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Initial in vitro biocompatibility of a bone cement composite containing a poly-ε-caprolactone microspheres

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Abstract The biocompatibility of a reinforced calcium phosphate injectable bone substitute (CPC-IBS) containing 30% poly-ε-caprolactone (PCL) microspheres was evaluated. The IBS consisted of a solution of chitosan and citric acid as the liquid phase and tetracalcium phosphate (TTCP) and dicalcium phosphate anhydrous (DCPA) powder as the solid phase with 30% PCL microspheres. The surface of the CPC-IBS was observed by SEM, and analyzed by EDX profiles. The initial setting of the sample was lower in the IBS containing 0% citric acid than in the IBS containing 10 or 20% citric acid. The compressive strength of the PCL-incorporated CPC-IBS was measured using a Universal Testing Machine. The 20% citric acid samples had the highest mechanical strength at day 12, which was dependent on both time and the citric acid concentration. The in vitro bioactivity experiments with simulated body fluid (SBF) confirmed the formation of apatite on the sample surfaces after 2, 7, and 14 days of incubation in SBF. Ca and P ion release profile by ICP method also confirmed apatite nucleation on the CPC-IBS surfaces. The in vitro biocompatibility of the CPC-IBS was evaluated by using MTT, cellular adhesion, and spreading studies. In vitro cytotoxicity tests by MTT assay showed that the 0 and 10% CPC-IBS was cytocompatible for fibroblast L-929 cells. The SEM micrograph confirmed that MG-63 cells maintained their phenotype on all of the CPC-IBS surfaces although cellular attachment was better in 0 and 10% CPC-IBS than 20% samples.

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1 Introduction

Because of their attractive biological and mechanical properties and bioactivity (i.e., their capacity to form a chemical bond with the surrounding tissues), calcium phosphate ceramics, such as hydroxyapatite ceramics, have gained acceptance clinically and have generated a great deal of interest regarding bone tissue engineering [1–5]. But there are sever shortages of donors for organ transplantation. Moreover, the often limited successes of transplantation because of the risk of viral transmission and immunological consequences, orthopedic surgery for trauma, tumors, fractures, and transplants require artificial tissue fabricated from biomaterials [6]. However, tissue engineering for replacement, filling, fixation, and osteointegration can also lead to implant failure if the biological, immunological, and mechanical properties do not match those of the physicochemical and bone microenvironment. Therefore, substantial efforts have been expended to develop calcium phosphate biomaterials that mimic and adapt to bone tissue microenvironments [7, 8].

The first calcium phosphate cement (CPC) was developed in the mid 1980s by Brown and Chow [9]. In general, CPCs quickly became a popular filler material because of their ability to self-set, their improved injectability, their ability to conform perfectly to the implant site, their increased reactivity, and their potential use in controlled drug delivery [10]. In addition, the osteconductivity and bone regeneration properties of CPC are reported to be excellent [11]. Since the initial development of CPC by Brown and Chow, several compositions have been formulated with the aim of improving its overall biocompatibility and its mechanical properties [12].

In recent years, improvements in the biocompatibility, bioactivity, and efficacy of CPC-Injectable Bone

Substitutes (CPC-IBS) have been attempted by varying the amount and relative concentration of their constituents. However, the development of an ideal IBS has been limited by various problems with mechanical and biological properties. We previously studied the surface reactivity of an IBS that was reinforced with hydroxypropyl methyl cellulose (HPMC) [13, 14]. The bioactivity and cellular compatibility of the HPMC-containing IBS system correlated directly with its surface chemistry and morphology [14].

In this study, the powder phase consisted of a tetracalcium phosphate (TTCP), dicalcium phosphate anhydrous (DCPA), and poly-ε-caprolactone (PCL) microsphere. The liquid phase of the CPC-IBS consisted of chitosan and citric acid (CA). Chitosan is a cellulose derivative that is used as food and cosmetic ingredients. It is non-toxic, biocompatible, and has been used in several bone tissue engineering applications [15–17]. Citric acid ($C_6H_8O_7$) plays an important role as an intermediate product of the Krebs cycle in humans, and it forms a chitosan-organic acid complex [18]. It was previously found that the mechanical properties of chitosan/hydroxyapatite composites were enhanced after the addition of a small amount of CA [19]. In this study, we used poly (ε-caprolactone) (PCL) microsphere to develop a new bone cement system which contains cement-polyester composite for improved biocompatibility and mechanical properties. Polyester polymers such as PCL or PLGA have been used in biomedical applications because of their excellent biocompatibility, biodegradability, and non-toxicity, especially in drug delivery carriers [20–25]. These polymers are tough, flexible, non-toxic, and completely or partially biodegradable inside the body when they interact with body fluid, enzymes, and cells as they undergo hydrolytic degradation and enzymatic attack. These properties make these polymers an interesting candidate for controlled drug release applications and as cement-polymer composites. The incorporation of PCL microsphere in IBS system is also hypothesized to enhance mechanical and biological properties of the total IBS system.

2 Materials and methods

2.1 Preparation PCL microspheres

Poly-ε-caprolactone (Mw: 80,000 kDa) was purchased from Sigma Aldrich Co. Ltd. The preparation of microsphere was done through solvent evaporation-extraction method as previously described in [26, 27] with slight modifications and optimizations.

