



ORIGINAL ARTICLE

Fabrication and characterization of polycaprolactone and tricalcium phosphate composites for tissue engineering applications



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KEYWORDS

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Abstract *Background/purpose:* β-Tricalcium phosphate (β-TCP) is an osteoconductive material which has been used for clinical purposes for several years, as is polycaprolactone (PCL), which has already been approved for a number of medical and drug delivery devices. In this study we have incorporated various concentrations of β-TCP into PCL with the aim of developing an injectable, mechanically strong, and biodegradable material which can be used for medical purposes without organic solvents.

Materials and methods: This study assesses the physical and chemical properties of this material, evaluates the *in vitro* bioactivity of the PCL/β-TCP composites, and analyzes cell proliferation and osteogenic differentiation when using human bone marrow mesenchymal stem cells (hBMSCs).

Results: The results show that weight losses of approximately 5.3%, 12.1%, 18.6%, and 25.2%, were observed for the TCP0, TCP10, TCP30, and TCP50 composites after immersion in simulated body fluid for 12 weeks, respectively, indicating significant differences ($P < 0.05$). In addition, PCL/β-TCP composites tend to have lower contact angles ($47 \pm 1.5^\circ$ and $58 \pm 1.7^\circ$ for TCP50 and TCP30, respectively) than pure PCL ($85 \pm 1.3^\circ$), which are generally more hydrophilic. After 7 days, a significant (22% and 34%, respectively) increase ($P < 0.05$) in alkaline phosphatase level was measured for TCP30 and TCP50 in comparison with the pure PCL.

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Conclusion: PCL/TCP is biocompatible with hBMSCs. It not only promotes proliferation of hBMSCs but also helps to differentiate reparative hard tissue. We suggest 50% (weight) PCL-containing β -TCP biocomposites as the best choice for hard tissue repair applications.

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Introduction

Effectively and uneventfully regenerating defective hard tissue in deeper and complex bone tissue has long been a goal for clinicians, but it is a complicated issue, and progress has been slow. Materials with varying degrees of bioactivity and degradation are required so as to conform to differing clinical requirements for bone tissue engineering.¹ In previous clinical work, the most successful treatment has been the use of autologous bone grafts for bone defects. Autografting meets the requirements which are indispensable for new bone formation, namely, osteogenesis, osteoconductivity, and osteoinductivity, and it is currently considered the gold standard in this field.

Natural bone is a composite material, consisting of organic matrixes such as collagen, and extracellular matrix (ECM) linked with glycoprotein and calcium phosphate ceramics.² In order to ameliorate its relative disadvantages in regards to material degradation, we experimented with the use of β -tricalcium phosphate (β -TCP) as an additive to see how it would affect the rate of decay of polycaprolactone (PCL). β -TCP is a bioceramic material that is widely used for hard tissue repair; it has a chemical composition similar to the apatite naturally present in bone tissue, and has been applied extensively as a bone grafting material.^{3–5} Although β -TCP has various favorable characteristics that recommend its clinical use, it also has drawbacks which have proven problematic. For example, it is difficult to deliver to the required site and hard to compact adequately due to it being relatively brittle. Efforts have been made in recent years to overcome these disadvantages, leading to the use of several polymers, such as poly(L-lactic acid),⁶ PCL,⁷ poly(lactide-co-glycolide),⁸ and poly(3-hydroxybutyrate),⁹ which have the potential to improve the handling properties of β -TCP.

PCL was chosen for this study. It is a semicrystalline linear aliphatic polyester with a high degree of crystallinity and hydrophobicity. PCL has already been approved for a number of medical and drug delivery devices and is now extensively used for tissue regeneration owing to its cost-effectiveness, durability, excellent biocompatibility, and biodegradability.¹⁰ In the human body, PCL breaks down over a period of time with no side-effects; it generally takes from 6 months to 2 years to degrade *in vivo*, depending on its molecular weight.¹¹ The high mechanical strength and low degradation rate of PCL give PCL-based biomaterials several advantages for application in long-term hard tissue engineering.¹² However, PCL-based biomaterials and composites also have some shortcomings due to their slow degradation rate and lack of bioactivity, restricting their application in hard tissue engineering.¹³ To overcome these limitations,

certain modifications will need to be discovered. The most promising approach presently being explored is incorporating different polymers into other inorganic materials. The work so far in this direction has been exciting, and is considered a promising strategy for designing useful scaffolds for bone tissue engineering.^{14,15}

In the present study we have incorporated various concentrations of β -TCP into PCL, with the aim of developing an injectable, mechanically strong, and biodegradable material while avoiding the use of organic solvents. This study assesses the physical and chemical properties of this material, and evaluates the *in vitro* bioactivity of the PCL/ β -TCP composites. Cell proliferation and osteogenic differentiation are considered using human bone marrow mesenchymal stem cells (hBMSCs).

