J Mater Sci: Mater Med (2011) 22:1257–1268
DOI 10.1007/s10856-011-4296-5

Alginate combined calcium phosphate cements: mechanical properties and in vitro rat bone marrow stromal cell responses

Gil-Su Lee · Jeong-Hui Park · Jong-Eun Won · Ueon Sang Shin · Hae-Won Kim

Received: 21 April 2010 / Accepted: 16 March 2011 / Published online: 2 April 2011

Abstract Here, we prepared self-setting calcium phosphate cements (CPCs) based on α-tricalcium phosphate with the incorporation of sodium alginate, and their mechanical properties and in vitro cellular responses were investigated. The addition of alginate enhanced the hardening reaction of CPCs showing shorter setting times within a range of powder-to-liquid ratios. When immersed in a body simulating fluid the alginate-CPCs fully induced a formation of an apatite crystalline phase similar to that of bare CPCs. The compressive and tensile strengths of the CPCs were found to greatly improve during immersion in the fluid, and this improvement was more pronounced in the alginate-CPCs. As a result, the alginate-CPCs retained significantly higher strength values than the bare CPCs after 3–7 days of immersion. The rat bone marrow derived stromal cells (rBMSCs) cultured on the alginate-CPCs initially adhered to and then spread well on the cements surface, showed an on-going increase in the population with culture time, and differentiated into osteoblasts expressing bone-associated genes (collagen type I, osteopontin and bone sialoprotein) and synthesizing alkaline phosphatase. However, the stimulated level of osteogenic differentiation was not confirmative with the incorporation of alginate into the CPC composition based on the results. One merit of the use of alginate was its usefulness in forming CPCs into a variety of scaffold shapes including microspheres and fibers, which is associated with the cross-link of alginate under the calcium-containing solution.

G.-S. Lee · J.-H. Park · J.-E. Won · U. S. Shin · H.-W. Kim (✉)
Biomaterials and Tissue Engineering Laboratory, Department of Nanobiomedical Science & WCU Research Center, Dankook University Graduate School, Cheonan, South Korea
e-mail: kimhw@dku.edu

G.-S. Lee · J.-H. Park · J.-E. Won · U. S. Shin · H.-W. Kim
Institute of Tissue Regeneration Engineering (ITREN), Dankook University, Cheonan, South Korea

H.-W. Kim
Department of Biomaterials Science, School of Dentistry, Dankook University, Cheonan, South Korea

1 Introduction

Calcium phosphate cements (CPCs) are currently gaining great interests due to their usefulness in the direct filling of bone defects and as cell matrices in tissue engineering [1–3]. When compared to other candidate bone replacement materials, the main merit of CPCs is their self-setting nature, which is particularly useful for the application as an injectable device [4]. However, some aspects must still be explored to identify more extended applications, which include improvement in mechanical strength and toughness, control over biodegradability and scaffolding technique, such as macropore generation for the tissue engineering purpose [2, 3].

Along with the powder sources of CPCs, many compositions involving the combination with degradable polymers have been exploited to attain better mechanical and biological properties [5–9]. Widely studied biopolymers include gelatin, collagen, chitosan and alginate [5–10]. The use of these biopolymers generally allows the setting reaction to be shortened while maintaining the CPC network much stronger. The incorporation of gelatin within CPCs has been shown to improve the mechanical properties [5]. CPC composites containing chitosan, as thoroughly studied by Xu et al. [7, 8] have been shown to retain greatly enhanced mechanical strength and bone cell responses. Due to the cohesive nature of these biopolymers,

the setting reaction could also be shortened when the concentration was properly applied. Another merit of the use of biopolymers is the possibility of controlling over the delivery potential of drugs such as antibiotics and growth factors, which can be specifically conjugated with the chemical structure of the biopolymers. For example, the addition of chitosan to CPCs was suggested to produce an effective composition for gene delivery [9].

Within the regime of the CPC-biopolymer systems, in this study we evaluated the CPC-alginate compound. Alginate, as one of the naturally derived polysaccharides, has been used in many biomedical applications, including cell encapsulation, drug delivery and wound dressing because of its non-toxicity, degradability and cross-linking ability [10, 11]. It is soluble in aqueous media, and becomes cross-linked with divalent cations including Ca^{2+} through the ionic interaction between the ions and glucuronate groups. However, compared to other biopolymers, the use of alginate has not often been conducted in conjunction with CPCs [9, 13], particularly with regard to a systematic study on the mechanical and in vitro cellular evaluations.

Therefore, here the effects of the addition of alginate to CPC based on α-tricalcium phosphate composition were investigated in terms of setting reaction, mechanical properties, and in vitro cellular responses. Specifically, stromal cells obtained from rat bone marrow were used to assess the cell population and osteogenic development upon the CPC-alginate substrate. The results provided herein may give some insight into the use of CPC-alginate as a 3D scaffold for bone tissue engineering with bone marrow stromal cells (BMSCs).

2 Materials and methods

2.1 Preparation of CPCs with alginate

The α-TCP cement powder was synthesized via a thermal reaction of $CaCO_3$ and dicalcium phosphate anhydrous (DCPA) at 1400°C, as described in our previous study [13]. The obtained α-TCP powder was added with 2% hydroxyapatite powder, mixed and sieved down to 40 μm to produce CPC powders with an average size of 4.79 (± 0.037) μm (Saturn DigiSizer 5200, Micromeritics, USA). As the liquid phase for CPC, 5% Na_2HPO_4 in distilled water was used. Moreover, 2% alginate solution in 5% Na_2HPO_4 (Na-alginate, Sigma–Aldrich) was used as the liquid phase for alginate-CPCs. The concentration of alginate was based on a pilot study using different concentrations of alginate (1, 2 and 3%), where the alginate was examined completely soluble in the liquid phase while preserving viscosity appropriate for mixing with cement powders. The mixing ratio of powder-to-liquid (PL) was varied (1.0, 1.2, 1.5, 2.0 and 2.5). The mixture was then molded and hardened in a 100% humidity chamber for 24 h. The designations and materials used for the preparation of alginate-CPCs are summarized in Table 1.

Table 1 Sample designations and materials used for the preparation of cements

Sample designation	Alginate concentration in liquid (%)	Liquid mixing ratio Alginate solution: 5% Na_2HPO_4 solution	CPC: liquid ratio
CP10	2	0:1	1.0:1
CP12			1.2:1
CP15			1.5:1
CP20			2.0:1
CP25			2.5:1
CPA10	2	1:1	1.0:1
CPA12			1.2:1
CPA15			1.5:1
CPA20			2.0:1
CPA25			2.5:1

2.2 Characterizations and mechanical tests

The Gillmore needle test (400 g weight used) was introduced to determine the setting time of the cements [14]. The phase transformation of α-TCP into apatite was monitored by immersion in a body simulating fluid for various time points. Morphological changes in the cements were observed by scanning electron microscopy (SEM; S-3000H, Hitachi). The compositional variation was monitored by energy dispersive spectroscopy (EDS; SNE-3000 M, Bruker). The phase change in the cements was characterized by X-ray diffraction (XRD; Ultima IV, Riguka). For the strengths tests, samples were prepared with a dimension of ϕ 5 mm × 3 mm using a plastic mold. The compressive strength (CS) of the samples was measured using an Instron 3344 before and after the immersion test. The tensile strength of the specimens was also measured by means of the diametral tensile strength (DTS) method, according to the equation: $2P/\pi dt$, where P = force applied, d = diameter and t = thickness of the test specimen, respectively. A load was applied to each specimen at a speed of 2 mm/min and the stress–strain curve was recorded and the maximum stress was determined from the data. A total of five specimens were tested for each condition.

