In vivo response of bioactive PMMA-based bone cement modified with alkoxysilane and calcium acetate

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KEYWORDS

Bone cement, Polymethylmethacrylate, Bioactivity, Tissue response, Hydroxyapatite, Calcium acetate.

ABSTRACT

The use of polymethylmethacrylate (PMMA)-based bone cement is popular in orthopaedics for the fixation of artificial joints with bone. However, it has a major problem with prostheses loosening because of coverage by fibrous tissue after long-term implantation. Recently, we developed a bioactive bone cement that shows direct bonding to living bone through modification of PMMA resin with γ-methacryloxypropyltrimethoxysilane (MPS) and calcium acetate. The cement was designed to exhibit bioactivity, through incorporation of silanol groups and calcium ions. Thus, it has the potential to form a layer of bone-like hydroxyapatite, which is essential for achieving direct bonding to living bone. This type of modification allowed the cement to show spontaneous hydroxyapatite formation on its surface in a simulated body fluid after one day, and there is evidence of osteoconduction of the cement in rabbit tibia for periods of more than three weeks. However, the influence of the dissolved ions from the modified cement has not yet been clarified. Thus, we focused on the dissolution of the modified PMMA-based bone cement and its tissue response in muscle and bone by comparison with the behaviour of non-modified

PMMA-based bone cement. One week after implantation in the latissimus dorsi of a rabbit, the modified PMMA-based bone cement showed more inflammatory width than the commercial cement. However, four weeks after implantation, the inflammatory width of both cements was essentially the same. The osteoconductivity around the modified cement was higher than for the conventional cement after four weeks implantation. These results indicate that the initial dissolution of calcium acetate from the modified cement to form the hydroxyapatite induced the acute inflammation around tissue, but also developed the osteoconductivity. It is suggested that the initial inflammation can be effective for inducing osteoconduction through a bone healing reaction when the material provides an environment that promotes bone formation.

INTRODUCTION

Polymethylmethacrylate (PMMA)-based bone cement is a self-curing acrylic polymer used in orthopaedic fields for the fixation of artificial joints with bone [1]. The cement is composed of two components. One component is a powder that includes PMMA and benzoyl peroxide (BPO) as a radical initiator of polymerization, and barium sulfate or zirconium oxide as a radiopacifier. The other component is a liquid that includes methylmethacrylate (MMA) monomer, *N*,*N*-dimethyl-*p*-toluidine (NDT) as an accelerator of polymerization and hydroquinone as stabilizer. These constituents are mixed in the surgery until a dough-like consistency is achieved. Then, the paste is injected into the bone cavity. The mixed paste of the starting components sets in approximately 15 min [2]. Therefore, it allows for easy fixation of artificial joints with bone. However, the most significant problem with use of this cement is an aseptic loosening of the implanted prosthesis, as the cement becomes capsulated by fibrous tissue after long-term implantation. The formation of this fibrous layer at the interface between the cement and bone causes micromotions, resulting in failure of the implanted

prosthesis [3]. Therefore, the development of a bioactive bone cement that is able to directly bond to living bone (i.e. bioactivity) has been a research focus to achieve a tight fixation, even after long implantation periods [4].

The essential prerequisite for an artificial material to bond to living bone is the formation a biologically active bone-like hydroxyapatite layer on its surface in bony defects [5,6]. The same type of hydroxyapatite formation can be observed on the surface of bioactive materials, even in an acellular simulated body fluid with ion concentration nearly equal to human body plasma, as described by Kokubo and colleagues [7,8]. Fundamental studies on the reactions of bioactive glass and glass-ceramics in a simulated body fluid indicate that the hydroxyapatite formation can be induced by the release of calcium ions (Ca^{2+}) from the material into the body fluid, and by catalytic effect of silanol (Si-OH) groups formed on the surface of the material [9,10]. These findings brought about the idea that incorporation of Si-OH groups and Ca^{2+} ions makes PMMA-based bone cement bioactive. Based on this idea, we developed a bioactive PMMA-based bone cement by incorporating γ-methacryloxypropyltrimethoxysilane (MPS) and calcium acetate [11–14]. In this

modification, the MPS provides Si-OH groups on exposure to an aqueous solution, and the calcium acetate provides the Ca^{2+} ions on dissolution from the cement. The cement modified with MPS and calcium acetate forms hydroxyapatite on its surface within one day in a simulated body fluid. The modified cement exhibits higher bonding strength with bone *in vivo* compared with a commercial PMMA-based bone cement after implantation for three weeks [15]. These findings support the proposition that the modified cement has a high bioactivity (i.e., osteoconductivity) after implantation in bone defects. It is suggested that the bioactivity of the modified cement depends on the dissolution behaviour of the cement.

In this work, the effect of the dissolution behaviour of the modified cements was evaluated by comparing it with a conventional PMMA-based bone cement. To clarify the tissue response, samples of cured cements were implanted into the latissimus dorsi muscle in rabbits. The cured samples were also examined for the release of any unreacted monomer and calcium ions by exposure to distilled water or saline. Moreover, a non-cured paste of the sample was implanted to evaluate the practical effectiveness for osteoconduction in bony defects, as previous work has

revealed osteoconduction in the cured modified cement [15].

MATERIALS AND METHODS

Preparation of the cement

Reagent grade chemicals were used as the starting materials. Calcium acetate monohydrate ($Ca(CH_3COO)_2 \cdot H_2O$, Wako Pure Chemicals Industries, Ltd, Osaka, Japan) was pulverized to a size of less than 44 μm, followed by calcination at 220 °C for 2 h to remove any water in the material. The PMMA powder (Sekisui Plastics Co., Ltd, Tokyo, Japan), with molecular weight of 100,000 and an average particle size of 14 μm was mixed with calcium acetate. Barium sulfate (Sakai Chemicals Industry Co., Ltd, Osaka, Japan) and BPO (Wako Pure Chemicals Industries Ltd) were then added to the powders. The prepared powder for use in the *in vivo* experiments was sterilized using 25 kGy of γ-rays.