Materials and methods

Preparation of PCL/ β -TCP composites

The PCL/ β -TCP composite material used in this study was obtained by mixing reagent grade PCL (molecular weight = 43,000–50,000; Polysciences, Warrington, PA, USA) and β -TCP (Sigma-Aldrich, St. Louis, MO, USA) powder with composite weight ratios of 100:0 (TCP0), 90:10 (TCP10), 70:30 (TCP30), and 50:50 (TCP50) weight-% at 1300 g for 15 minutes using a hybrid-defoaming mixer, after which the mixture was ball-milled in ethyl alcohol using a centrifugal ball mill (S 100; Retsch, Hann, Germany) for 6 hours. The PCL/ β -TCP powder was then molded in a Teflod mold (diameter, 12 mm; height, 3 mm) and placed in an oven at 90°C for 30 minutes. The composite quantities were sufficient to fully cover each well of the 24-well plate (GeneDireX, Las Vegas, NV, USA) to a thickness of 2 mm for cell experiments.

Setting time and injectability

The setting time of the composites was tested according to standards set by the International Standards Organization 9917-1. For evaluation of the setting time, each material was analyzed using Gilmore needles (456.5 g). Records were made when the needle failed to create a 1-mm deep indentation in three separate areas.

The injectability of PCL/ β -TCP composite paste was determined by pressing 2.5 g of as-prepared paste by hand through a 5-mL syringe with an opening diameter of 2.0 mm. This suggests that injection by hand has a slightly lower standard deviation than injection by machine with a preset load. After hydration at 37°C in 100% relative

humidity for different incubation times, the paste was extruded from the syringe until no more could be forced out. The weight of the paste which had passed completely through the syringe was then measured. The injectability was calculated as:

$$I = m_{\text{injected}} / m_{\text{initial}} \times 100\%, \quad (1)$$

where I is the injectability, m_{injected} and m_{initial} are the weight of the paste forced through the syringe and the paste initially contained in the syringe. All values were derived from an average of 10 tests performed for each group.

Characterization

The phase composition of the cements was analyzed using X-ray diffractometry (Bruker D8 SSS, Karlsruhe, Germany), run at 30 kV and 30 mA with a scanning speed of $1^\circ/\text{min}$. Raman measurements were performed using B&W Tek's Raman spectrometer (B&W Tek Inc., Newark, DE, USA). Three-hundred milliwatts of 78-nm radiation from a diode pumped laser was used for excitation. All the spectra reported here were collected under an exposure time set to a single 5-second accumulation in a range from 400 cm^{-1} to 1800 cm^{-1} , for a total of 905 data points. Rayleigh scattering was blocked using a holographic notch filter, and the tilted baselines of some surface-enhanced Raman scattering spectra were flattened using commercial Raman software (B&W Tek Inc.). The β -TCP content of the composite was determined using thermogravimetric analysis (Netzsch STA 449C, Bavaria, Germany). The samples were analyzed in aluminum pans under a nitrogen purge and heated from 30°C to 700°C with a heating rate of $10^\circ\text{C}/\text{min}$.

Weight loss

To evaluate the *in vitro* bioactivity, the PCL/ β -TCP composites were immersed in a 10-mL simulated body fluid (SBF) solution at 37°C . The SBF solution, of which the ionic composition is similar to that of human blood plasma, consisted of 7.9949 g of NaCl, 0.3528 g of NaHCO_3 , 0.2235 g of KCl, 0.147 g of K_2HPO_4 , 0.305 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.2775 g of CaCl_2 , and 0.071 g of Na_2SO_4 in 1000 mL of distilled H_2O and was buffered to a pH of 7.4 with hydrochloric acid (HCl) and tris(hydroxymethyl) aminomethane ($\text{CH}_2\text{OH}_3\text{CNH}_2$).¹⁶ All chemicals used were of reagent grade. The solution in the shaker water bath exhibited no change under static conditions. After soaking for different time durations (from 3 days to 3 months), specimens were removed from the tube and evaluated for various physicochemical properties. The degree of degradation was determined by monitoring the weight change of the specimens. After drying at 45°C , the composites were weighed using a balance both before and after immersion. The 10 specimens were examined at each time point for each of the materials being investigated.

Mechanical properties

After being taken out of the mold, the specimens were incubated at 37°C in 100% humidity for 1 day. Diametral tensile strength (DTS) testing was conducted on an EZ-Test

machine (Shimadzu, Kyoto, Japan) at a loading rate of 1 mm/min. The maximum compression load at failure was obtained from the recorded load-deflection curves.

Cell adhesion and proliferation

Before performing the cell experiments, all samples were sterilized by being soaked in 75% ethanol and then exposed to UV light for 30 minutes. The hBMSCs were obtained from Sciencell Research Laboratories (Sciencell, Carlsbad, CA, USA) and grown in mesenchymal stem cell medium (Sciencell) at passage 3–6. The hBMSCs were directly cultured on the sterilized specimens at a density of 10^4 cells per well in a 24-well plate and incubated at 37°C in a 5% CO_2 atmosphere for various numbers of days. After different culturing times, cell viability was evaluated using the PrestoBlue (Invitrogen, Grand Island, NY, USA) assay. At the end of the culture period, the medium was discarded and the wells were washed twice with cold phosphate-buffered saline (PBS). Each well was then filled with a 1:9 ratio of PrestoBlue in fresh Dulbecco's Modified Eagle Medium and incubated at 37°C for 60 minutes. The solution in each well was then transferred to a new 96-well plate and read using Tecan Infinite 200 PRO microplate reader (Tecan, Männedorf, Switzerland) at 570 nm with a reference wavelength of 600 nm. hBMSCs cultured on tissue culture plates without materials were used as a control (Ctl). The results were obtained in triplicate from three separate experiments in terms of optical density.

