lithium fluoride made it possible to break the polymerized PMMA layers and contact the surrounding SBF through the broken polymerized PMMA layers. It can be seen from the results of TF-XRD, SEM and XPS that CAC–P showed no apatite formation on its surface after 60 days of soaking in the SBF. On the contrary, $LiAl_2(OH)_7 \cdot 2H_2O$ and HAp were formed on the surface of CAC–LP after 7 and 15 days of immersion, respectively. Therefore, our suggestion can be confirmed from the results of surface analyzes.

Furthermore, the interlayer containing hydroxyl (OH) groups such as Si–OH layer [35] and Ti–OH layer [36], the dissolution rate of calcium ion from substrate into the SBF solution [37, 38], surface structure and pore size on the surface of substrate [39] play a leading part in the formation of HAp. In CAC–LP, $LiAl_2(OH)_7 \cdot 2H_2O$ acted

as nuclei of HAp formation and made it possible to form HAp on the surface of CAC–LP. In addition, calcium ions released from calcium aluminate enabled CAC–LP to accelerate the formation of HAp. In this regard, it seemed no wonder that lithium fluoride promoted the release of calcium ions as shown by the results of ICP-AES.

Conclusion

We prepared CA–PMMA composite cement containing lithium fluoride to make up for low bioactivity of PMMA-based cement. Results of biocompatibility and bioactivity tests indicated that there was no cytotoxicity related to lithium fluoride and non-reacted monomers such as MMA and TEGDMA. The addition of 1.0% by weight of lithium fluoride enabled CA–PMMA composite cement to form HAp on its surface within 15 days soaking of a SBF solution. Further investigations such as long-term *in vivo* animal assay is required to obtain further information of the bioactivity of CA–PMMA composite cement including lithium fluoride.

Acknowledgment

The authors would like to thank Korea Research Foundation Grant (KRF-2001-042-E00112) for financial support.

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Received 7 February 2002
and accepted 10 July 2003