The liquid components were prepared by mixing MMA (Wako Pure Chemicals Industries Ltd), MPS (Chisso Industry Co., Ltd, Tokyo, Japan) and NDT (Kanto Chemical Co. Inc., Tokyo, Japan). The MMA monomer, MPS and NDT were used without any further purification. Then, the prepared liquids for use in the

in vivo evaluation were sterilized using a 0.22 μm polytetrafluoroethylene (PTFE) syringe filter. The composition of the powder and the liquid is given in Table 1. Commercially available PMMA-based bone cement (Zimmer® dough-type radio-opaque cement, Zimmer Inc., Warsaw, IN, USA) was used as a reference material.

Evaluation of the muscle tissue response

Male Japanese white rabbits weighing about 3.0 kg were used in our experiments. The guidelines of the Japan food Research Laboratories, Tokyo, Japan, for animal experiments were carefully observed. The sterilized powder and liquid were mixed aseptically in a powder-to-liquid ratio of 2.0 at ambient temperature to form a paste. Then, the pastes were shaped to form cylindrical samples with a diameter of 1 mm and a length of 10 mm before they completely cured. The cylindrical samples were inserted into the latissimus dorsi muscles of rabbits under anaesthesia using a 15-gauge needle with a stylet and pentobarbital sodium solution. The rabbits were killed at one or four weeks after the operation to extract the implanted cements and the surrounding muscle tissue. The extracted muscle tissue

was fixed using a neutrally buffered formaldehyde aqueous solution. The muscle tissues were decalcified, dehydrated, embedded in paraffin, and cut to obtain 1–2-μm sections. These sections were then subjected to haematoxylin and eosin staining.

The inflammatory width was measured using an image analysis system (Quantimet 500+, Leica Microsystems GmbH, Wetzlar, Germany). For each section, the inflammatory width, *W*, was calculated using the following equation

$$W = \frac{l - \sqrt{l^2 - 16A}}{4} \quad (1)$$

where l is the total length around the inflammatory region and A is the area of the inflammatory region. Eight to 17 sections were used for each measurement of individual groups. The values were displayed as the mean ± standard deviation (SD) and compared using Student's *t* test. Differences at $P < 0.05$ were regarded as significant.

Evaluation of the bone tissue response

Male Japanese white rabbits weighting about 2.0 kg were anesthetized using

pentobarbital sodium solution. Defects with an area of 5 × 10 mm^2 were made on the cortical bone at the medial aspect of the proximal metaphysis of the tibia. The sterilized powder and liquid were mixed to form a paste, and injected into the intramedullar canals from the bone defect to be cured *in situ*. The rabbits were killed four weeks after the operation. This experiment was carried out at Japan SLC Inc., Shizuoka, Japan. The guidelines of the company for animal experiments were carefully observed. Segments of the tibia containing the cement were observed using peripheral quantitative computed tomography (pQCT, XCT Research SA+, Stratec Medzintechnik Gmbh, Germany).

Measurement of the concentration of monomer and calcium ions released from the cement

The concentration of monomer released from the cement was measured quantitatively according to the method of Kühn [16]. The mixed pastes were shaped to a size of 3 × 10 × 15 mm^3 before they completely cured. One hour after mixing, the cured rectangular samples were each exposed to 5 cm^3 of distilled water in glass vials. The vials containing the samples were stored at 37 °C for various periods (1, 3,

7 and 14 days). The content of the MMA monomer was measured using high performance liquid chromatography (HPLC, LC-10Avp, Shimadzu Corp., Kyoto Japan) with a reverse-phase column (CAPCELLAPAK C_{18} MG, Shiseido Co., Ltd, Tokyo, Japan) and an MMA standard. The mobile phase was composed of 0.05% trifluoroacetic acid with acetonitrile (50:50), and the flow rate was 1.0 cm^3/min, with UV detection occurring at a wavelength of 220 nm. The dimensions of the results were in micrograms of MMA per gram of bone cement.

The release of calcium ions in saline (142.0 mmol/dm^3) was measured from the cured sample. Cured samples with a size of 10 × 15 × 1 mm^3 were immersed in 35 cm^3 of saline at 37 °C. After they had been kept for various periods (1, 3 and 7 days), the calcium concentration in the saline was measured using a tip-type selective calcium electrode (#7683, Horiba Ltd., Kyoto, Japan).

RESULTS

Figure 1 shows muscle sections around the cement modified with MPS and calcium acetate at one week after implantation, for comparison with the reference sample (commercial PMMA-based bone cement). The formation of layers of fibrous tissues was observed in both samples at the interface between the muscle tissue and the sample. This behaviour indicates a foreign-body reaction caused by the proliferation of fibroblasts and infiltration of macrophages around the implanted samples. The micrographs show that the degree of proliferation and infiltration on the modified cement was higher than that on the reference. There seemed to be a calcification layer at the interface between the muscle tissue and the modified cement, whereas this was not observed at the interface on the reference sample.

Figure 2 shows muscle sections around the cement modified with MPS and calcium acetate four weeks after implantation, for comparison with the reference sample (commercial PMMA-based bone cement). A decrease in the number of fibroblasts and macrophages around both cements was observed compared with the results after one week's implantation. The thickness of the fibrous layer also

decreased for both samples, compared with that at one week's implantation. These results indicate that the inflammation had healed with increasing implantation time. The calcification layer observed for the modified cement at one week's implantation had almost disappeared.

Table 2 shows the inflammatory width of the modified cement and the reference sample (commercial PMMA-based bone cement). The modified cement showed a significantly higher inflammatory width than the reference sample at one week after implantation ($P < 0.05$). However, no significant difference was observed in the inflammatory width between the two types of cement four weeks after implantation ($P = 0.81$). These results indicate that the acute inflammation around the modified cement healed to a level similar to that around the commercial sample after four weeks implantation.

Figure 3 shows pQCT images of a rabbit tibia around the bone defect and the cements four weeks after implantation. Osteoconduction was observed along the surface of the modified cement around the bone defect, but it was not observed on the conventional cement. The induced bone largely filled the bone defect around the

modified cement four weeks after implantation. This indicates that the modified cement exhibited a high osteoconductivity *in vivo*.