Collagen and fibronectin adsorption on substrates

After being cultured for different periods of time, the amounts of collagen (COL) and fibronectin (FN) secreted from the cells onto the composite's surface were analyzed and measured using enzyme-linked immunosorbent assay. The cells were detached using a trypsin-EDTA solution (Caisson Laboratories, North Logan, UT, USA) after being washed three times with cold PBS. Specimens were then washed three times with PBS containing 0.1% TWEEN-20 (PBS-T), and were then blocked with 5% bovine serum albumin (Gibco) in PBS-T for 1 hour. Dilutions of primary antibodies were set at 1:500. Following this procedure, samples were incubated with antihuman β -actin, antihuman COL I antibody or antihuman FN antibody (Gene-Tex, San Antonio, TX, USA) for 3 hours at room temperature. Afterwards, samples were washed three times with PBS-T for 5 minutes and incubated, while being shaken with horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature. The samples were then washed three times with PBS-T for 10 minutes each and then One-Step Ultra 3,3',5,5'-Tetramethylbenzidine substrate (Invitrogen) was added to the wells and developed for 30 minutes at room temperature in the dark, after which an equal volume of 2M H_2SO_4 was added to stop and stabilize the oxidation reaction. The colored products were then transferred to new 96-well plates and read using a multiwell spectrophotometer at 495 nm with reference at 620 nm, according to the manufacturer's recommendations. All experiments were carried out in triplicate. Additionally, β -actin antibodies were used as a control.

Cell morphology

After the cells had been seeded for 1 day and 3 days, the PCL/ β -TCP composites were washed three times with cold PBS and fixed in 1.5% glutaraldehyde (Sigma-Aldrich) for 2 hours, after which they were dehydrated using a graded ethanol series for 20 minutes at each concentration and dried with liquid CO₂ using a critical point dryer device (LADD 28000; LADD, Williston, VT, USA). The dried specimens were then mounted on stubs, coated with gold, and viewed using scanning electron microscopy (JEOL JSM-7401F, Tokyo, Japan).

Calcium concentration

After being cultured for different time periods, the Ca ion concentrations released from the PCL/ β -TCP composites in the medium were analyzed using an inductively coupled plasma-atomic emission spectrometer (Perkin-Elmer OPT 1MA 3000DV, Shelton, CT, USA). Three samples were measured for each data point. The results were obtained in triplicate from three separate samples for each test.

Osteogenesis assay

The level of alkaline phosphatase (ALP) activity was determined after the cells had been seeded for 3 days and 7 days. The process was as follows: the cells were lysed from discs using 0.2% NP-40 and centrifuged for 10 minutes at 2000 rpm after washing with PBS. ALP activity was determined using *p*-nitrophenyl phosphate (Sigma-Aldrich) as the substrate. Each sample was mixed with *p*-nitrophenyl phosphate in 1M diethanolamine buffer for 15 minutes, after which the reaction was stopped by the addition of 5N NaOH and quantified by absorbance at 405 nm. All experiments were done in triplicate. The osteogenic differentiation medium was Advance Minimum Essential medium (Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10⁻⁸M dexamethasone (Sigma-Aldrich), 0.05 g/L L-Ascorbic acid (Sigma-Aldrich), and 2.16 g/L glycerol 2-phosphate disodium salt hydrate (Sigma-Aldrich).

The osteocalcin (OC) protein released from the pulp cells was cultured on different substrates for 7 days and 14 days after cell seeding. Following the manufacturer's instructions, an OC enzyme-linked immunosorbent assay kit (Invitrogen) was used to determine the OC protein content. The OC protein concentration was measured by correlation with a standard curve. The analyzed blank disks were treated as controls. All experiments were done in triplicate.

Alizarin Red S stain

Accumulated calcium deposition was observed after 7 days and 14 days using Alizarin Red S staining, as described in a previous study.¹⁷ To summarize briefly, the cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) for 15 minutes and then incubated in 0.5% Alizarin Red S (Sigma-Aldrich) at pH 4.0 for 15 minutes at room temperature in an orbital shaker (25 rpm).

Statistical analysis

A one-way analysis of the variance statistical data was used to evaluate the significance of the differences between the means in the measured data. Scheffe's multiple comparison test was used to determine the significance of the deviations in the data for each specimen. In all cases, the results were considered statistically significant with a *P* value < 0.05.

Results

Characterization of PCL/ β -TCP biocomposites

The setting time is one of most important factors in the clinical use of this compound.¹⁸ Figure 1A shows that as the β -TCP content is increased, so does the working and setting times of the composites, going from 3.4 minutes and 9.5 minutes (TCP0 group) all the way up to 6.2 minutes and 18.2 minutes (TCP30 group), a significant difference (*P* < 0.05). We were able to inject Considerably more PCL/ β -TCP paste than PCL paste (Figure 1B). Furthermore, the injectability of the PCL/ β -TCP paste rose in proportion with the increase in β -TCP content. The addition of β -TCP both decreases the setting time and impairs injectability in comparison to PCL paste. The TCP30 paste's setting time (19 minutes) gives an advantage to surgeons by allowing more time for the operation before the paste sets.