2.3 Cell proliferation

Bone marrow stromal cells (BMSCs) were obtained from male Sprague–Dawley rats (aged 4–8 weeks, Korean), as described previously [15, 16]. Bone marrow was aspirated from the tibiae and femora of rats and the mononuclear cells were obtained using an enzymatic solution in

conjunction with centrifugation at 1500 rpm. The isolated BMSCs were then cultured in the normal growth medium (α-minimal essential medium (α-MEM), 1% penicillin/streptomycin, and 10% fetal bovine serum, from Gibco) under a humidified atmosphere of 5% CO_2 at 37°C. For the cellular tests, samples were prepared with a dimension of ϕ 12 mm ×1 mm using a plastic mold. The prepared cement sample was sterilized with 70% ethanol and dried under a laminar flow overnight and then placed in each well of a 24-well plate for the cell tests. Before cell seeding, the cements were soaked in phosphate buffered solution and then in culture medium (serum-free) each for 10 min. A 20 μl aliquot of cells (4×10^4) was loaded onto the surface of each sample and then left for 1 h to allow an initial cell adherence, after which 1 ml of the normal cell growth medium was added.

The cell proliferation on the cements was measured by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium MTS (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega) method, as described previously [15, 16]. When the MTS reagent is applied to living cells, it is reduced by cells into a color formazan product which is soluble in culture medium. After culturing cells for 3, 7 and 14 days, the medium was removed and 200 μl of MTS reagent was added to each sample. After 3 h of incubation at 37°C, the quantity of formazan product was measured at an absorbance of 490 nm, which is directly proportional to the number of living cells, using an ELISA plate reader (iMark, Biorad).

The cell morphology was observed by scanning electron microscopy (SEM; S-3000H, Hitachi). After culturing, the cells were fixed with 2.5% glutaraldehyde, dehydrated with a graded series of ethanol (50, 70, 90, 95 and 100%), treated with hexamethyldisilazane (HMDS) solution. After coating the samples with gold, scanning electron microscope was operated at normal mode with an accelerating voltage of 15 kV.

2.4 mRNA expression

The osteogenesis of BMSCs was induced by a treatment with 100 nM dexamethasone, 50 μg/ml ascorbic acid, and 10 mM β-glycerol phosphate. After culturing the cells on the cement samples for 5, 10 and 15 days in the osteogenic medium, the total RNA of each sample was extracted from the cells using Qiazen RNeasy Mini kit (Qiagen). Then, 2 μg of total RNA were reverse-transcribed in order to synthesize cDNA using an AccuPower RTPReMix kit (Bioneer). The differentiation of BMSCs was monitored by means of detecting differentiation markers, including collagen type I (Col I), osteopontin (OPN) and bone sialoprotein (BPS). The sense and antisense primers were designed according to published cDNA sequences of GenBank, and β-actin was used as housekeeping gene to normalize RNA expression (as presented in Table 2). The synthesized cDNAs were used as templates in polymerase chain reaction (PCR). PCR was carried out under the following conditions: denaturation at 94°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 60 s. The number of cycles was 35 cycles. Electrophoresis was carried out on a 2% agarose gel and was visualized by ultraviolet-induced fluorescence.

Table 2 Primers used for gene expression by PCR and real time PCR

Gene	Primer sequence
β-actin	(f)GCTACGAGCTGCCTGACGG
	(r)GAGGCCAGGATGGAGCC
Col I	(f)TGTTCGTGGTTCTCAGGGTAG
	(r)TTG TCG TAG CAG GGT TCT TTC
OPN	(f)ATCTGATGAGTCCTTCACTG
	(r)GGGATACTGTTCATCAGAAA
BSP	(f)ATAGGCAACGAGTACAACAC
	(r)GTATCCAGATGCAAAGACAG

Quantitative real-time PCR was performed using SYBR Green PCR kit (Quantace) in a spectrofluorometric thermal cycler (Rotor-Gene 3000, Corbett Research). After the real-time PCR run, the Ct value showed how many PCR cycles were necessary to obtain a certain level of fluorescence. The mRNA in each sample was calculated by the comparative Ct value method. Each measurement was assessed in triplicate.

2.5 Alkaline phosphatase activity

The alkaline phosphatase (ALP) activity of the cells was measured using the ALP assay kit (Procedure No. ALP-10, Sigma) [16]. After culturing BMSCs for 7, 14 and 21 days in the osteogenic medium, the cells were gathered and disrupted in 10% Triton X-100 with further cyclic freezing/thawing steps. The total protein content of each sample was determined using a DC protein assay kit (BioRad), which was used to normalize the sample quantity to be reacted. The protein standard curve was based on the bovine serum albumin (Aldrich). The ALP activity, which involves the enzymatic reaction of ALP on a *p*-nitrophenyl phosphate substrate, was assessed colorimetrically. The *p*-nitrophenol product was measured by the absorbance at 405 nm.

2.6 Scaffold formulations with alginate-CPCs

The alginate-CPC solution was shaped into three-dimensional (3D) scaffolds such as fibers or microspheres for use in bone tissue engineering. Within a bath containing 50 mM $CaCl_2$, the alginate-CPC solution kept into a

syringe was injected through a needle with intervals to produce spherical particulates. On the other hand, the alginate-CPC solution was pumped through a needle with continuous force to form fibers. In both cases the alginate-CPC solidified immediately upon injection and coming in contact with the $CaCl_2$ bath, preserving the initial shape of the microspheres and continuous fibers.

2.7 Statistical analysis

Data shown represent the mean ± one standard deviation. Statistical analyses were conducted using a student's *t*-test and the results were considered significant at a P value < 0.05.

3 Results

3.1 Physicochemical properties

The effects of the addition of alginate on the setting time of the α-TCP based CPCs were observed by the Gillmore needle test, as shown in Fig. 1. The setting times were measured while varying the powder-to-liquid (PL) ratio from 1.0 to 2.5. At a PL ratio of 1.0, the setting time was over a day (data not shown). However, when the PL ratio was 1.2, the setting time was greatly reduced (about 1 h). When the PL ratio increased further the setting time decreased gradually for both types of cements. Moreover, the setting times were significantly lower in the CPCs with the addition of alginate (CPA samples) than those without it (CP samples) at all PL ratios.