The beginning of microsphere preparation was started with preparing two phases separately. The first was an organic phase containing 1 g of PCL dissolved in 15 ml of methylene chloride (Sigma Aldrich, Inc.). This solution was prepared in a glass bottle with rubber sealer, ultrasonicated for 30 min and poured and kept in a glass syringe, closed with a rubber cup, and held at 4°C for 15 min. The second was an aqueous phase containing 0.1% methylcellulose which was used as a suspensive agent. The emulsion was formed by pouring the organic phase drop wise into the aqueous phase under mechanical stirring for 2 h at a constant rate of 450 rpm. To facilitate fast solvent extraction and methylene chloride elimination, the emulsion was dropped slowly into a large volume of distilled water and kept for another 2 h in room temperature. Cellulosic filter papers were to separate microspheres from liquid, then washed with distilled water (3 times) and dried at room temperature in darkness for 24 h. The morphology of the PCL microsphere was observed using both light microscopy (LM) and scanning electron microscope (SEM) images, and the purity of the polymer material was verified by FTIR analysis.

2.2 Preparation of CPC-containing PCL microspheres

To fabricate the CPC-IBS system, we first incorporated the PCL microsphere into the powder phase and then mixed it with the liquid phase. The powder phase of our CPC consisted of TTCP and DCPA. TTCP powder was prepared as previously described using a solid state method [28, 29]. In brief, $2CaHPO_4$ and $CaCO_3$ were reacted in a furnace at a holding temperature of 15,000°C for 6 h. DCPA powder was purchased from Sigma Aldrich Co. Ltd. 30% w/v PCL microspheres were uniformly mixed with CPC powder with a spatula. In the liquid phase, we used 0, 10 and 20% citric acid (CA) with 2% chitosan. In this study, the CPC-IBS samples were therefore designated as 0% CA, 10% CA and 20% CA respectively. The liquid phase was added to CPC powder at a ratio of 0.4 ml g^{-1} and mixed well using a spatula until chewing gum-like slurry appeared.

2.3 Morphology of cements

As soon as the solid and liquid phases were mixed, the cement slurry was molded into tubes (9.5 diameters and open at both ends). Morphology of the cements was observed under an SEM after setting (JSM-635, JEOL, Tokyo, Japan).

2.4 Setting time measurements

Following the international standard ISO 9917 for dental silicophosphate cement and using the Gilmore needle method, the initial setting times of the cements were measured [30]. Cements were kept in relative humid

conditions (98%) in 37°C and when a 400-g mass loaded to a needle with a tip diameter of 1 mm failed to make a perceptible circular indentation on the surface of the cements, the time was considered initial setting time and recorded.

2.5 Compressive strength measurements

Samples of each of the three types of cements were molded into columns (9.0 mm in diameter and 18.0 mm in length). The specimens were incubated in air at 37°C and 98% humidity for 1, 3, 7, and 14 days, after which compressive strength was measured using a computer-controlled Universal Testing Machine (UTI, Korea). At least five specimens of each three samples were measured and the upper most and the lower most values were discarded (n = 3). The crosshead speed was 1 mm min^{-1}. The compressive strength was calculated by using the fracture load divided by the specimen's cross-sectional area.

2.6 In vitro bioactivity in simulated body fluid

In vivo bone bonding ability i.e. bioactivity of a material can be predicted in vitro from the formation of bone like apatite on the material surfaces in simulated body fluid (SBF) [31]. Therefore in this study, the bioactivity of 0, 10, and 20% CA cement specimens after setting were evaluated by observing the apatite formation ability of the cements in SBF. Cement specimens were immersed and incubated in a CO_2 incubator with 5% CO_2 and 100% relative humidity at 37°C for 2, 7 and 14 days intervals. The detailed recipe of SBF solution preparation were followed according to the methods described elsewhere by Kokubo et al. [31, 32]. The volume of SBF solutions for each sample were determined by $V_s = S_a/10$, where V_s is the volume of solution and S_a is the surface area of the samples [32] and the samples were kept in static condition in suitable plastic bottle until the immersion time was over. However, SBF solutions were changed with the same volume of SBF every 24 h when apatite formations on CPC-IBS surfaces were observed using scanning electron microscopy (SEM, JSM-635, JEOL, Japan). To check the ion concentrations (Ca and P) in SBF solutions, solutions were collected after finishing each immersion time and subjected to measure Ca and P ion concentrations by ICP (ELAN® 6000 ICP-Mass Spectrometer, PerkinElmer, USA) analysis.

2.7 Cell culture

In this study fibroblast-like L-929 cells derived from mouse fibroblasts were used for cell cytotoxicity assay. To study cellular attachment and adherence to the samples, human osteoblast-like MG-63 cells derived from human osteosarcoma were used. L-929 cells were cultured with RPMI media and MG-63 cells were cultured with DMEM media. Both RPMI and DMEM media were further supplemented with 10% fetal bovine serum (Hyclone), 1% antibiotics, 50 μg ml^{-1} ascorbic acid (Sigma), and 10 mM β-glycerophosphate (Sigma) to facilitate faster growth and inhibit bacterial infections.