The X-ray diffractometry analysis shows that the diffraction peaks at 21.44°, 22.06°, 23.76°, and 36.32° are typical for PCL (Figure 2). The presence of these narrow peaks makes sense when we consider the semicrystalline nature of this polymer.¹⁹ The diffraction peaks at 25.7°, 27.7°, 31°, and 34.3° are present in the composite, which correspond to the characteristic peaks of β -TCP.²⁰ This confirms that β -TCP has been incorporated into the PCL. The reduction in the intensity of the peaks is due to microstructural changes in PCL and changes in its crystallinity.

A Raman spectra of the PCL/ β -TCP composite reveals significant differences in their molecular structure compared with pure PCL, particularly as 1725 cm⁻¹ is assigned for the C=O stretching vibration (Figure 3). The peaks at 1730 cm⁻¹ and 1736 cm⁻¹ correspond to the amorphous phase, while 1720 cm⁻¹ is assigned to the crystalline phase, suggesting the crystallinity of the composites becomes reduced in the presence of nanoparticles, as confirmed by the respective peak intensities.²¹ In addition, the other peaks at 1470–1415 cm⁻¹ (δ CH₂), 1303–1281 cm⁻¹ (ω CH₂), 1107–1033 cm⁻¹ (skeletal stretching), and 912 cm⁻¹ (ν C–COO) occur during the crystalline phases. There is a Raman spectrum of β -TCP over the entire optical frequency range. The peaks at 949 cm⁻¹ and 970 cm⁻¹ have been associated with internal vibrations of the PO₄³⁻ ions.²²

Figure 4 shows the thermogravimetric analysis profiles used to examine the contents of β -TCP within the prepared materials. As indicated in the thermograms, the maximum rate of weight loss occurred when the temperature was approximately 407 ± 2 °C for all the samples, which actually corresponds to the degradation of PCL phase. The

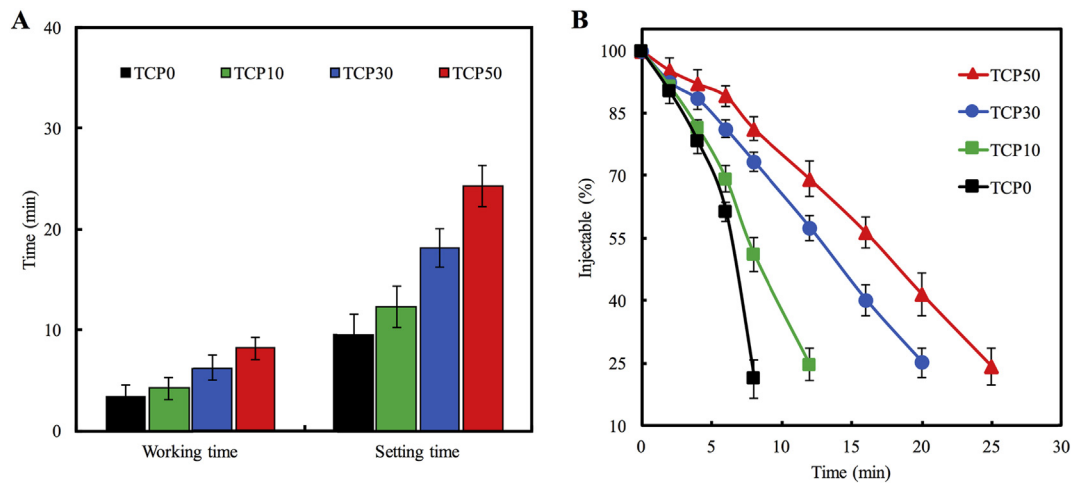


Figure 1 The (A) setting time and (B) injectability of various polycaprolactone/ β -tricalcium phosphate (β -TCP) composites.

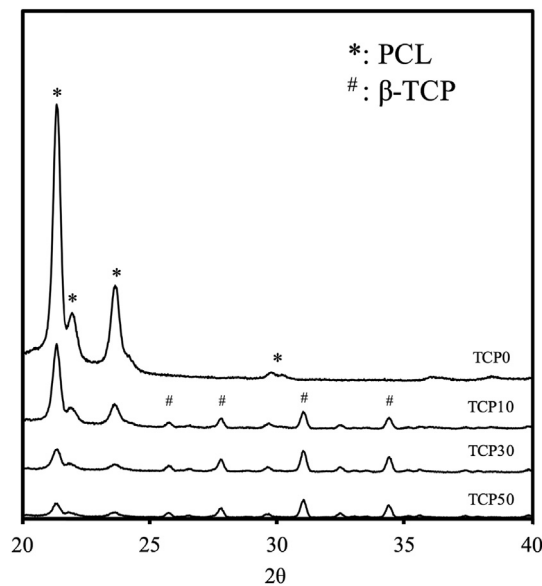


Figure 2 X-ray diffractometry patterns of four specimens with different ratios of polycaprolactone/ β -tricalcium phosphate (PCL/ β -TCP).

remaining weights of TCP0, TCP10, TCP30, and TCP50 were 0.59%, 9.95%, 29.97%, and 45.30%, respectively.