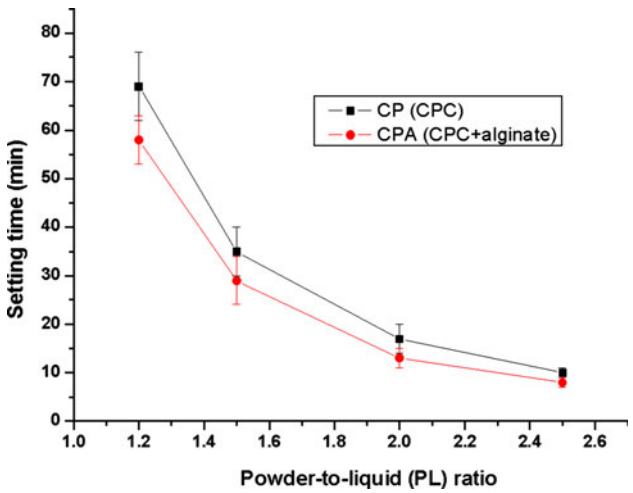

Fig. 1 Setting time of the CPCs with alginate (CPA series) and without it (CP series) while varying the powder-to-liquid (PL) ratio (from 1.2 to 2.5). Setting time was measured by the Gillmore needle test on five different samples, and the data shown are the mean ± std. Increasing the PL ratio enhanced the setting reaction, and the setting time was lower in the CPA series

The surface morphologies of CPCs obtained with and without the addition of alginate were observed by SEM, as shown in Fig. 2. While the CP series (CP12 and CP20 are shown representatively) had a somewhat porous microstructure (more porous in CP12), the CPA series were almost dense, where the alginate polymeric phase filled the pore spaces of the CPC particles.

The CPCs with and without the alginate were soaked in a body simulating medium (SBF) for various time periods to investigate the morphological changes associated with phase transformation. Figure 3 shows the SEM morphologies of the cements after incubation of the samples in SBF for 1, 3 and 7 days. CP20 and CPA20 are shown as the representative samples. At day one, very tiny crystallites began to appear on both types of cements. As the incubation time increased (from 3 to 7 days), the crystal size became larger, suggesting enhanced crystallization.

The produced crystalline phase was analyzed as shown in Fig. 4. The EDS composition analysis of the cements (Fig. 4(a, b)) showed that the initial Ca/P of ~1.50 was increased to ~1.67–1.68 after 7 days of incubation for both types of cements, demonstrating that the initial α-TCP phase was transformed to apatite under the moisture condition. There were no significant differences between CP20 and CPA20 with respect to the Ca/P compositional change. The XRD results of the cement samples before and after the immersion for 7 days also confirmed that a complete phase transformation from α-TCP to HA occurred during the immersion (Fig. 4(c)).

3.2 Mechanical properties

The mechanical properties of the cement samples with different compositions were investigated based on compressive strength and diametral tensile strength tests. As shown in Fig. 5, the compressive strengths of the CPA series increased as the PL ratio increased (CPA10 < CPA15 < CPA20 < CPA25), and this trend was more conspicuous in the samples treated in SBF. The strength continued to increase with incubation time, with the maximum value being obtained at 3 days and a similar value being maintained for up to 7 days of incubation. Particularly in the CPA25, although the initial hardened cements had a compressive strength of ~10 MPa, the value increased significantly up to ~60–70 MPa after 3–7 days of incubation. In the CP series (CP20 and CP25), the compressive strengths were shown to increase after 3 days of incubation, which however was reduced after 7 days. As a result, the compressive strength of CPA25 was significantly higher than that of CP25 after 7 days.

In addition to the compressive strengths, the tensile strengths of the cements were measured by the diametral tensile strength (DTS) method, as shown in Fig. 6. The

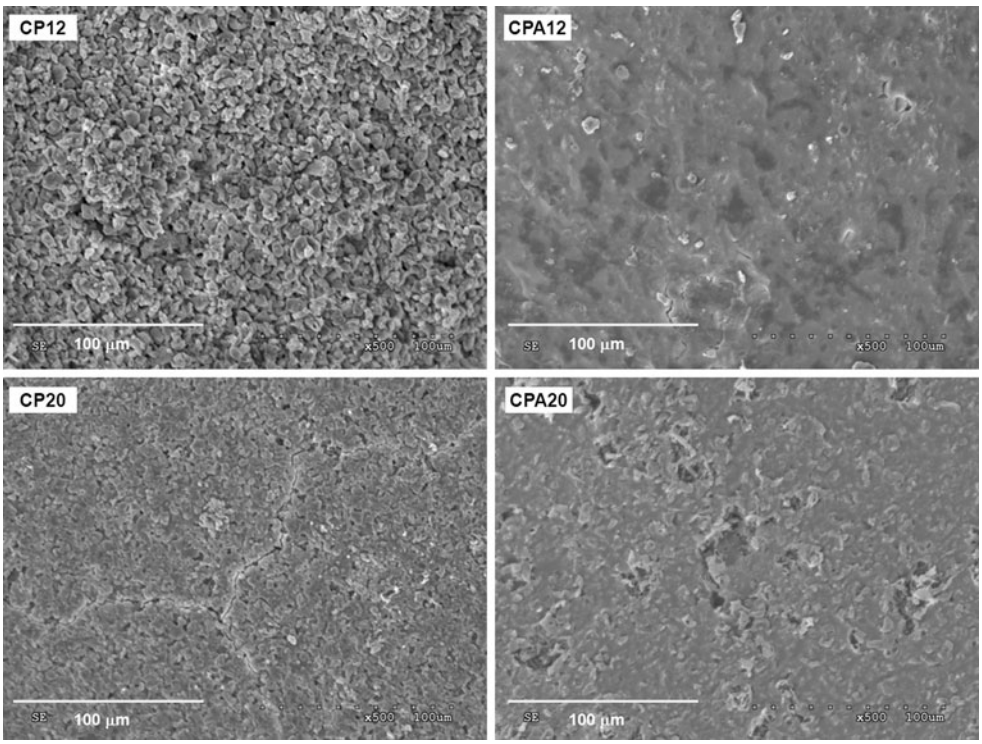

Fig. 2 SEM surface morphology of the CPC (CP12 and CP20) and CPC-alginate (CPA12 and CPA20) after hardening. The CPA series containing alginate exhibited much denser morphology where the alginate phase was shown to surround the CPC particles and fill the gaps between the particles

change in DTS values with respect to incubation time was similar to that of the compressive strength data. The DTS values of the CPA series were shown to increase as the PL ratio increased. Moreover, incubation in SBF for 1 day led to significant improvement of the DTS values, while prolonged incubation led to maintained (3 days) or slightly decreased (7 days) strengths. It should be noted that the strength enhancement with incubation in SBF was higher in the CPA series than in the CP series.

3.3 rBMSCs growth and osteogenic differentiation

Based on the physicochemical properties of the cements we selected one composition (PL ratio = 2.0) for each group to study the in vitro cellular responses. CP20 and CPA20 were chosen as they have relatively short setting times (10–20 min) and high mechanical strengths (especially tensile strength values) and showed complete transformation into a hydroxyapatite phase in a biological fluid. Although CP25 and CPA25 also have similar properties, they were found to be too viscous or sticky to allow ease-of-preparation of samples. Rat bone marrow stromal cells (rBMSCs) were cultured directly on the cements with (CPA20) and without the alginate (CP20) for periods of up to 14 days, after which the cell growth morphology was observed by SEM (Fig. 7). At day 3, cells exhibited highly elongated cytoskeletal processes on both types of cements, suggesting good adherence and spreading (Fig. 7(a)). When the cells were cultured on the cements for 7 days the cell numbers appeared to increase greatly, and some cells fully spread and permeated into the underlying surface (Fig. 7(b)). With prolonged culture of 14 days, the cell numbers increased further, and the cells in the CPA group covered the surface almost completely, reaching almost a confluence (Fig. 7(c)). The cell proliferation on the cements was assessed by an MTS assay (Fig. 8). The similar cell growth level at day 3 became different at days 7 and 14 between the two cement groups with statistical significance ($P < 0.05$), reflecting the cell growth image. The involvement of alginate was considered to improve the cell proliferative potential upon the CPC substrate.