Figure 4 shows the amount of unreacted monomer released from the cement with and without modification (commercial PMMA-based bone cement). The results of the modified cement included the released MMA and the MPS, as we determined contents by UV peak detection at 220 nm in the HPLC. The releasing behaviour of unreacted monomer from both cements was almost the same up to 14 days, where the amount of unreacted monomer released from the modified cement showed no significant difference from of the commercial cement for all periods.

Figure 5 shows the change in calcium ion concentration due to immersion of the modified cement and reference sample (conventional PMMA-based bone cement) in saline. The calcium ion concentration released from the modified cement increased sharply up to 1.98 ± 0.10 mmol/dm^3 after one day, and later on, continued to increase slowly, reaching 2.62 ± 0.24 mmol/dm^3 after seven days. On the other hand, the calcium ion concentration in saline did not change, even after soaking the reference up to seven days.

Figure 6 shows the change in pH of the saline due to immersion of the cured samples. An increase in pH was detected after immersion of the modified cement, but the pH of the saline decreased slightly after immersion of the conventional PMMA-based bone cement.

DISCUSSION

We have previously reported that modification of PMMA-based bone cement with MPS and calcium acetate shows the potential for osteoconduction. The present results clarify that modification provides osteoconduction in PMMA-based bone cement, although the modified cement induces acute inflammation in muscle tissue one week after implantation. However, the inflammation healed after four weeks to be similar to commercial PMMA-based bone cement. Vigorous osteoconduction was observed in the modified cement at a bone defect after four week's implantation. These results led us to expect that the osteoconductivity of the modified cement was closely related to the self-healing reaction following the acute inflammation.

Previously, we have reported that a cement modified with MPS and calcium acetate forms hydroxyapatite on its surface within one day's immersion in a simulated body fluid [11–14]. The hydroxyapatite formation is achieved by release of Ca^{2+} ions from the cement and by the production of Si-OH groups on the cement surface. The release of Ca^{2+} ions increases the degree of supersaturation with respect

to hydroxyapatite and accelerates hydroxyapatite nucleation and growth, whereas Si-OH groups can induce heterogeneous nucleation of hydroxyapatite on the surface of the material. Therefore, the modified cement was designed to dissolve the calcium acetate from the cement in the body fluid to form the hydroxyapatite on its surface. The concentration of the dissolved Ca^{2+} ions from the modified cement was 2.62 ± 0.24 mmol/dm^3 after soaking in saline for seven days (Fig. 5). An increase in the extracellular Ca^{2+} concentration to more than about 3 mM promotes the proliferation and migration of osteoblastic cells [17,18]. Our study suggests that dissolution of Ca^{2+} ions from the modified cement allowed hydroxyapatite formation on the sample surface and provided an environment that simulated osteoblastic cells. It strongly supports the idea that osteoconduction around the modified cement was attributable to the dissolution of Ca^{2+} ions from the modified cement.

After one week's implantation, foreign-body reaction was observed in both cements at the muscle tissue (See Fig. 1). In general, the insertion of a foreign body in tissue triggers a response by the organism to isolate and destroy the exogenous

material [19]. This response leads to the release of cytokines and growth factor, inflammatory initiation, and healing responses. In particular, the disadvantages of using a PMMA-based bone cement were pointed out long ago, such as thermal necrosis of the surrounding tissue [20], and chemical necrosis around the tissue due to the release of unreacted monomer [21]. We have previously demonstrated that the maximum temperature during setting of commercial PMMA-based bone cement reaches about 83 °C, but the maximum temperature during setting of modified cement is lower than this (about 51 °C) [15]. The amount of unreacted monomer released from the cements after soaking in distilled water for seven days was nearly identical for both cements (Fig. 4). The inflammation around the commercial PMMA-based bone cement is a disadvantage of the cement. Although the modified cement has fewer disadvantages than the commercial PMMA-based bone cement, it shows massive inflammation in the muscle tissue with calcification one week after implantation. The modified cement releases MMA, MPS, and Ca^{2+}, as well as acetate ions. When the acetate ions are released into the body fluid from the modified cement, they increase the pH to weakly alkaline levels in the body fluid. It

is suggested that a local increase in pH induces the acute inflammation in the muscle tissue involving the formation of the granulation tissue around the modified cement one week after implantation. The same reaction must have occurred at the bone defect.

When the bone fracture occurred, the stages of repair induced the inflammation, and involved the formation of the granulation tissue synchronized with the cleansing of its tissue by macrophages, the proliferation of fibroblasts, and the formation of a soft callus. Once the soft callus filled the wound, calcification of the callus began within three to four weeks. The calcified callus was sufficiently rigidity to prevent gross motion of the fracture [22]. In the tissue around the modified cement, we observed identical cascades of bone healing that led to the formation of granulation tissue at one week and osteoconduction at four weeks. These results indicated that the osteoconduction around the modified cement was promoted by inflammation (i.e., bone healing reaction) due to dissolution of the calcium acetate. That is, it is suggested that the initial inflammation can be effective for inducing osteoconduction through a bone healing reaction when the material

provides an environment that promotes osteoconduction.

Malard *et al*. reported that a brief inflammatory process observed in the early stages after implantation with various grains sizes of biphasic calcium phosphate is favourable to the osteoconduction process [23]. This phenomenon provided us with the fundamental idea for a novel material design for inducing osteoconduction using a self-healing reaction. However, we still have to clarify the optimum composition of the modified cement that can induce an appropriate acute inflammatory reaction to guide the bone healing reaction and provide an effective environment for osteoconduction by controlling the local dissolution of calcium acetate.

CONCLUSIONS

We have carried out experiments to examine the biological response of muscle and bone tissue to bioactive PMMA-based bone cement modified with γ-methacryloxypropyltrimethoxysilane and calcium acetate, and have compared the results with non-modified PMMA-based bone cement. Although the modified cement induced acute inflammation in the muscle tissue one week after implantation, the inflammation healed to a level similar to that seen with commercial bone cement, and vigorous osteoconduction was observed at the bone defect four weeks after implantation. The osteoconduction around the modified cement was promoted by inflammation (i.e., a bone healing reaction) due to the dissolution of the calcium acetate. This phenomenon provided us with the fundamental idea of a novel material design to induce osteoconduction using a self-healing reaction.