Immersion studies of PCL/ β -TCP biocomposites

The degradation rates of the PCL/ β -TCP in SBF solution have been recorded for various periods of time ranging from 3 days to 84 days. As shown in Figure 5, the pure PCL material (TCP0) shows much less degradation than the composites, exhibiting a weight loss of 1.9% after soaking in the SBF for 4 weeks. At the end of the immersion period, weight losses of approximately 5.3%, 12.1%, 18.6%, and 25.2% were observed for the TCP0, TCP10, TCP30, and TCP50 composites, respectively, indicating significant differences ($P < 0.05$).

The DTS values of the biocomposites were 8.0 MPa, 7.4 MPa, 6.7 MPa, and 6.1 MPa for TCP0, TCP10, TCP30, and

TCP50, respectively (Figure 6). Otherwise, TCP50 has a lower DTS value. This may be due to the large surface area and pore volume of TCP50. There was little difference in the values of TCP0 after 4 weeks, 8 weeks, and 12 weeks of immersion (the values were 7.8 MPa, 7.6 MPa, and 7.4 MPa, respectively). It should be noted that the strength of TCP50 declines significantly from the prepared strength of 6.1 MPa to 4.1 MPa after soaking for 12 weeks ($P < 0.05$), a reduction of approximately 31.4%. By contrast, MgO decreased by about 7.1%, which is not significantly different ($P > 0.05$) from its original strength. We suggest that the reduction of the DTS values with increasing time is caused by the degradation phenomena. Moreover, PCL which contains a β -TCP composite has shown great potential for hard tissue repair applications.

Cell adhesion and proliferation

Initial cell attachment is usually responsible for cellular behavior and eventual tissue integration, while cell proliferation and differentiation are closely correlated with the amount of new bone generation. The facilitation of cell adhesion and proliferation on the PCL/ β -TCP composite was confirmed and observed using PrestoBlue assay (Figure 7). The results show that more cells adhere to TCP50 than to TCP0 and Ctl at all culture time-points (Figure 7A). There may be several explanations for the relative abundance of cell adhesion observed. First of all, PCL/ β -TCP composites tend to have lower contact angles ($47^\circ \pm 1.5^\circ$ and $58^\circ \pm 1.7^\circ$ for TCP50 and TCP30, respectively) than pure PCL ($85^\circ \pm 1.3^\circ$), which are generally more hydrophilic. Cell proliferation gradually increases along with the amount of β -TCP in PCL, which indicates a significant difference ($P < 0.05$) compared with the PCL (TCP0). For example, TCP30 and TCP50 saw increases of approximately 19.5% and 23.8%, respectively, in the optical density value compared with TCP0 on Day 7 (Figure 7B).

Figure 8A shows the amounts of COL protein adsorbed on composites from cells cultured with different groups. At 3 hours, the FN adsorption on TCP50 is 1.94 and 1.71 times higher ($P < 0.05$) than on TCP0 and TCP10, respectively.

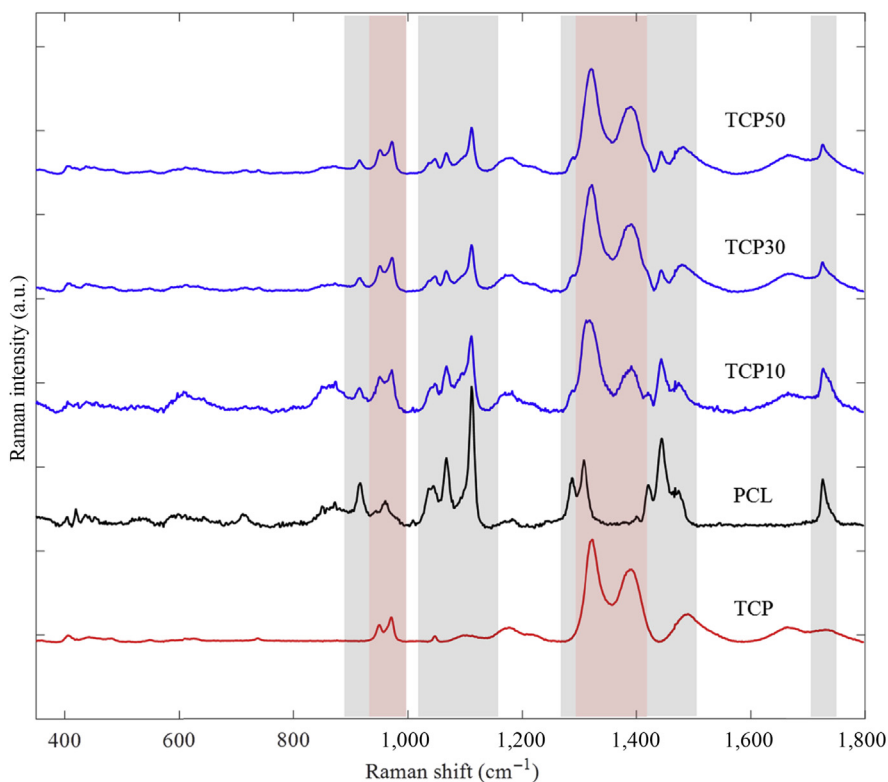


Figure 3 Raman patterns of four specimens with different ratios of polycaprolactone/ β -tricalcium phosphate (PCL/ β -TCP).