The osteogenic development of the rBMSCs upon the CPC substrates was investigated based on the gene expressions. Bone-related genes, including type I collagen, osteopontin and bone sialoprotein, were shown to be well expressed, and their levels increased with culture time as deduced from the PCR bands (Fig. 9(a)). However, there was no noticeable difference observed in the bands between the two cement groups. Quantification of the mRNA levels by real time PCR also showed similar results on both types of cements, demonstrating that the addition of alginate to CPCs did not have a significant effect on the

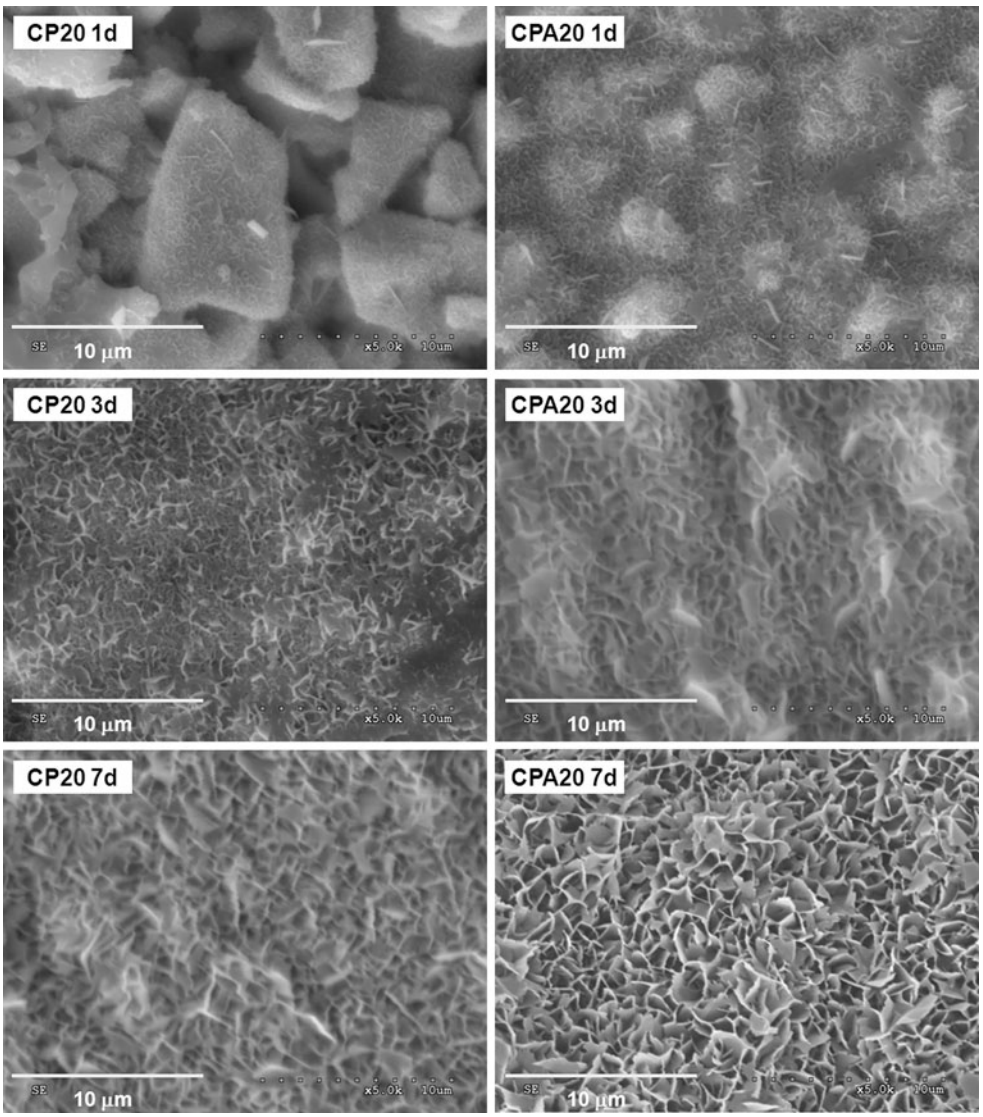

Fig. 3 SEM morphology, showing the surface change of the CPCs when incubated in SBF for various time points (1 to 7 day). Apatitic crystallites were well developed after the incubation, and the crystallite size increased with incubation time

expression of a series of genes by the rBMSCs. The osteoblastic differentiation of the cells was also evaluated in terms of the alkaline phosphatase (ALP) activity, as shown in Fig. 10. The ALP level was normalized to the total protein content of the cells during culture for up to 21 days, which is considered to be representative of the osteoblastic differentiation of rBMSCs [17, 18]. Initially (7 days), the ALP activity was significantly higher on the CPA20, but it became similar at 14 and 21 days when compared to that on the CP20.

3.4 Alginate role in formulating CPC scaffolds

The addition of alginate into the CPC composition was shown to play an important role in the formulation of the cements. As presented in Fig. 11, the alginate-CPC solution could be emulsified into microspheres or injected into a fibrous matrix by applying appropriate tools. These processes were primarily facilitated by the rapid hardening of alginate after it came into contact with the calcium ions in the $CaCl_2$ medium used in the present study.

4 Discussion

In this study, the effects of the alginate addition to the α-TCP based CPCs were investigated in terms of setting reaction, mechanical properties and in vitro cellular behaviors using rat bone marrow stromal cells (rBMSCs). As one of the natural polymers used in biomedical