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TABLE AND FIGURE CAPTIONS

Table 1 Composition of modified cement with MPS and calcium acetate compared with cement without any modification (reference).

Table 2 The inflammatory width around the cements in rabbit latissimus dorsi muscles.

Figure 1 Histological section of the interface between a rabbit latissimus dorsi muscle and cement modified with MPS and calcium acetate (left) after one week's implantation, for comparison with a commercial PMMA-based bone cement (right). C: cement, M: muscle, Arrow: calcification region. (Original magnification 100×, haematoxylin and eosin stain).

Figure 2 Histological section of the interface between a rabbit latissimus dorsi muscle and the cement modified with MPS and calcium acetate (left) after four week's implantation, for comparison with a commercial PMMA-based bone cement (right). C: cement, M: muscle. (Original magnification 100×, haematoxylin and eosin stain).

Figure 3 pQCT images of a rabbit tibia around the bone defect four weeks after operation. Left: cement modified with MPS and calcium acetate. Right: reference sample (conventional PMMA-based bone cement). Arrow: osteoconduction.

Figure 4 Amount of monomer released from the cement during immersion in distilled water. Open circles: cement modified with MPS and calcium acetate. Closed circles: reference sample (commercial PMMA-based bone cement).

Figure 5 Calcium ion concentration of the saline after soaking the modified cement and reference to saline for various periods up to seven days. Open circles: cement modified with MPS and calcium acetate. Closed circles: reference sample (conventional PMMA-based bone cement).

Figure 6 pH of the saline after soaking the modified cement and reference for various periods up to seven days. Open circles: cement modified with MPS and calcium acetate. Closed circles: reference sample

(conventional PMMA-based bone cement).

Table 1 Composition of modified cement with MPS and calcium acetate compared with cement without any modification (reference).

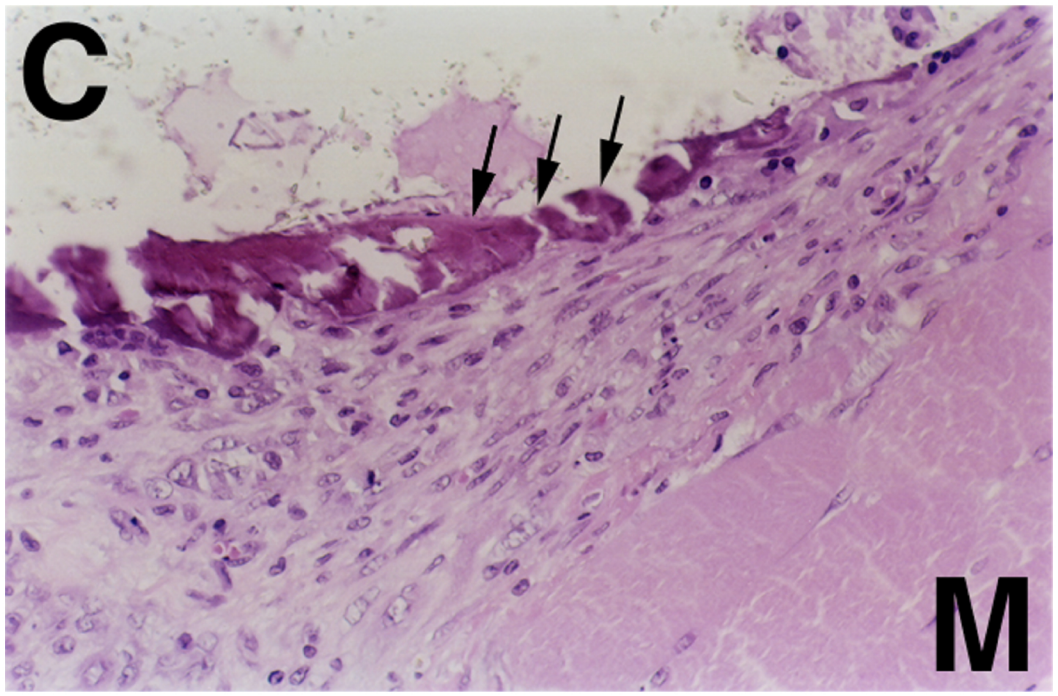
Sample	Powder / mass ratio				Liquid / mass ratio		
	PMMA	$Ca(CH_3COO)_2$	$BaSO_4$	BPO	MMA	MPS	NDT
Modified cement	0.680	0.194	0.097	0.029	0.794	0.198	0.008
Reference (Conventional PMMA-based bone cement)	0.874	0	0.097	0.029	0.992	0	0.008

Table 2　The inflammatory width around the cements in rabbit latissimus dorsi muscles.