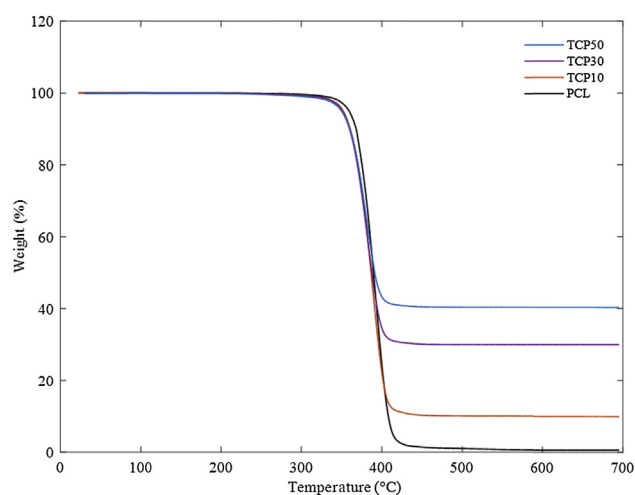


Figure 4 Thermogravimetric analysis of four specimens with different ratios of polycaprolactone/ β -tricalcium phosphate (PCL/ β -TCP).

There are no significant differences ($P > 0.05$) between TCP0 and TCP10. The effect of substrates on the adsorption of FN has also been examined. As shown in Figure 8B, the value for the TCP50 is significantly ($P < 0.05$) higher than Ctl and pure PCL.

Figure 9 shows the hBMSCs cells after Day 1 and Day 3 of being cultured on the PCL/ β -TCP composite obtained using scanning electron microscopy. On Day 1, the hBMSCs are flat with intact, well-defined morphology and extending filopodia, indicating survival of the cells on all composites.

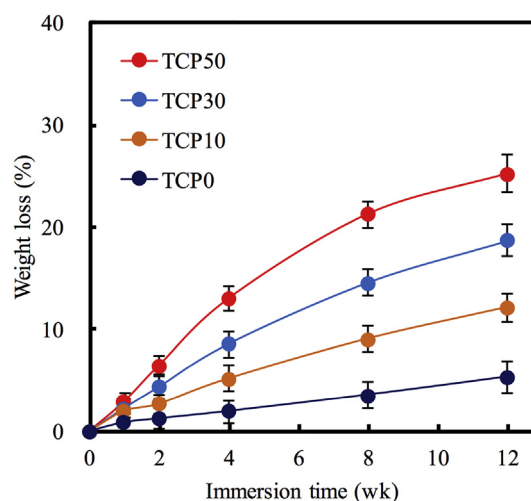


Figure 5 Weight loss of various specimens after immersion in simulated body fluid for predetermined time durations. TCP = tricalcium phosphate.

After being cultured for 3 days, the hBMSCs grow well on four kinds of PCL-based substrates, and there appears to be more cells than on Day 1. Furthermore, all of the composite materials seem to boost cell growth more than pure PCL.

Osteogenesis protein secretion

Osteogenic differentiation of stem cells is one of the key steps in determining whether bone formation is successful,

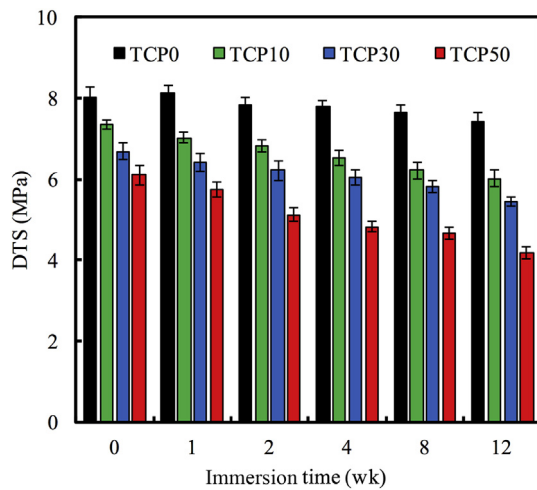


Figure 6 Diametral tensile strength of various cements after immersion in simulated body fluid for predetermined time durations. DTS = diametral tensile strength; TCP = tricalcium phosphate.

so the ALP expression of hBMSCs cultured on different composites has also been considered. Figure 10A shows the analysis of quantitative examination data and the ALP activity of cells cultured on different composites after 3 days and 7 days. Increasing the β -TCP content leads to increased ALP levels in the composites at all incubation times. A significant (22% and 34% increase ($P < 0.05$) in the ALP level was measured for TCP30 and TCP50 in comparison with the pure PCL after 7 days. ALP enzyme activity is also associated with bone formation, and it occurs in high levels during the bone formation phase.²³ Similarly, the OC secretion by cells cultured on TCP50 is significantly higher ($P < 0.05$) than the levels secreted by cells on other substances (Figure 10B). After 14 days, the OC secretion was found to have increased by 25% and 43% in the cells cultured on TCP30 and TCP50, respectively, in comparison with the PCL substrate.