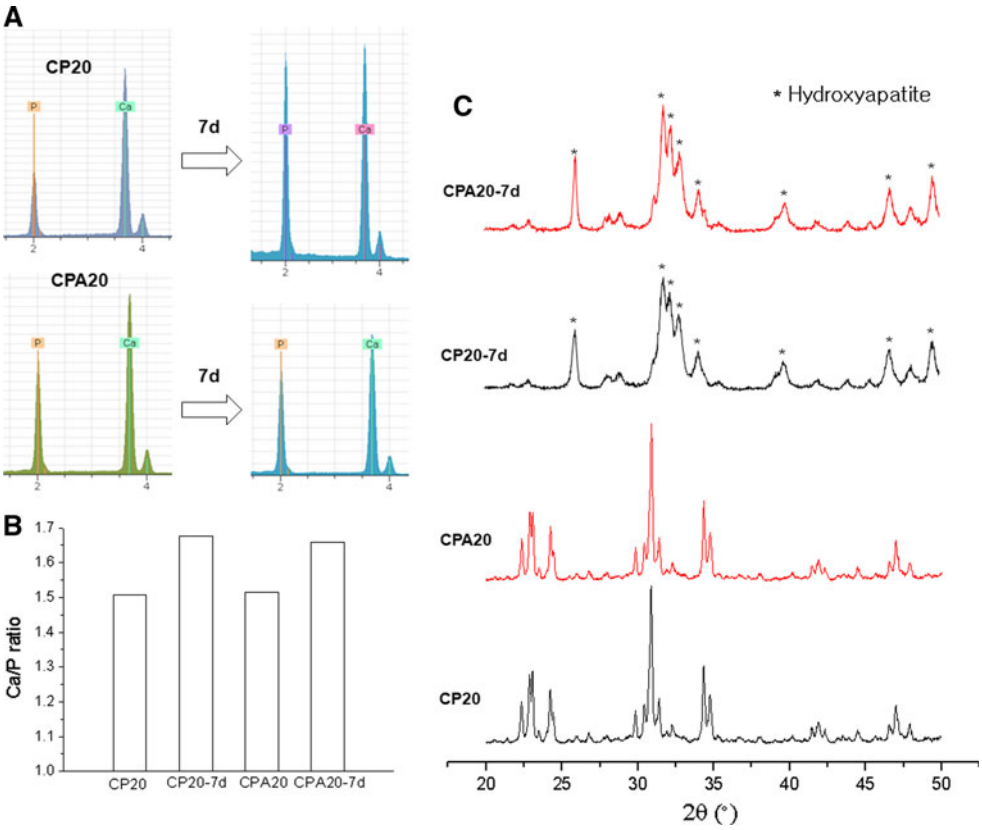

Fig. 4 Analyses of the crystallites formed after the SBF-incubation of the cements. CP20 and CPA20 samples are shown as representative examples. **a–b** Energy dispersive spectrum and Ca/P compositional ratio of the samples before and after SBF-incubation for 7 days. The initial Ca/P of 1.5 changed to 1.67–1.68, which is characteristic of hydroxyapatite. **c** X-ray diffraction pattern showing a complete transformation of the initial α-TCP into HA with SBF-incubation for 7 days

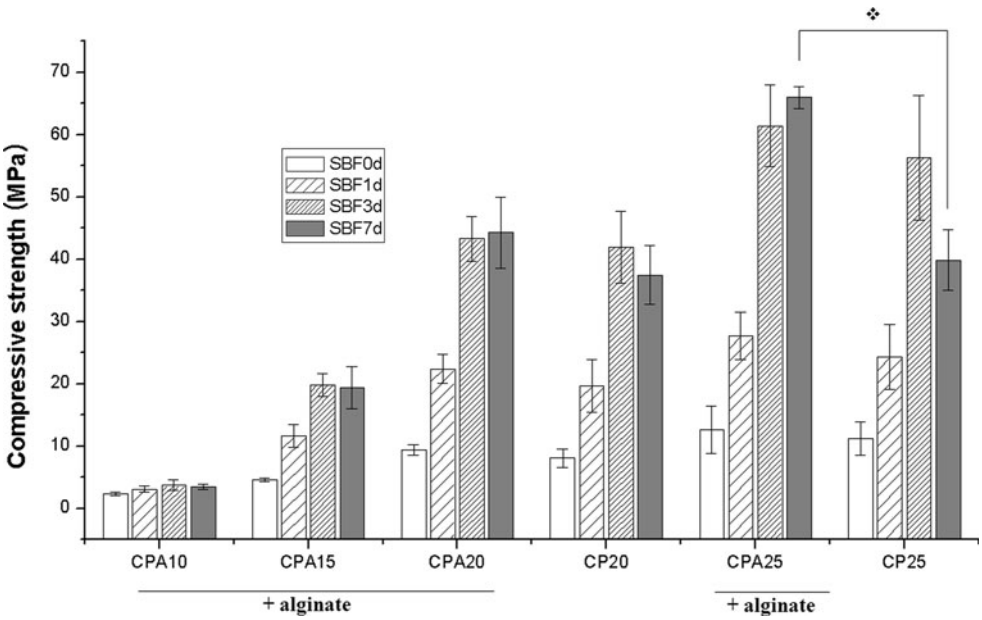

Fig. 5 Compressive strength of the cement samples (CP and CPA series) during incubation in SBF for various periods (1, 3 and 7 days). Cements with a higher PL ratio showed a higher compressive strength value, and the SBF-incubation led to even greater strength. In particular, the CPA25 sample had significantly higher strength than the CP25 after incubation for 7 days (* $P < 0.05$ by student's *t*-test)

applications, alginate is considered to play some roles in those properties of the CPCs. The setting times of the cements (both with and without alginate) were observed to be in a wide range from minutes to about an hour within the PL ratios used herein (1.2–2.5), and there was significant enhancement in the setting reaction with the addition of alginate at all PL ratios (Fig. 1). It is well known that the cementation involves the hydrolysis of α-TCP into a calcium-deficient HA (CDHA) under moisture conditions according to the reaction [19, 20]:

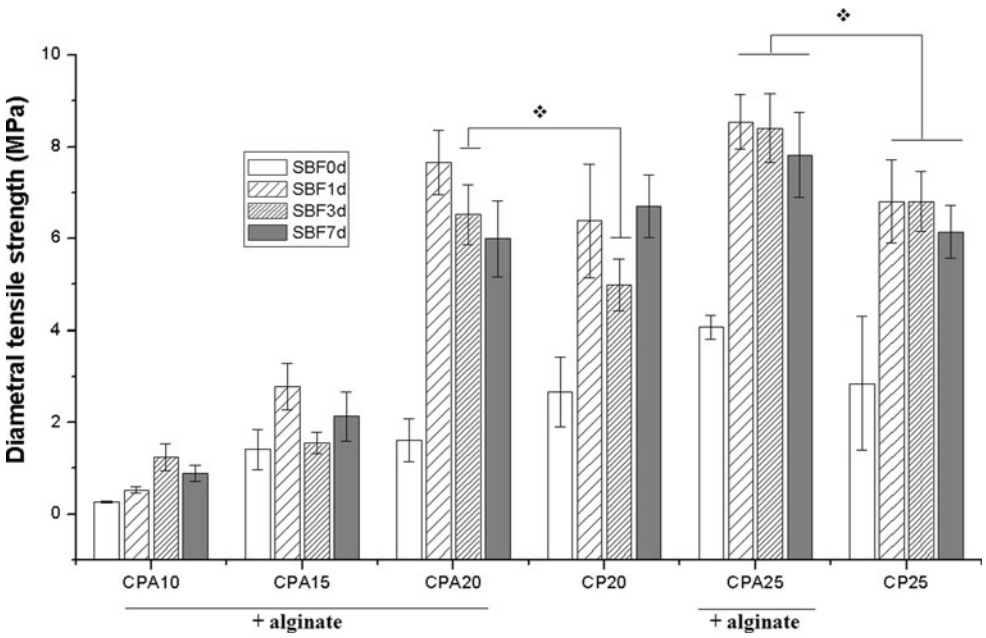

Fig. 6 Diametral tensile strength of the cement samples (CP and CPA series) during incubation in SBF for various periods (1, 3 and 7 days). Significantly higher strength values were observed for the CPA series when compared to the CP series, particularly after the SBF-incubation (* $P < 0.05$ by student's *t*-test)

$$\alpha - Ca_3(PO_4)_2(\alpha - TCP) + H_2O = Ca_9(HPO_4)(PO_4)_5OH(CDHA)$$

Herein, the addition of alginate is considered to help the hardening reaction of CPCs. From the above reaction, the alginate added should come into contact with the calcium ions, which are abundant in the CPCs (in the form of α-TCP or/and CDHA), consequently being cross-linked through the ionic exchange of sodium by calcium.