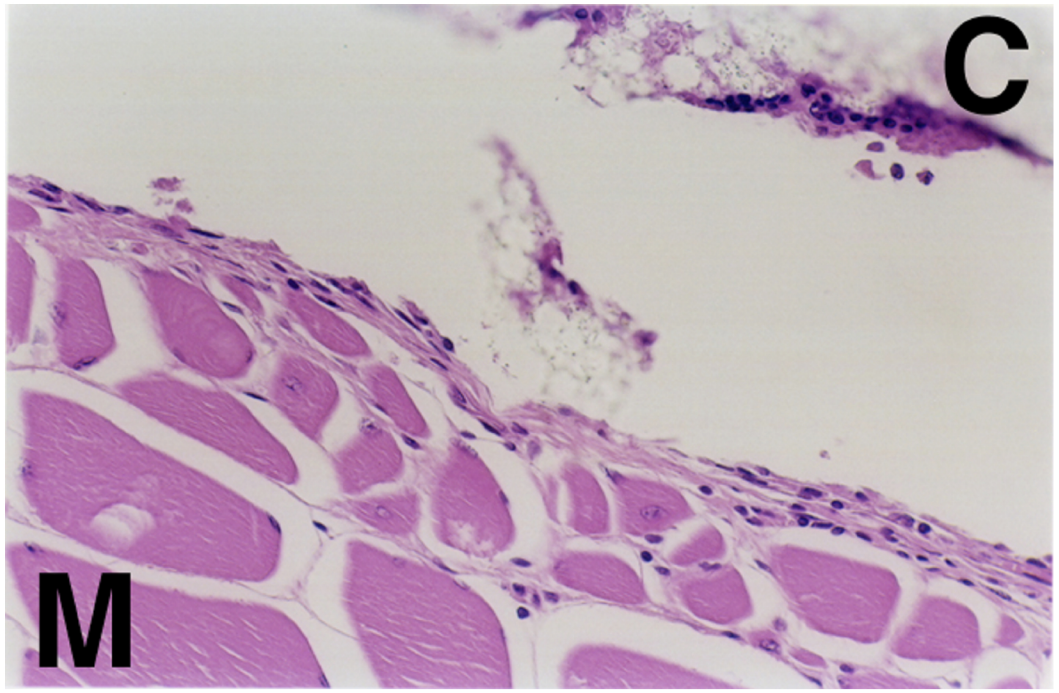
Sample	Inflammatory width / μm	
	1 week	4 weeks
Modified cement (with MPS and calcium acetate)	140.7 ± 31.1*	30.9 ± 19.5
Reference (Commercial PMMA-based bone cement)	67.2 ± 11.7	32.2 ± 10.8

Values are expressed as mean ± S.D.

* Significantly different from the value of Reference (Commercial PMMA-based bone cement) at one week ($P < 0.05$).



Modified cement with MPS and$Ca(CH_3COO)_2$



Reference (Commercial PMMA-based bone cement)

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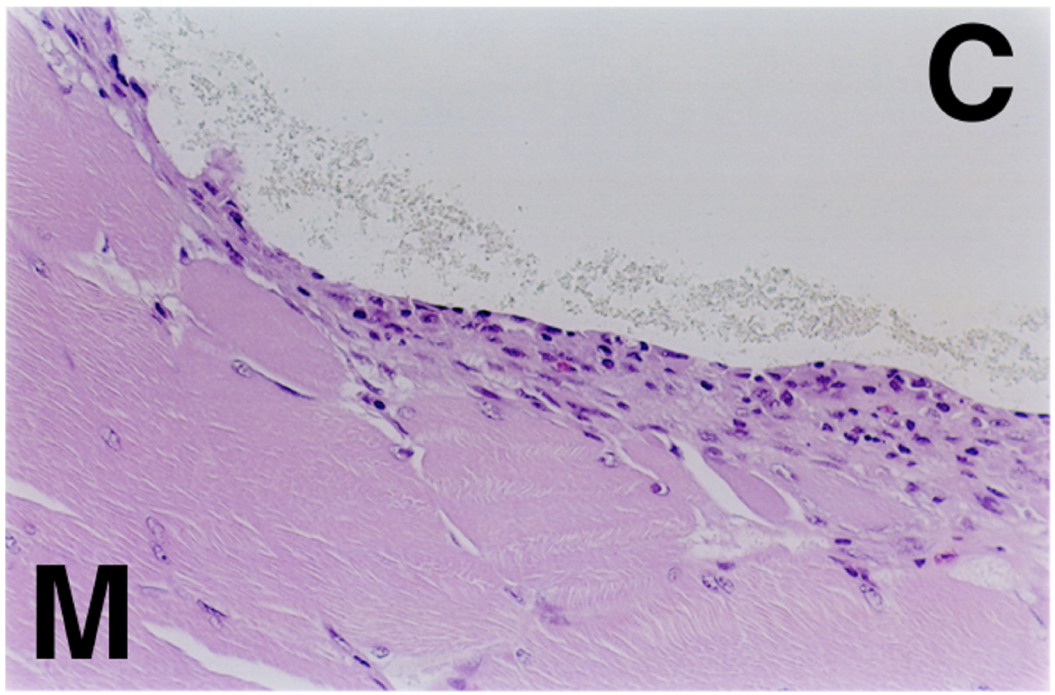
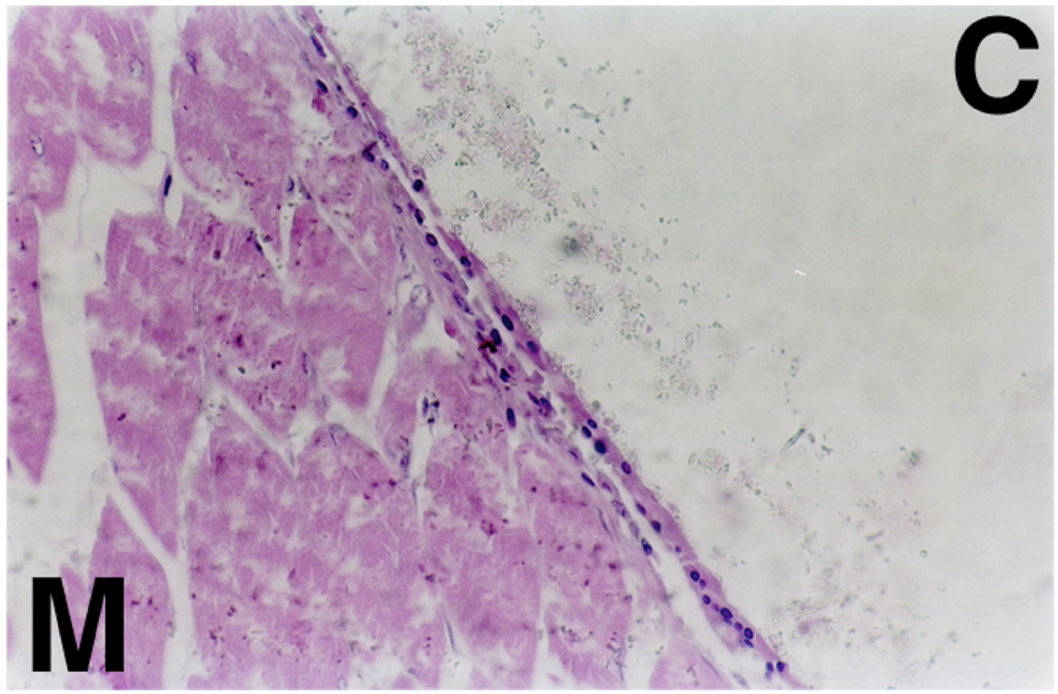