2.8 MTT assay

The cell viability against of each of the CPC samples (0, 10 and 20%) was determined using the MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay [33]. To quantify the cell viability percentage against each of the fabricated cements, fibroblast like L-929 cells were used and subcultured in a 24 well plate at 1×10^4 cells per ml per well. Sample extract was prepared following the standard testing protocol (ISO 10993-5) [34]. RPMI medium was used as the diluent to obtain the diluted extracts (100, 50, 25, and 12.5%). After adding 1 ml of diluted extract solutions in the subsequent wells, 24-well culture plate was then placed in a CO_2 incubator and incubated for 72 h at 37°C. Then 100 μl of MTT solutions (prepared and stocked in 5 mg ml^{-1} phosphate buffer saline) was added to every well and re-incubated at 37°C in 5% CO_2 for 4 h. When this final incubation was over, the supernatant was removed from the wells carefully without disturbing purple crystalline formazan at the bottom of each of the wells. To dissolve the purple formazan, 1 ml of dimethyl sulfoxide (DMSO) was added to each well with gentle agitation. Then the solution was collected by pipetting into a 96-well plate and absorbance was measured at a wavelength of 595 nm with an ELISA reader. The cell viabilities against each of the CPC-IBS samples were measured as the mean percentage of optical densities of medium containing the extract solutions to the optical densities of the fresh medium.

2.9 One cell morphology

To study cell growth behavior and attachment on the 0, 10 and 20% CA cements, osteoblast-like cells MG-63. Cells were grown and allowed to settle down onto the cement surfaces for 30 min in DMEM media and attached with 2% gluteraldehyde after subsequent washing in 0.1 M phosphate buffer (PB) or phosphate buffer saline (PBS). After fixing the cells, cell-sample constructs were then dehydrated using 50, 70, 90, 95 and 100% ethanol. Reagent-grade hexamethyldisialazane (HMDS) (Sigma H4875) was used as chemical dryer to dry the CPC-IBS samples and kept in room temperature at relatively dry place Finally, cellular attachment to the material surfaces was observed

by SEM (JSM-5410-V, Jeol) following carbon coating with the SPI-module Sputter Coater at 7 mA for 20 min.

3 Result and discussion

Calcium phosphate cements (CPCs) are approved for clinical applications because of their excellent biocompatibility, bioactivity, and osteoconductivity. Targeting to develop minimally invasive surgical techniques, several IBS systems containing calcium phosphate cements, therefore, have been researched by biomaterial investigators from various corners (10–14, 27). In some of our previous studies, we fabricated calcium phosphate bone cements and studied their mechanical and biological properties in vitro [13]. However, the objective of this work was to fabricate a new IBS system that contains a poly-ε-caprolactone microsphere. Because PCL polymers are non-toxic, biocompatible, and bioresorbable, they are used in bone tissue engineering and drug delivery systems. We hypothesized that incorporation of PCL microsphere into injectable bone substitute containing calcium phosphate cements could be beneficial in case of developing new IBS system and drug delivery to bone microenvironment for improved tissue regenerations.

Three types of IBS systems were developed in the array, which had different citric (CA) concentrations but the same amount of PCL microsphere and hence in this paper was denoted as 0% CA, 10% CA and 20% CA accordingly. To develop the IBS, we added 30% PCL microsphere into the powder phase of the CPC-IBS system. PCL microspheres were prepared using a solvent evaporation-extraction process and were evaluated by light microscope (data not shown), SEM, and FTIR (Fig. 1). Morphologically, the PCL microspheres were round in shape and ranged in size from 80 to 300 μm. The surfaces of the PCL microsphere were irregularly rough and had micro-sized pores (Fig. 1). FTIR analysis showed that the C=O band presented at 1722.2 cm^{-1}, which confirmed the purity of the polymer material (Fig. 1d).

The surfaces of three kinds of samples were examined under SEM after setting. In Fig. 2, we observed the SEM images of CPC-IBS system with and without the incorporation of PCL microsphere. All of the CPC-IBS systems in this study had rough surfaces. However, Fig. 2a showed several micro to submicron particles with obvious pore structures.

On the other hand, the PCL microsphere incorporated very well into each of the IBS systems (Fig. 2c–e). The microsphere incorporated well in the 20% CA samples