The hardened cements showed a complete phase transformation of the initial α-TCP into a nanocrystallite HA in a simulated biological fluid, as deduced from the changes in SEM morphology and the analyses of EDS atomic composition and XRD phase (Figs. 3, 4). According to the phase transformation, there were significant changes in the mechanical properties of the cements, including compressive strength and diametral tensile strength (Figs. 5, 6). The properties of crystalline HA formed during the incubation in a fluid, such as the degree of crystallization and the crystal morphology should play important roles in the mechanical properties of the cements [21, 22]. The HA nanocrystallites form an interconnected network within the porous structure of the CPCs, leading to an improvement in strength. Therefore, the increase in strength with the incubation in a fluid is highly associated with the HA nanocrystalline formation. In addition to the HA crystallization, the material degradation during the incubation, particularly with prolonged incubation, should also occur and determine the mechanical properties; thus the reduction in strength with prolonged incubation may be due in part to the material degradation. Therefore, the mechanical properties need to be interpreted within both regimes of crystallization and degradation of cements, and more study is required to fully elucidate the compressive and tensile strength changes depending on the composition (CPC vs. CPC-algiante).

Based on the rBMSCs responses to the cement samples, particularly on the cell growth morphology and MTS results, all the CPCs including alginate-added ones were demonstrated to provide favorable substrate conditions for cells to initially adhere and spread and to further proliferate (Figs. 7, 8). Moreover, the cell differentiation into an osteoblastic lineage was confirmed on both types of cements by the observation of ALP increase and the expression of bone-associated genes (Figs. 9, 10). Particularly at an early stage (day 7), the osteoblastic differentiation appeared to be highly stimulated with the alginate addition based on the ALP determination, which however was not supported by the gene expression levels (Col I, OPN and BSP) from the PCR analysis. In fact, it is not easy to elucidate this discrepancy in the cellular differentiation behaviors because of the differences in the assessment tools and phenotypes used for the ALP and PCR analyses. One possibility is that whilst the PCR assay uses the same quantity of RNA extracts, the ALP activity was the value normalized to the total protein content, which thus should reflect a larger population of cells (referring to the MTS result) at day 7 in the alginate-containing group. Therefore, based on this study it is not possible to confirm the up-regulatory roles of alginate addition to the CPC composition in the osteoblastic differentiation of rBMSCs. In this respect, more systematic study may be needed to elucidate

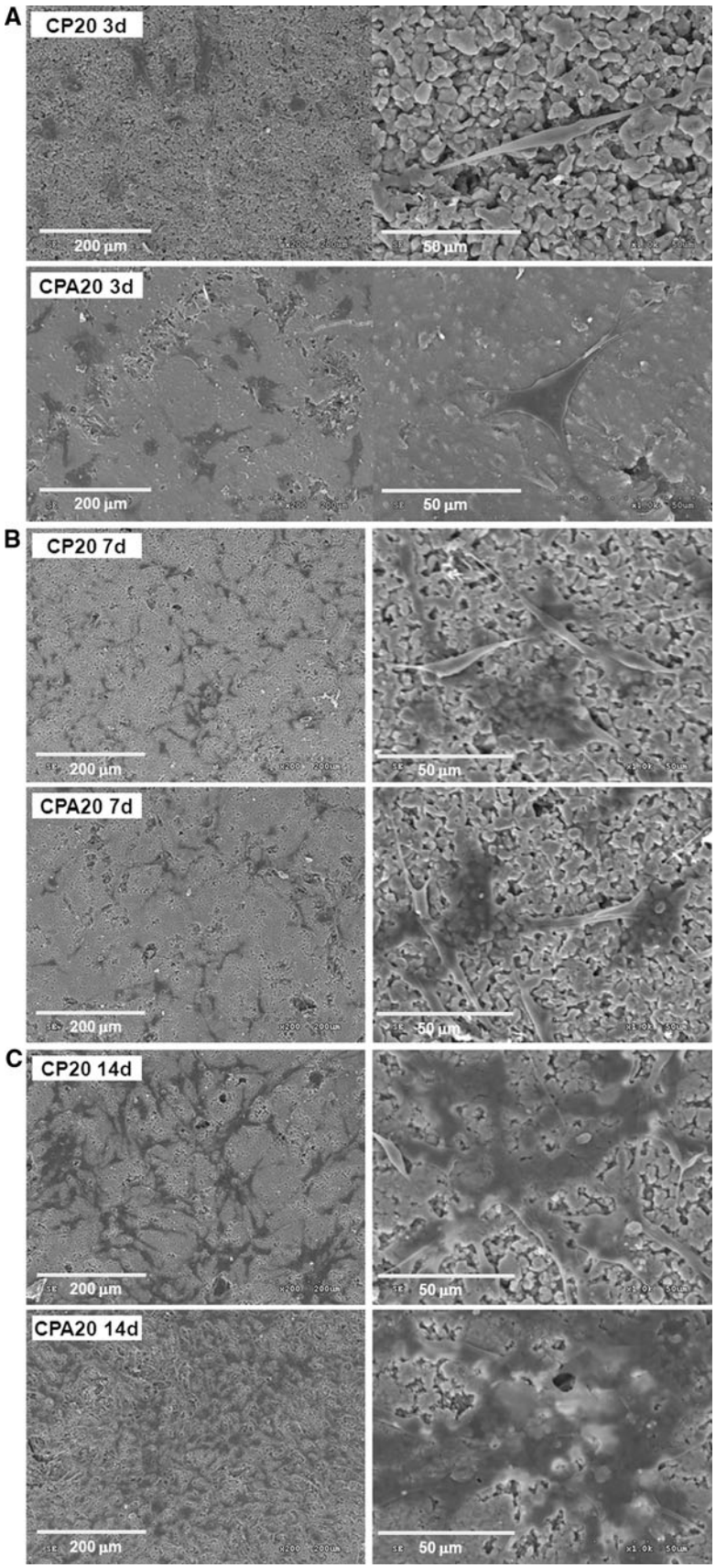

Fig. 7 Growth morphology of the rat bone marrow stromal cells (rBMSCs) on the CPC (CP20) and CPC-alginate (CPA20) during culture for 3, 7 and 14 days. Low and high magnifications are shown for each sample. Cells adhered and grew actively on both CPCs with initial filopodia protrusions and spreading followed by increased population with time and almost full coverage of the underneath surface

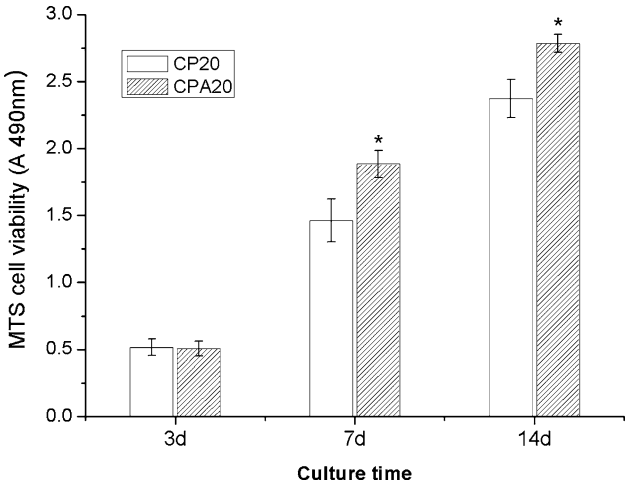

Fig. 8 MTS cell viability measured on the CPC (CP20) and CPC-alginate (CPA20) during culture for up to 14 days. Statistical significance was noticed between the cement groups at day 7 and 14 (* $P < 0.05$, student's *t*-test)