Modified cement with MPS and $Ca(CH_3COO)_2$



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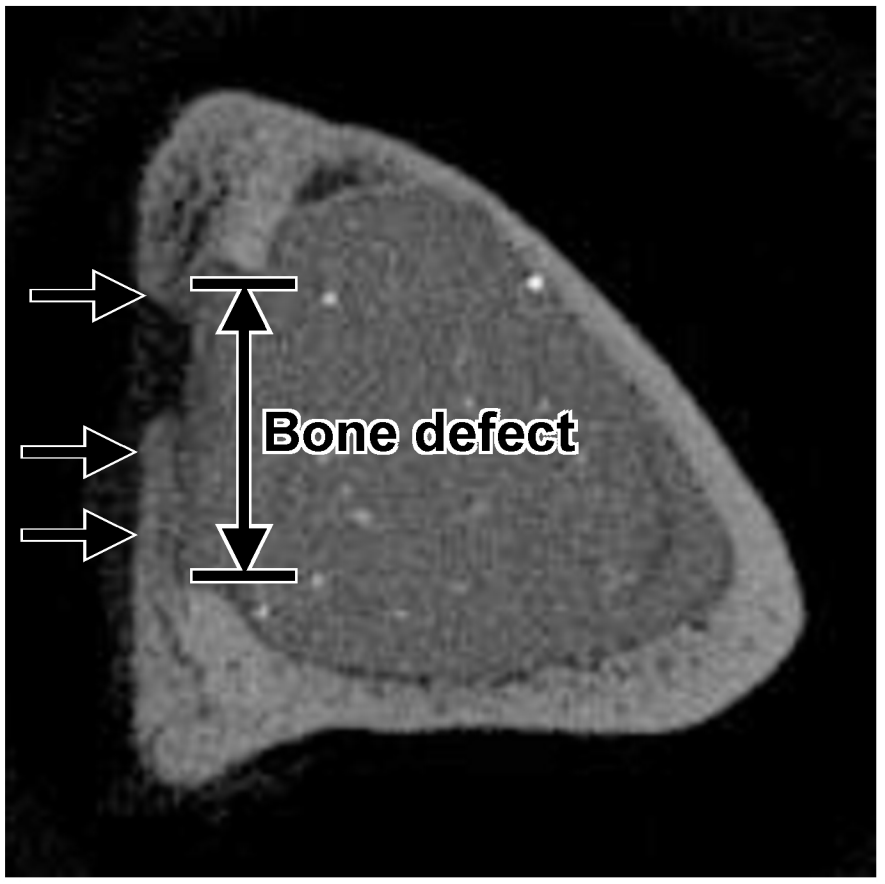
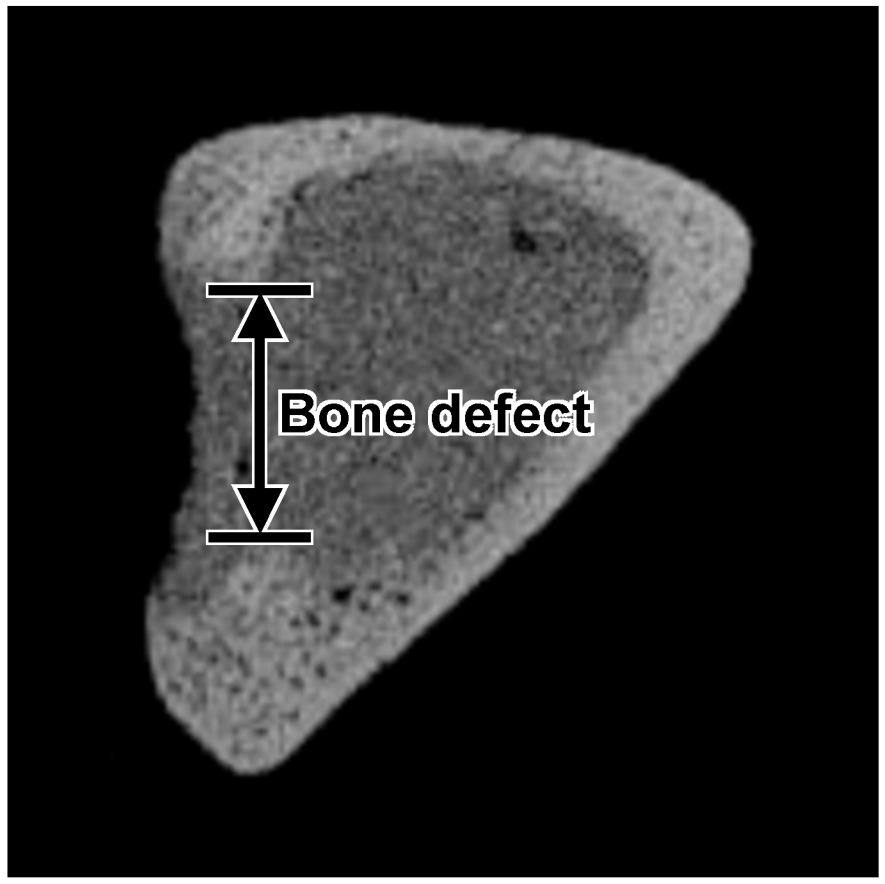


Modified cement with MPS and $Ca(CH_3COO)_2$



Reference (Conventional PMMA-based bone cement)

Figure 3 pQCT images of a rabbit tibia around the bone defect four weeks after operation. Left: cement modified with MPS and calcium acetate. Right: reference sample (conventional PMMA-based bone cement). Arrow: osteoconduction.