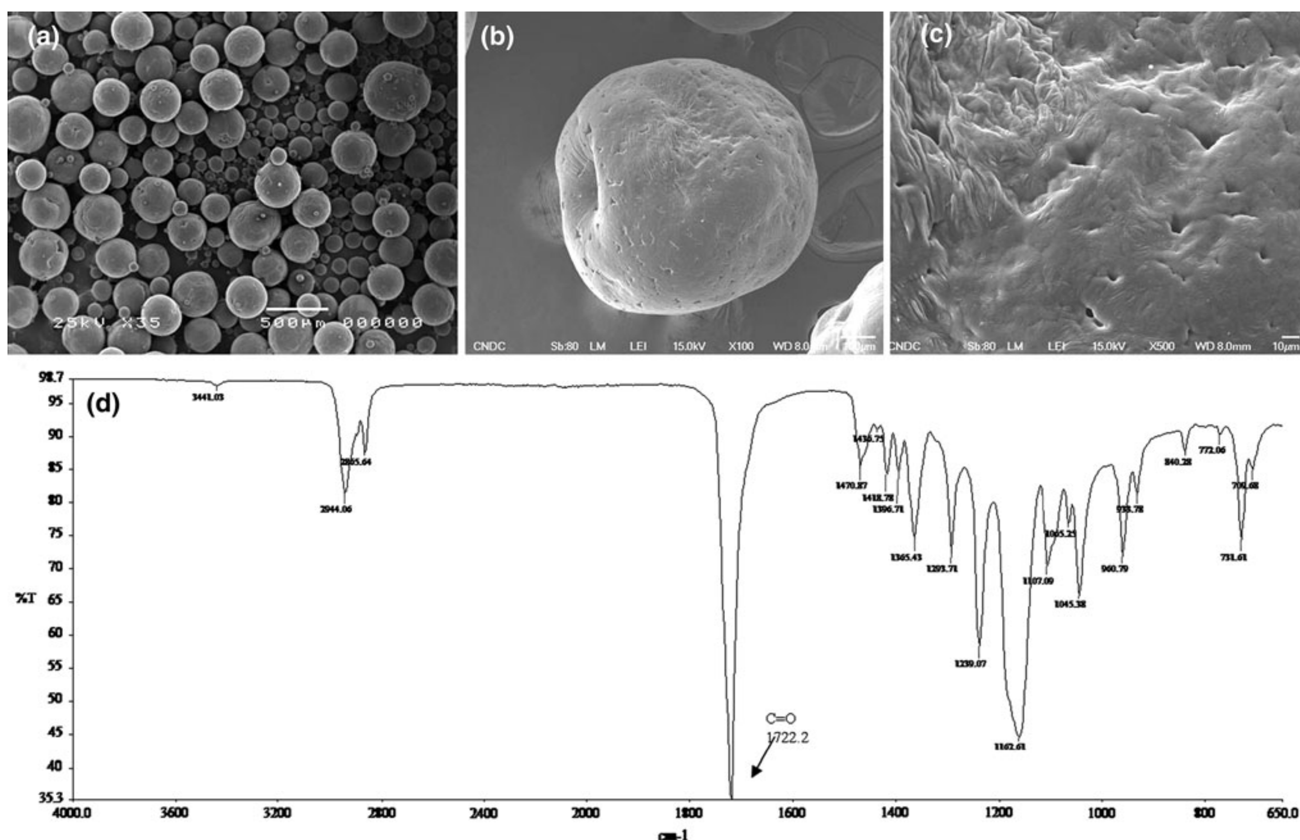

Fig. 1 **a–c** SEM images of the PCL microsphere. **b** Surface morphology and enlarged view. **d** FTIR results for the PCL microsphere

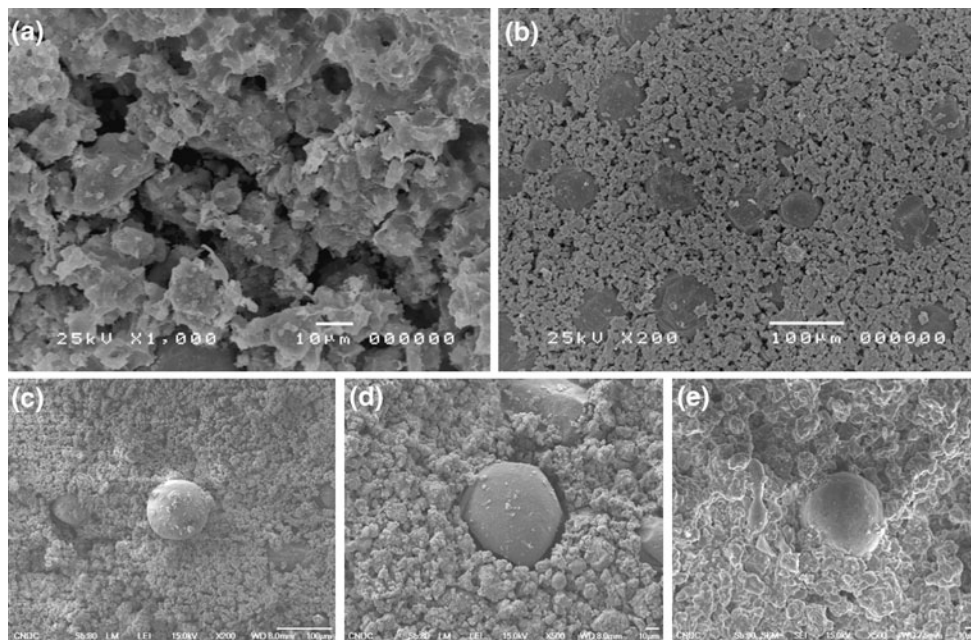

Fig. 2 SEM images of the surface morphology of different CPC-IBS systems. **a** Surface morphology of CPC without a PCL microsphere. **b** Surface morphology of CPC with a PCL microsphere. **c** 0% CA sample. **d** 10% CA sample. **e** 20% CA sample

(Fig. 2e), because citric acid (CA) played a role in the reaction between the PCL surfaces and CPC samples. In this study, all of the CPC-IBS systems had a rough surface morphology. Biomaterial surfaces play an important role in osteogenesis and osteointegration in vivo. Surface morphology and properties of a biomaterial play important role in cellular attachment, proliferation and differentiation [35–39]. The surface roughness has been shown to increase the attachment and proliferation of human osteoblast cells in titanium and titanium based alloys [39]. The degree of surface roughness increases the total surface area of the implant and hence thereby facilitates osteoblast adsorption and proliferation by increasing the osteogenic protein adsorption on the surface [40, 41].

However, surface chemistry is also important for cellular proliferation. Several previous studies have shown that CPCs are osteoconductive and biocompatible [11, 14, 42, 43], other studies reported osteo-disintegration or no bone formation after implant. EDX profiles of the samples showed that Ca and P peak high on the CPC portion of the sample, whereas Ca and P peak low on the PCL portion (Fig. 3).