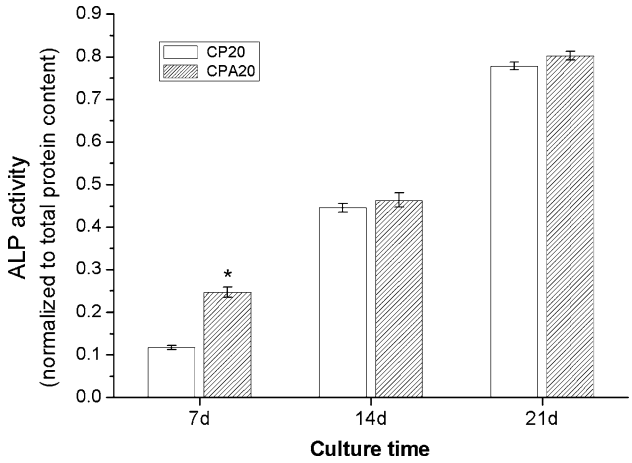

Fig. 10 ALP activity of cells cultured on the CPC (CP20) and CPC-alginate (CPA20) during culture for up to 21 days. ALP level differed significantly between the cement groups at day 7 (* $P < 0.05$, student's *t*-test)

the roles of alginate in the differentiation of rBMSCs. However, the results of gene expressions and ALP activity in tandem with cell proliferation demonstrate the alginate-added CPC is considered to provide good substrate conditions for rBMSCs to grow and undergo osteoblastic differentiation. In other words, the addition of alginate did not negate the efficacy of α-TCP based cement, which in itself has been known as a potential bone substitutes due to its good osteogenic and bone-forming ability [23–25].

Along with the mechanical aspects, particularly when kept under moist conditions, one important merit of the addition of alginate to the CPC composition was found in the ease of formulation into complex-shaped 3D scaffolds. Although CPCs have been shown to be useful as injectable matrices for bone regeneration, they have been difficult to formulate into specific shapes that can be useful for tissue engineering [26]. Herein, we demonstrated the involvement of alginate within CPC composition was effective in

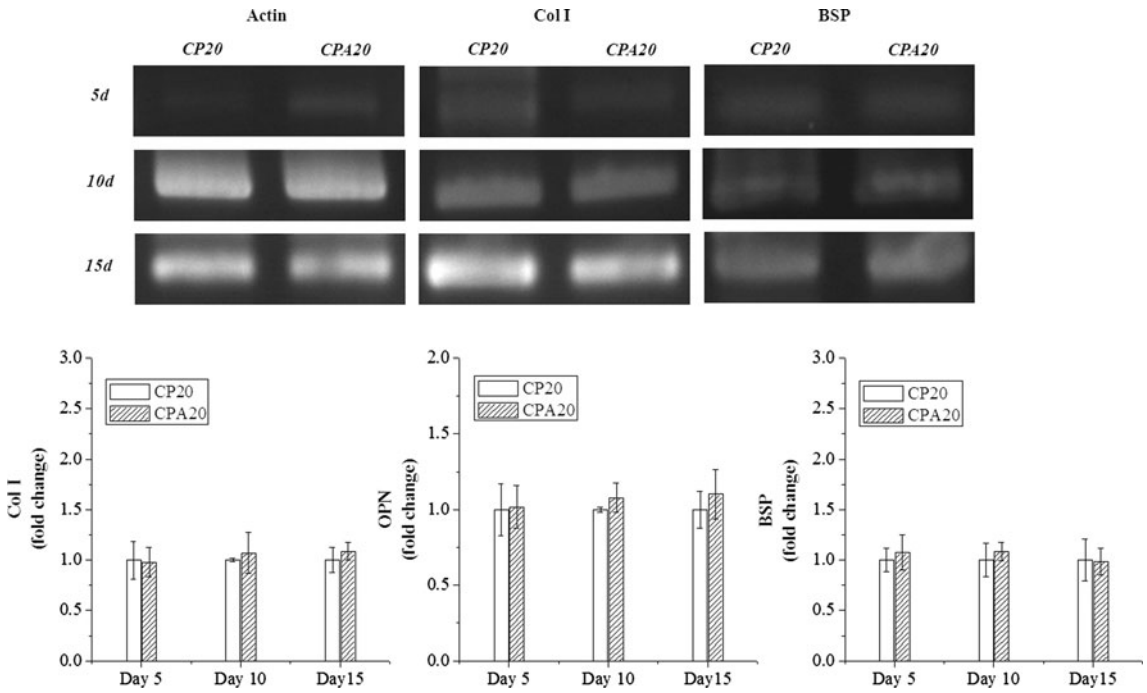

Fig. 9 **a** RT–PCR gene expressions of cells cultured on the CPC (CP20) and CPC-alginate (CPA20) for 5, 10 and 15 days, and **b** real time PCR quantification of the gene expressions. Bone associated genes, including collagen type I (Col I), osteopontin (OPN) and bone sialoprotein (BSP), were expressed at similar levels in both cement groups

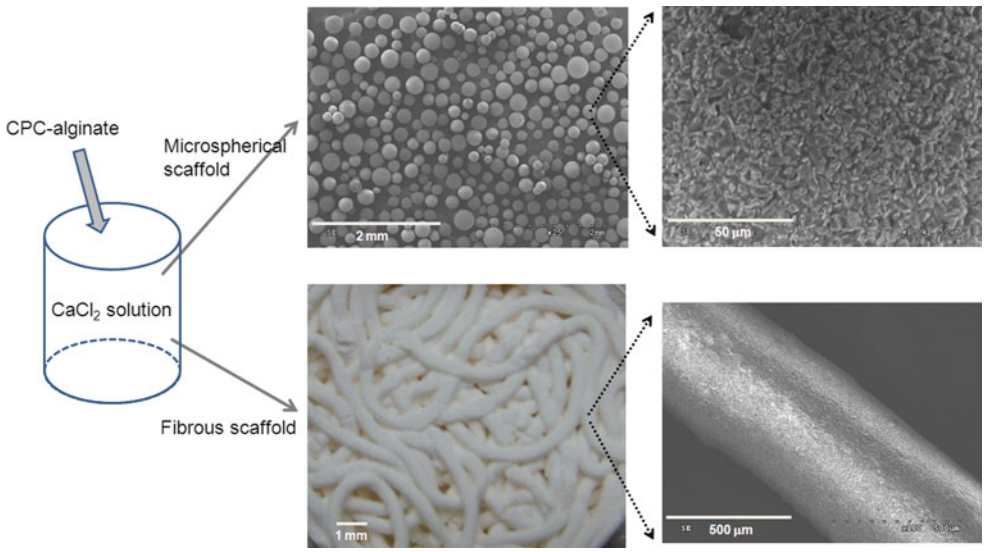

Fig. 11 Schematic illustration, showing the application of the alginate-containing CPCs as bone regenerative scaffolds when formulated into specific shaped 3D scaffolds, e.g. spherical and fibrous shape, which is associated with the alginate hardening within the Ca-containing medium

the fabrication of 3D scaffolds such as microspheres and fibers. Because the microspheres and fibrous scaffolds have been shown to provide sufficient 3D matrix conditions for tissue cells to adhere to, spread and multiply, the produced CPC-based scaffolds are considered to be potentially useful as tissue engineering matrices targeting hard tissues. We are currently investigating the in vitro cell culture of such alginate-CPC 3D scaffolds for bone tissue engineering.