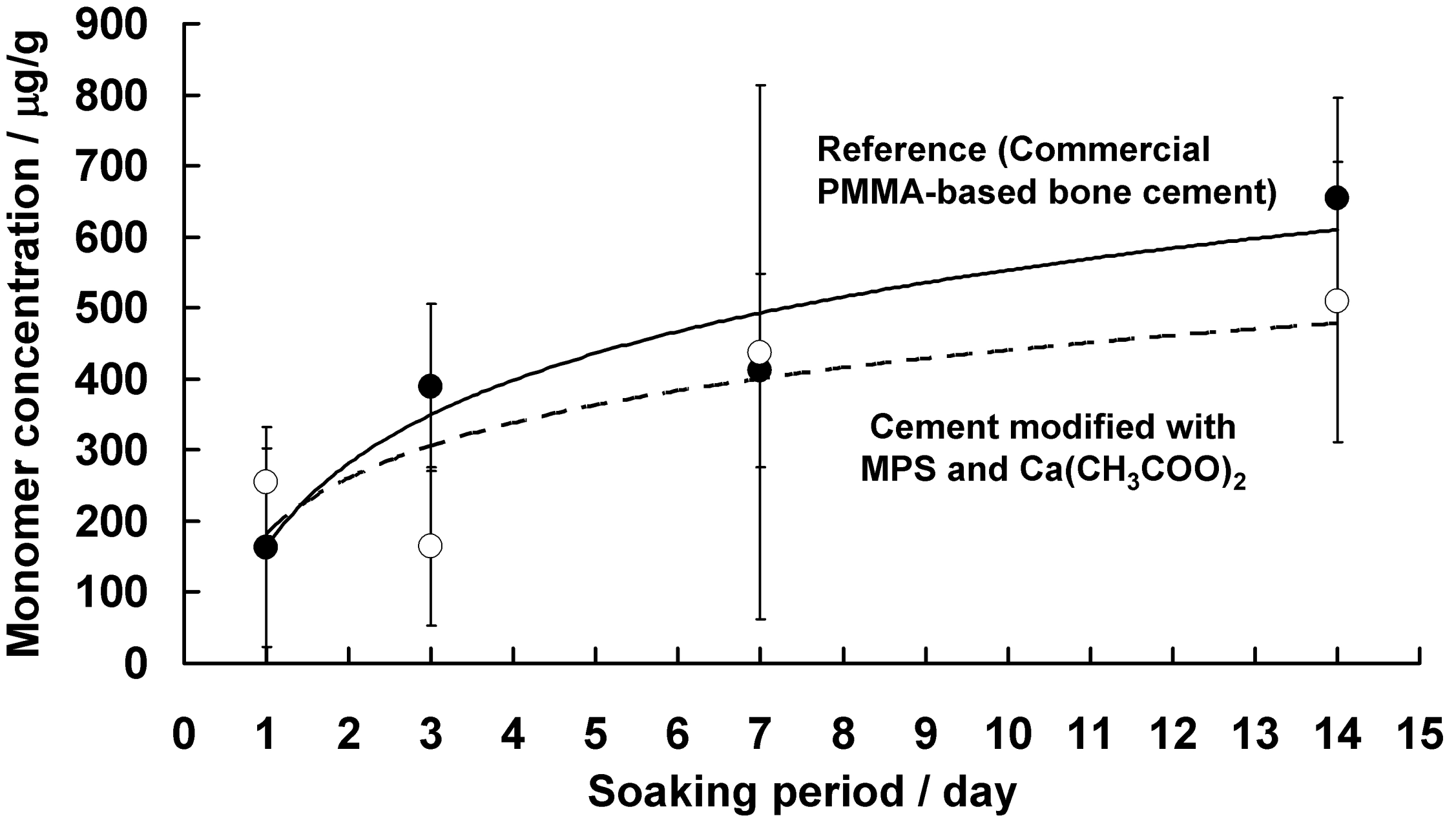


Figure 4 Amount of monomer released from the cement during immersion in distilled water. Open circles: cement modified with MPS and calcium acetate. Closed circles: reference sample (commercial PMMA-based bone cement).