Figure 4 shows the setting time of the CPC-IBS systems with PCL incorporation. The setting time, or hardening, of the cement was dependent on the CA concentration. The setting time for the 0% CA sample was 37 min. A quick decrease in the setting time was observed as soon as the setting time of the 10% CA sample was measured. The setting time for 10% CA samples were measured to be 25 min which was even lower in case of 20 or 25% CA being 15 and 10 min respectively. In this study, the setting time was described as the initial process of hardening. The cements were mainly hardened because of hydration of the salts in the powder component and chelating reactions between the one or two constituents. Another means of hardening is thought to be the transformation of the composites of the cements to Hap. Fernandez et al. demonstrated the effect of citric acid on CPC, which acted as a water-reducing agent [44]. In addition, the citric acid molecules were shown to absorb on the surfaces of the powder nuclei. This adsorption was explained by the chelating reaction between citric acid and calcium in the powder component. However, in this study we assumed that, addition of the 30% PCL microsphere to the cement system is found beneficial in reaction to their neighboring particles (TTCP/DCPD particles).

Figure 5 shows the compressive strength of the three IBS systems. The compressive strength of the 0, 10, and 20% CA samples was influenced by both time and the citric acid concentrations. The compressive strength of the samples was measured on days 1, 3, 7, and 14. The 0% CA cement had the lowest compressive strength (2 ± 0.5 MPa), which increased as the time of incubation increased. However, compressive strength also increased as the CA concentration increased. The compressive strength of the 10% CA cement on day 1 was 4.67 MPa, which was gradually increased until day 14. At day 14, the compressive strength of the 10% CA cement was 12.3 ± 1.22 MPa. The compressive strength of the 20% CA cement was much higher than that of the other samples: 6 MPa on day

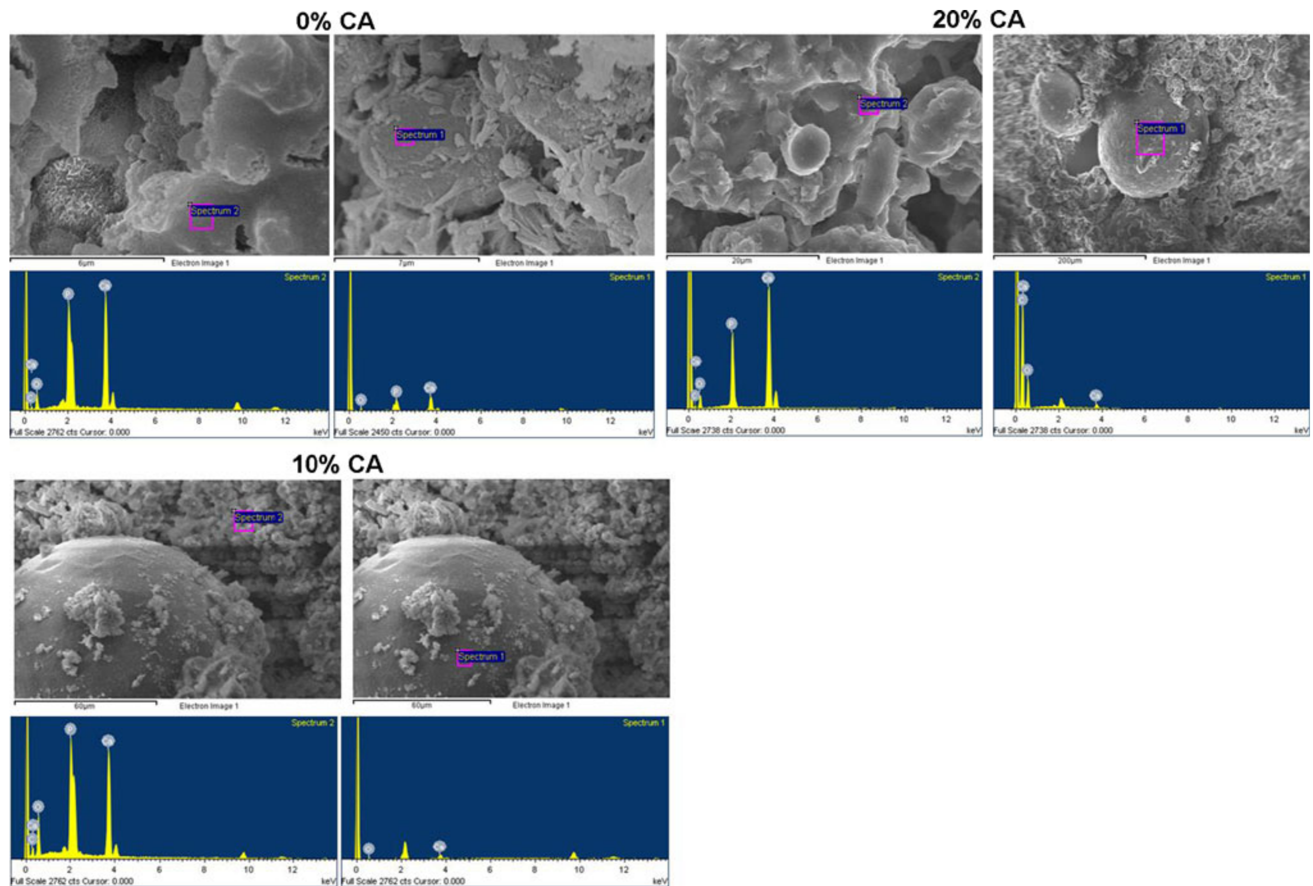

Fig. 3 EDX profile of the 0, 10, and 20% CA samples. Higher Ca and P peaks were observed in the CPC portion than in the PCL microsphere