Mineralization

The aim of this assay is to determine and show the effects of various ratios of PCL/ β -TCP composites on bone matrix

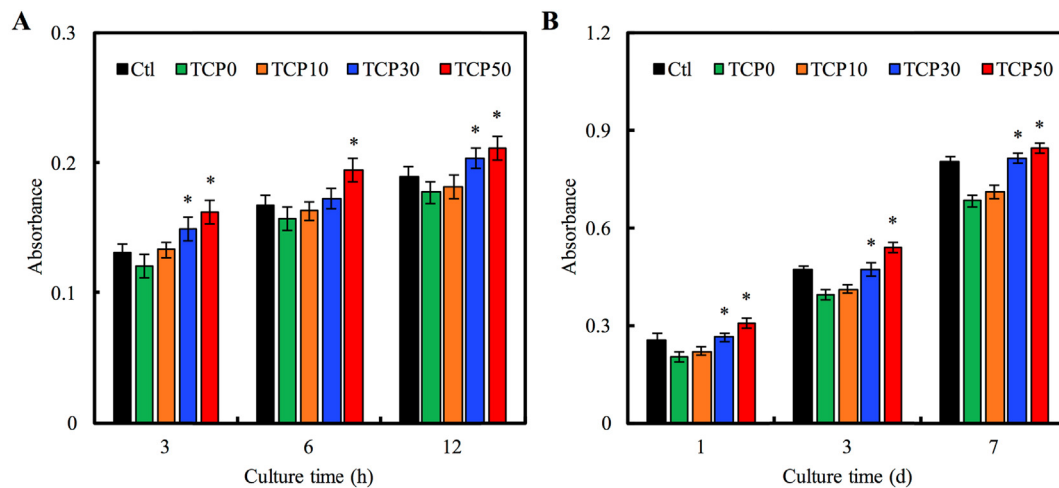


Figure 7 (A) Adhesion; and (B) proliferation of human bone marrow mesenchymal stem cells cultured on various specimens for different time-points. * A significant difference of $P < 0.05$ compared with TCP0. Ctl = control; TCP = tricalcium phosphate.

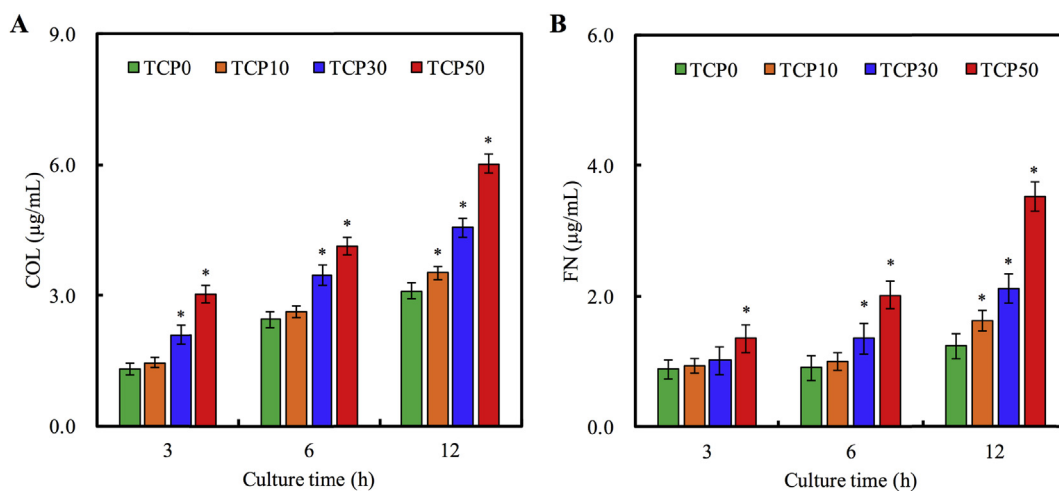


Figure 8 (A) Collagen (COL); and (B) fibronectin (FN) adsorbed on polycaprolactone/ β -tricalcium phosphate (TCP) composites for different time-points. * A significant difference of $P < 0.05$ compared with TCP0.