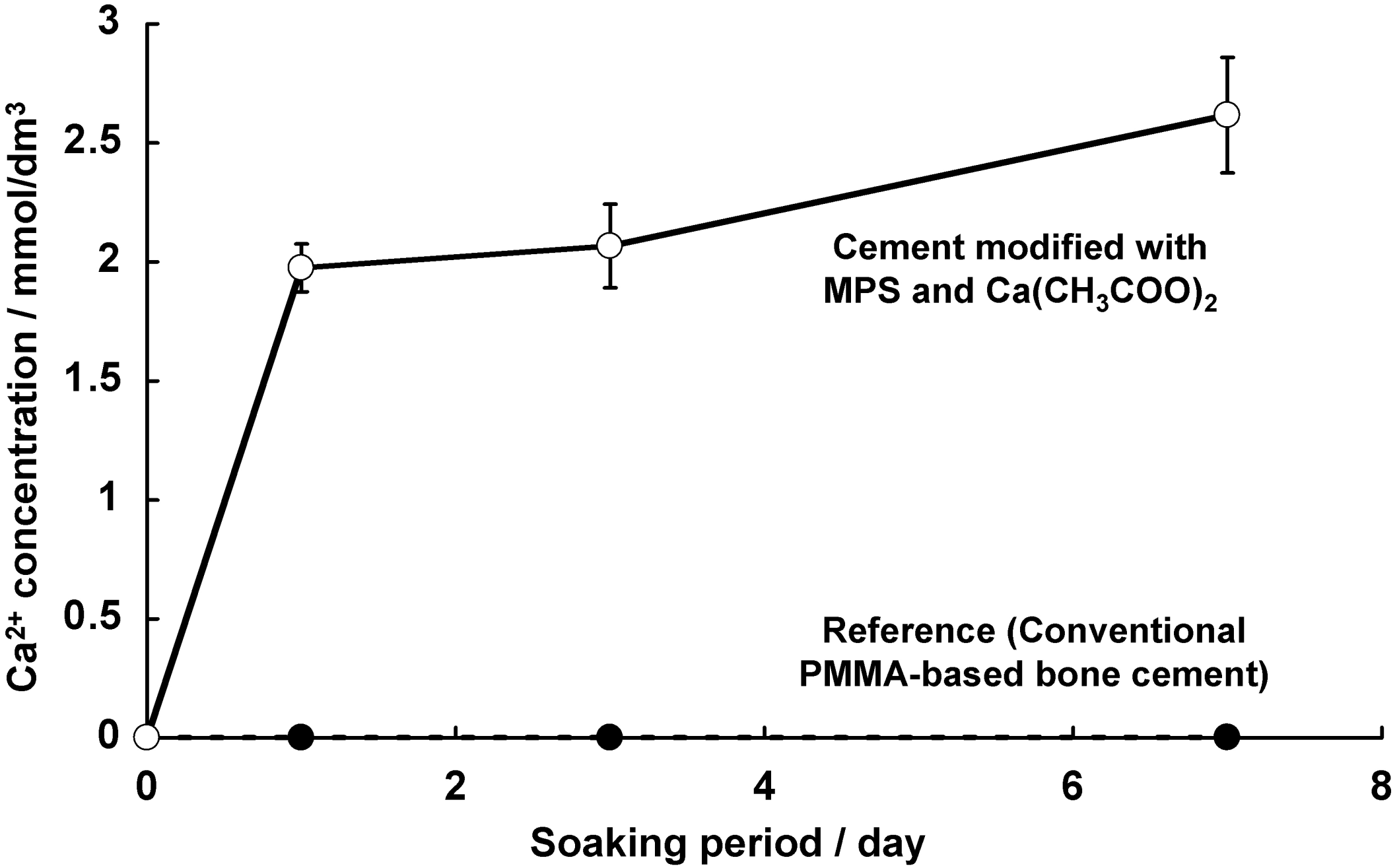


Figure 5 Calcium ion concentration of the saline after soaking the modified cement and reference to saline for various periods up to seven days. Open circles: the cement modified with MPS and calcium acetate, Closed circles: reference sample (conventional PMMA-based bone cement).

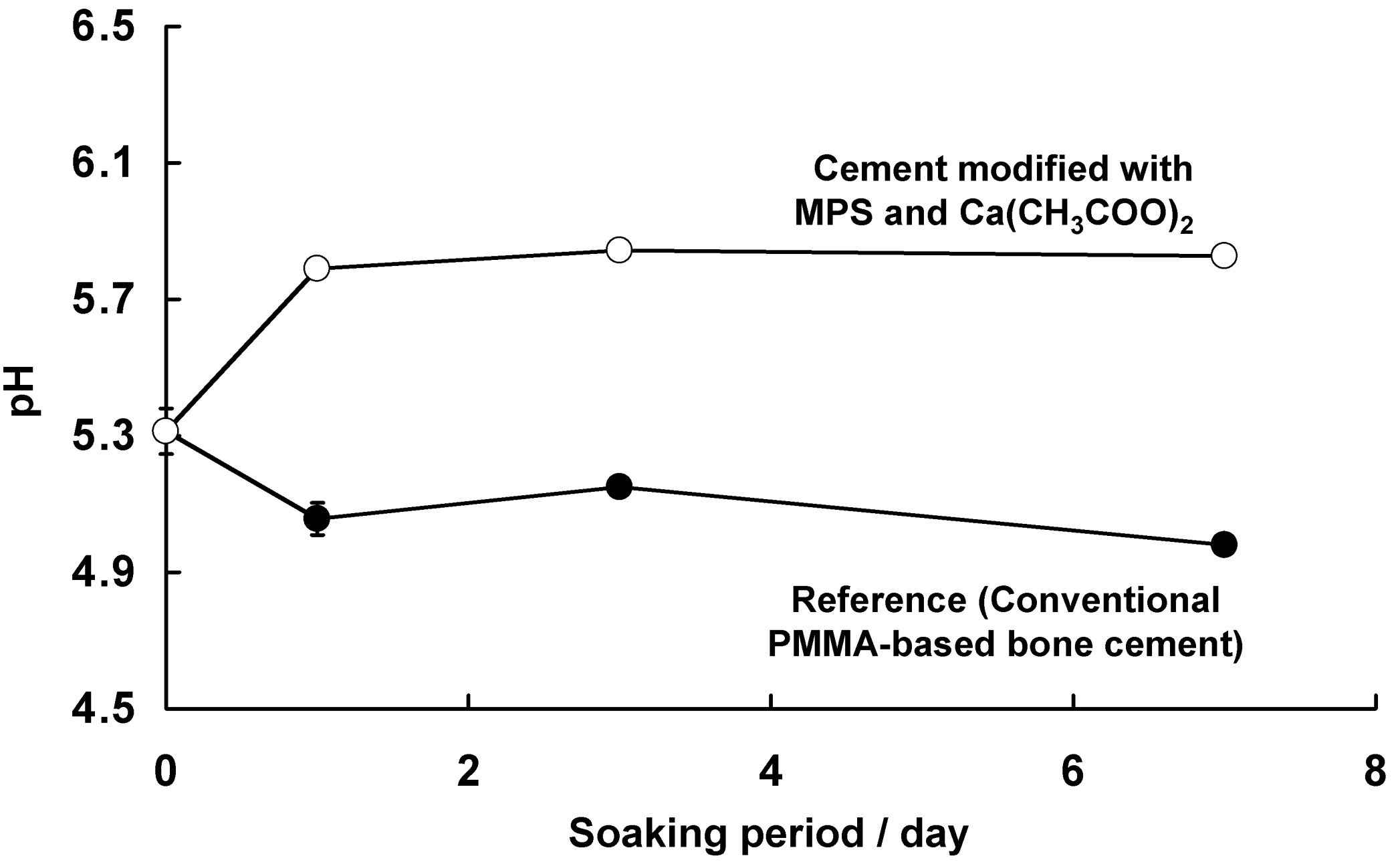


Figure 6 pH of the saline after soaking the modified cement and reference for various periods up to seven days. Open circles: the cement modified with MPS and calcium acetate. Closed circles: reference sample (conventional PMMA-based bone cement).