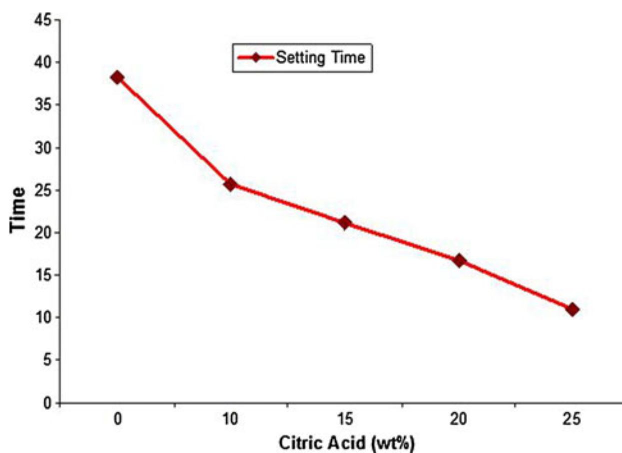

Fig. 4 Initial setting time of the cements. Setting time decreased with increasing concentrations of citric acid (wt%)

1, which increased to 12.45 MPa on day 14. The compressive strength of the samples was also thought to be affected by the reaction of the solid and liquid phases. The water-reducing capability of CA improved the degree of particle packing, which resulted in increasing compactness with increasing time. An increase in the incubation time also led to chelation and transformation (to Hap), which makes the IBS system convenient for bone regeneration and osteointegration. However, the overall compressive strength of the PCL-containing CPC-IBS system also increased by means of strong integration of CPC particles and microsphere surfaces. The improved mechanical strength of the CPC matrix with the addition of CA, the uniform dispersion of the PCL microsphere in the CPC matrix, and good bonding between the CPC and PCL microsphere all contributed to the compressive strength of the PCL-containing CPC-IBS system.

The surface bioactivity of the newly fabricated IBS system containing the PCL microsphere was evaluated. The CPC-IBS samples containing 0, 10, or 20% CA were immersed in SBF for 2, 7, and 14 days, as described previously by Kokubo et al [32] Apatite formation on the bioactive surfaces, resulting from the immersion of the samples in SBF, improves bone-bonding ability. In this study, bioapatite formation on the surfaces of the CPC-IBS samples was observed periodically using SEM, and nucleation of the ionic concentration was measured in SBF by ICP. Figure 6 shows typical apatite layer formation on the surfaces of the CPC-IBS system. The apatitic crystals

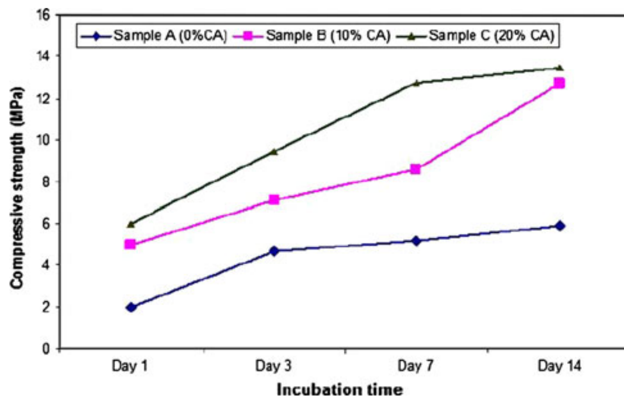

Fig. 5 Compressive strength of the three CPC-IBS systems. Average compressive strength (in MPa) increased as the time of incubation increased

deposited on each of the surfaces in a time-dependent manner, as evident by the growth in size and volume of the crystals from 2 to 14 days of incubation. Moreover, the growth of the apatite crystals was more concentrated in the 10 and 20% CA samples than in the 0% CA sample. These data also confirmed that apatite deposition was greater with an increasing concentration of citric acid in the CPC system.

Figure 7 shows Ca and P ion concentrations in the SBF after 2, 7, and 14 days of incubation. The Ca and P ions tended to deposit on the surfaces of the bioactive materials, resulting in time-dependent bioapatite formation. As the incubation time of the sample in SBF increased, the concentration of Ca and P ions tended to decrease in the SBF. This ionic nucleation is generally favored by the electrostatic forces of the sample surfaces. Because SBF is supersaturated toward hydroxyapatite and octacalcium phosphate, any ionic dissociation in SBF will break this equilibrium, resulting in the start of ionic nucleation on the bioactive surfaces. In this study, the Ca and P ion concentrations in each of the samples decreased rapidly from day 2 to day 14. A several-fold decrease in concentration (in ppm) was observed in SBF for the 0, 10, and 20% CA samples. This rate of decrease in ion concentration was fastest in the 20% CA sample (Fig. 6); pH might also have played a pivotal role.