5 Conclusions

Calcium phosphate cements (CPCs) based on α-tricalcium phosphate (α-TCP) were incorporated with alginate. The alginate helped the setting reaction of the cement and led to the production of a much denser microstructure when compared to alginate-free CPCs. After the immersion in a water-based medium, apatite nanocrystallites were formed easily, exhibiting characteristics typical of α-TCP based cements. The addition of alginate improved the compressive and tensile mechanical strengths of the CPCs, particularly when immersed in the medium. The in vitro cell responses to the alginate-CPCs, as observed using rat bone marrow stromal cells (rBMSCs), showed significantly increased cell growth but did not demonstrate confirmative simulation of osteoblastic differentiation based on the ALP activity and PCR gene expressions (Col I, OPN and BSP). One merit of the addition of alginate to the CPCs is that it facilitates the formation of tissue engineering 3D matrices, including microspheres and fibrous scaffold.

Acknowledgments This work was supported by Priority Research Centers Program (No. 2009-0093829) and WCU Program (No. R31-10069) through National Research Foundation (NRF) funded by the Ministry of Education, Science and Technology (MEST).

References

1. Schmitz JP, Hollinger JO, Milam SB. Reconstruction of bone using calcium phosphate bone cements: a critical review. Oral Maxil Surg. 1999;57:1112–26.
2. Ambard AJ, Mueninghoff LE. Calcium phosphate cement: review of mechanical and biological properties. Prosthodontics. 2006;15:321–8.
3. Chow LC. Next generation calcium phosphate-based biomaterials. Dent Mater. 2009;28:1–10.
4. Jones MA, Messersmith PH. In situ forming biomaterials. Oral Maxillofac Sur Clin North Am. 2002;14:29–38.
5. Shie MI, Chen DA, Wang CH, Chiang TI, Ding SH. Immersion behavior of gelatin-containing calcium phosphate cement. Acta Biomater. 2008;4:646–55.
6. Bigi AD, Bracci BA, Panzavolta SI. Effect of added gelatin on the properties of calcium phosphate cement. Biomaterials. 2004; 25:2893–9.
7. Carey LI, Xu HO, Simon CA, Takagi SH, Chow LA. Premixed rapid-setting calcium phosphate composites for bone repair. Biomaterials. 2005;26:5002–14.
8. Liu HU, Li HO, Cheng WE, Yang YU, Zhu MI, Zhou CH. Novel injectable calcium phosphate/chitosan composites for bone substitute materials. Acta Biomater. 2006;2:557–65.
9. Martino AL, Sittinger MI, Risbud MA. Chitosan: a versatile biopolymer for orthopaedic tissue-engineering. Biomaterials. 2005;26:5983–90.
10. Barralet JE, Wang L, Lawson M, Triffitt JT, Cooper PR, Shelton RM. Comparison of bone marrow cell growth on 2D and 3D alginate hydrogels. J Mater Sci Mater Med. 2005;16:515–9.
11. Tonnesen HH, Karlsen J. Alginate in drug delivery systems. Drug Dev Ind Pharm. 2002;28:621–30.
12. Alves HU, Santos LU, Bergmann CA. Injectability evaluation of tricalcium phosphate bone cement. J Mater Sci Mater Med. 2008; 19:2241–6.
13. Oh SA, Lee GS, Park JH, Kim HW. Osteoclastic cell behaviors affected by the α-tricalcium phosphate based bone cements. J Mater Sci Mater Med. 2010;21:3019–27.
14. ASTM C266–89. Standard test method for time of setting of hydraulic cement paste by the Gillmore needles. In: Annual Book of ASTM Standards, Vol. 04.01: Cement, Lime, Gypsum. Philadelphia: ASTM; 1993 p. 189–191.

15. Hong SJ, Jeong IS, Noh KT, Yu HS, Lee GS, Kim HW. Robotic dispensing of composite scaffolds and in vitro responses of bone marrow stromal cells. J Mater Sci: Mater Med. 2009;20:1955–62.
16. Oh SA, Kim SH, Won JE, Kim JJ, Shin US, Kim HW. Effects on growth and osteogenic differentiation of mesenchymal stem cells by the zinc-added sol-gel bioactive glass granules. J Tiss Eng. 2011;2010:475260-1-10.
17. Yu HS, Hong SJ, Park JH, Jeong IS, Kim HW. Bioactive and degradable composite microparticulates for the tissue cell population and osteogenic development. Adv Eng Mater. 2009;11:10.
18. Yu HS, Hong SJ, Kim HW. Surface-mineralized polymeric nanofiber for the population and osteogenic stimulation of rat bone-marrow stromal cells. Mater Chem Phy. 2009;113:873–7.
19. TenHuisen KE, Brown PA. Phase evolution during the formation of a-tricalcium phosphate. J Am Ceram Soc. 1999;82:2813–8.
20. Leamy PA, Brown PA, TenHuisen KE, Randall CL. Fluoride uptake by hydroxyapatite formed by the hydrolysis of a-tricalcium phosphate. J Biomed Mater Res. 1998;42:458–64.
21. Tamai MA, Isshiki TO, Nishio KO, Nakamura MI, Nakahira AT, Endoh HI. Transmission electron microscopic studies on an initial stage in the conversion process from a-tricalcium phosphate to hydroxyapatite. J Mater Res. 2003;18:11.
22. Durucan CA, Brown PA. α-tricalcium phosphate hydrolysis to hydroxyapatite at and near physiological temperature. J Mater Sci: Mater Med. 2000;11:365–71.
23. Hao HI, Amizuka NO, Oda KI, Fujii NO, Ohnishi HI, Okada AT, Nomura SH, Maeda TA. A histological evaluation on self-setting a-tricalcium phosphate applied in the rat bone cavity. Biomaterials. 2004;25:431–42.
24. Fini MI, Giavaresi GI, Aldini NI, Torricelli PA, Botter RO, Beruto DA, Giardino RO. A bone substitute composed of polymethylmethacrylate and α-tricalcium phosphate: results in terms of osteoblast function and bone tissue formation. Biomaterial. 2002;23:4523–31.
25. Santos LA, Carrodéguas RG, Rogero SO, Higa OZ, Boschi AO, Arruda AC. α-Tricalcium phosphate cement: ''in vitro'' cytotoxicity. Biomaterials. 2002;23:2035–42.
26. Ginebra M, Espanol MT, Montufar E, Perez R, Mestres G. New processing approaches in calcium phosphate cements and their applications in regenerative medicine. Acta Biomater. 2010;6:2863–73.