The apatite formation ability of several bioactive materials has been studied by several other researchers. It is generally agreed upon the bone-bonding ability of this needle-shaped crystalline apatite could augment osteoblastic proliferation. In the current study, we tested the cell viability against each of the materials by using an MTT assay. The MTT assay is a widely accepted assay procedure to measure metabolically active cells in diluted extracts of biomaterials by which the percentile of survived cells can be assumed and interpreted in vitro in understanding primarily the bio-functionality of a material [33, 45, 46]. Hence fibroblast-like cells were used because of

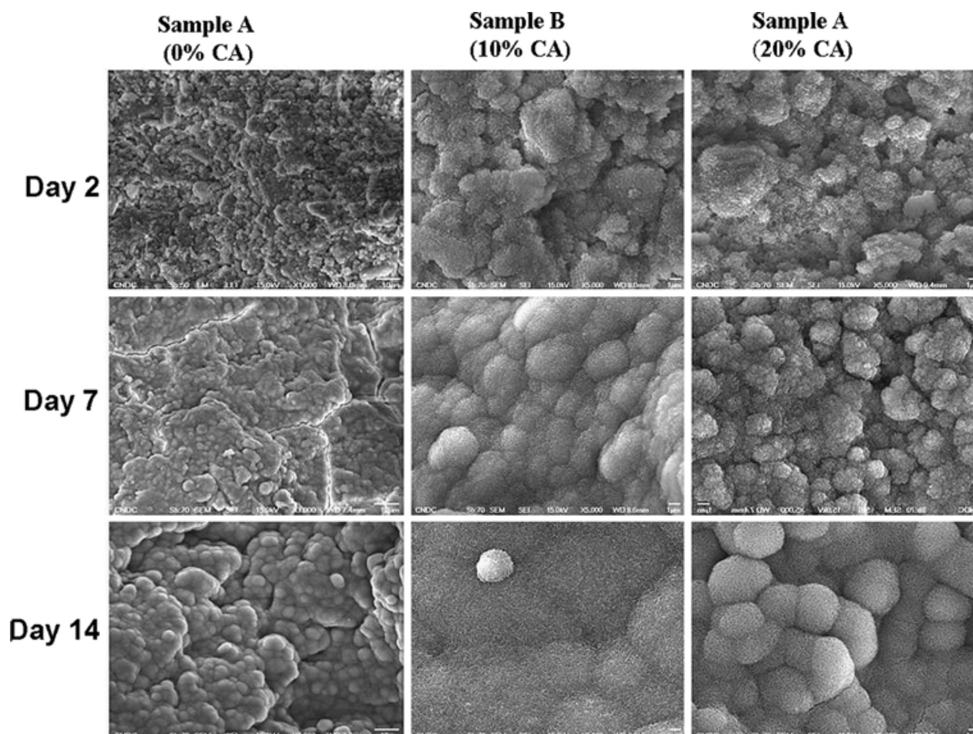

Fig. 6 SEM images of the surface morphology of the CPC-IBS systems containing 0, 10, and 20% CA after incubation in SBF for 2, 7, and 14 days

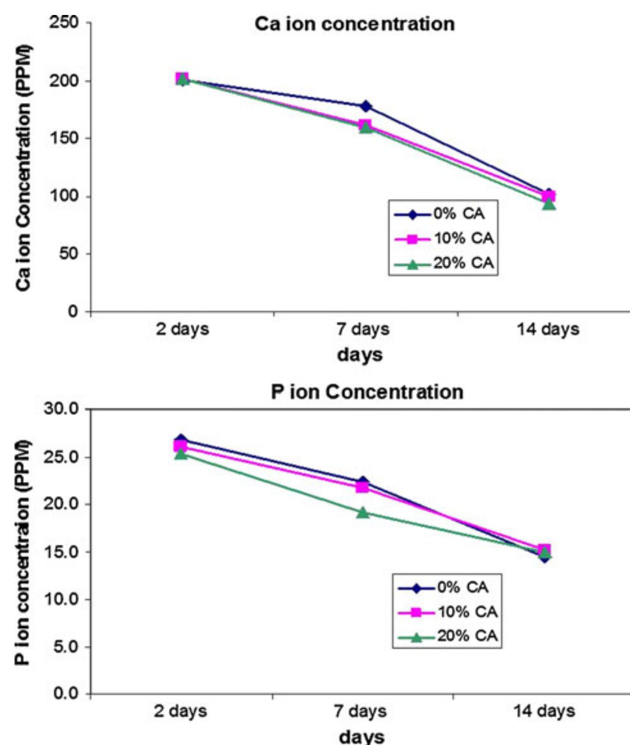

Fig. 7 ICP data showing the variation of Ca and P ion concentrations in SBF of the cements (0, 10, and 20% CA) after immersing for 2, 7, and 14 days

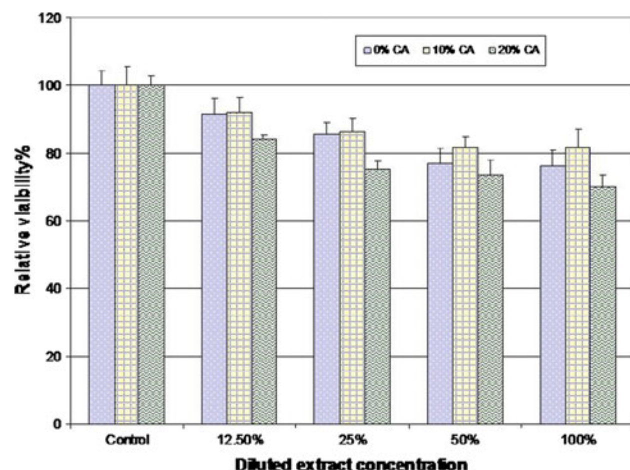

Fig. 8 Cellular viability (%) of samples A (0% CA), B (10% CA), and C (20% CA)

their roles and activities during wound healing and angiogenesis [47]. Survivability of these cells in vitro with tested biomaterials can be useful to postulate in vivo carcinogenesis of a material indirectly [46, 48, 49]. Figure 8 shows the percentage cellular viability of the fibroblast-like L-929 cells in each of the tested materials. Cell viability was greater than 80% in the 10% CA sample, less than 80% in the 0% CA sample, and greater than 70% in the 20% CA sample. The viability of the 20% CA sample was disappointing relative to that of the 0 and 10% CA samples. We assumed that the higher acidic concentration in the culture media of the 20% CA sample was somewhat toxic and resulted in the lower viability of fibroblast cells. One-cell morphologic analysis of the surface of the samples showed that cellular growth of the osteoblast-like cells was better in the 0 and 10% CA samples than in the 20% CA sample (Fig. 9). Osteoblast cells were fixed to the CPC samples in gluteraldehyde for 30 min and cellular morphology was observed by scanning electron microscopy. At the beginning of cellular attachment, cells were round in shape which is very common to normal morphological expression on biomaterial surface. Small bleb-like appendages were also observed on the surfaces of osteoblast cells grown on each CPC-IBS system. However, philopodial expression and attachment between cells and surfaces were better in the 0 and 10% CA samples than in the 20% CA sample. The results obtained from one-cell morphology showed a good agreement with that of the MTT results. The bioactivity of the CPC samples could play an essential role in enhancing biocompatibility of the samples revealing the fact that the more bioactivity, the more biocompatibility. However, detailed in vitro and in vivo studies regarding cell proliferation, material degradation and other histological/histochemical analyses are necessary prior to clinical application of this system.

4 Conclusions

In this study, we fabricated three CPC systems with a 30% PCL microsphere at different CA concentrations (0, 10, and 20% CA). Incorporation of the PCL microsphere in the IBS system improved its mechanical properties, i.e., setting time and compressive strength. The PCL microsphere is also widely used for controlled drug delivery. In this study, the PCL microsphere was successfully applied to the IBS system, but no drug was loaded. However, this new CPC-IBS system appears to be useful for in situ drug delivery in bone tissue engineering because of its bioactivity and biocompatibility. Incorporation of drugs, growth factors or proteins and their release in the bone microenvironment from such CPC-IBS systems could be beneficial for repairing bone trauma and tumor. Both hard and soft tissue regeneration can also be achievable by controlling the desired mechanical properties. Further research with detailed in vivo studies is needed in the development of a controlled drug delivery system prior to clinical applications.

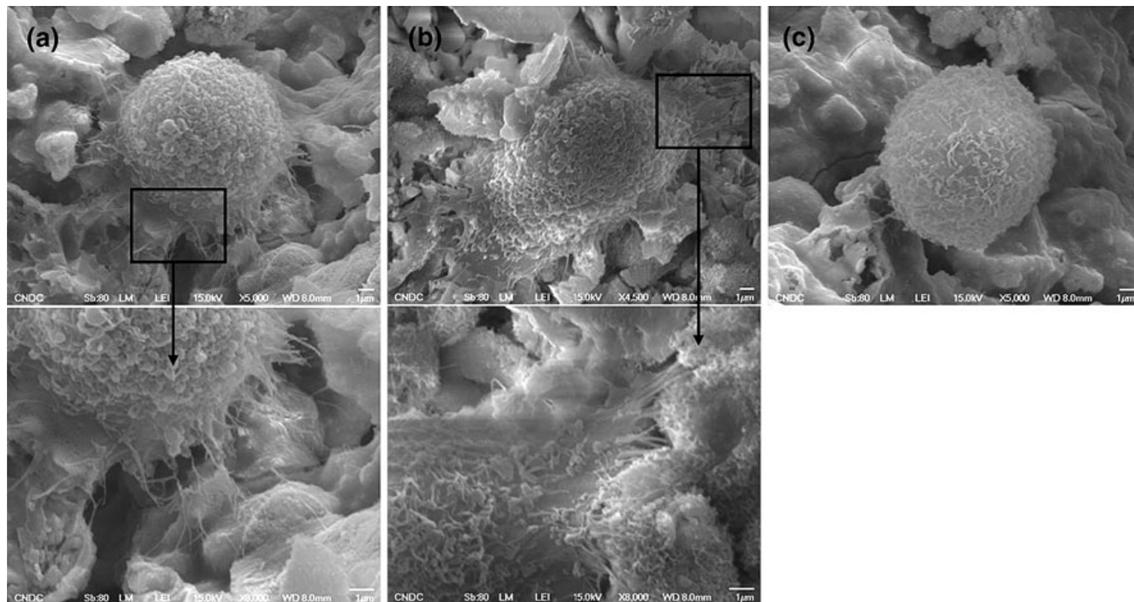

Fig. 9 SEM images of osteoblast morphology. Osteoblasts were grown on the surfaces of PCL-incorporated CPC-IBS systems containing 0% CPC (**a**), 10% CPC (**b**), and 20% CPC (**c**)

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