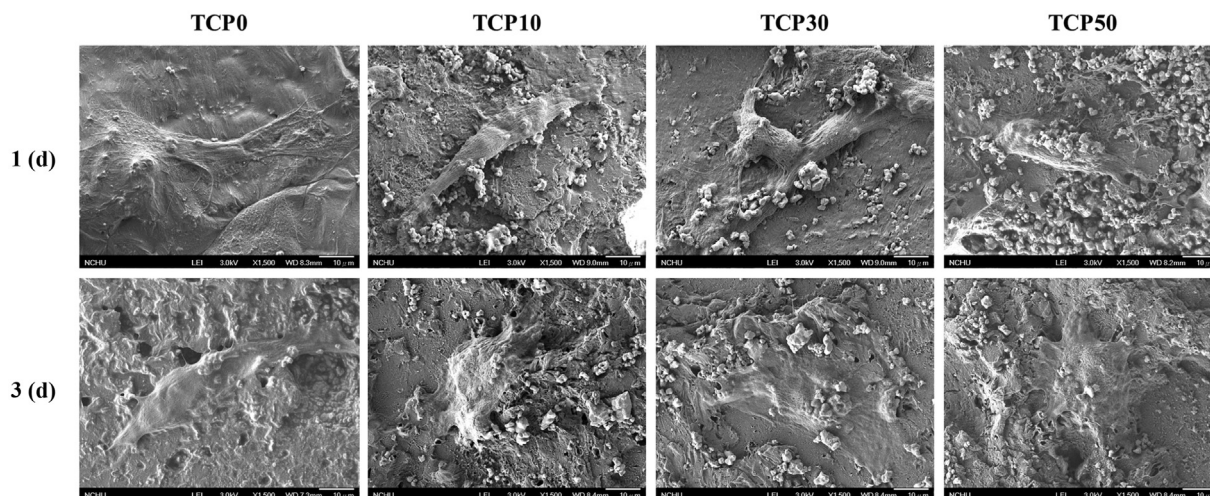


Figure 9 The morphology of human bone marrow mesenchymal stem cells cultured on polycaprolactone/ β -tricalcium phosphate (TCP) specimens after different time-points.

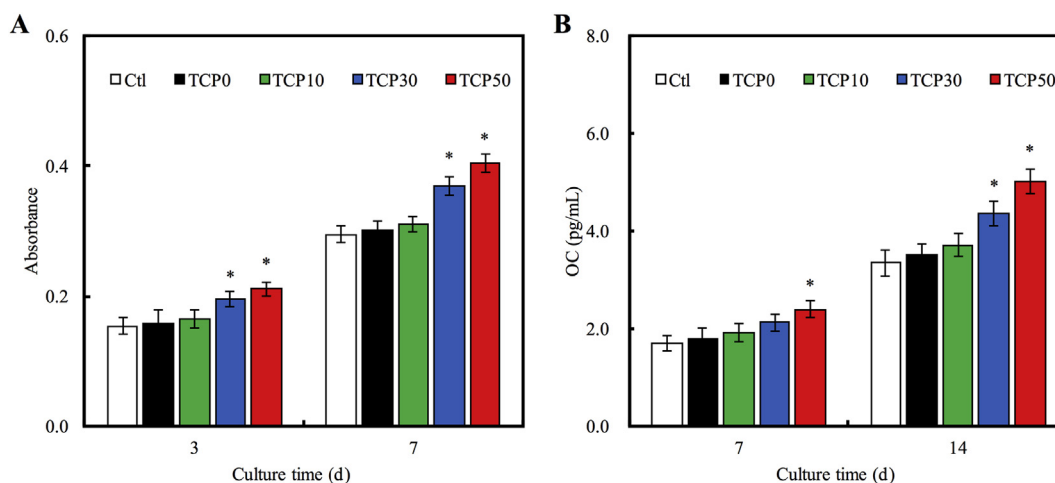


Figure 10 (A) Alkaline phosphatase activity; and (B) osteocalcin (OC) amount of human bone marrow mesenchymal stem cells cultured on various specimens for different time-points. * Indicates a significant difference of $P < 0.05$ compared with TCP0. Ctl = control; TCP = tricalcium phosphate.

formation following analysis using Alizarin Red S staining to identify calcium deposition, as seen in Figure 11. The results show that after 7 days TCP30 and TCP50 clearly increase the area of calcified nodules compared with TCP0. These observations correlate very well with the higher values of the osteogenesis markers for bone matrix maturation detected in the TCP30 and TCP50. In fact, the observed Ca concentration was close to 1.84mM for TCP30 and close to 2.35mM for TCP50, compared with 1.42mM of the standard complete culture medium. There was no difference in Ca concentration between the Ctl, TCP0, and TCP10 groups.

Discussion

In the present study we fabricated organic polymer/inorganic particle mixtures into composites using various techniques and then incorporated various concentrations of

β -TCP powders into PCL and investigated its physical and biological properties, with the aim of producing a bioactive, highly flexural strength bone substitutes for hard tissue regeneration. Calcium phosphate-based cement consists of a powder containing one or several solid compounds of calcium phosphate salts and a liquid, which can be either water or a solution.²⁴ Calcium phosphate cement, especially β -TCP, has wide applications in reconstructive bone surgery. Physiologically, β -TCP quickly degrades through both chemical dissolution and osteoclastic resorption, affecting the bone-remodeling process.²⁵ Setting time is a key factor, and a long setting time will lead to clinical problems under clinical conditions. Fernandez et al.²⁶ proposed that 10–15 minutes is a suitable setting time interval in the clinic. When composites are used for bone repair, the mechanical properties of the hardened materials are another important index. Our results show that a setting time of approximately 20 minutes is possible for injectable bone substitutes for use in vertebroplasty and

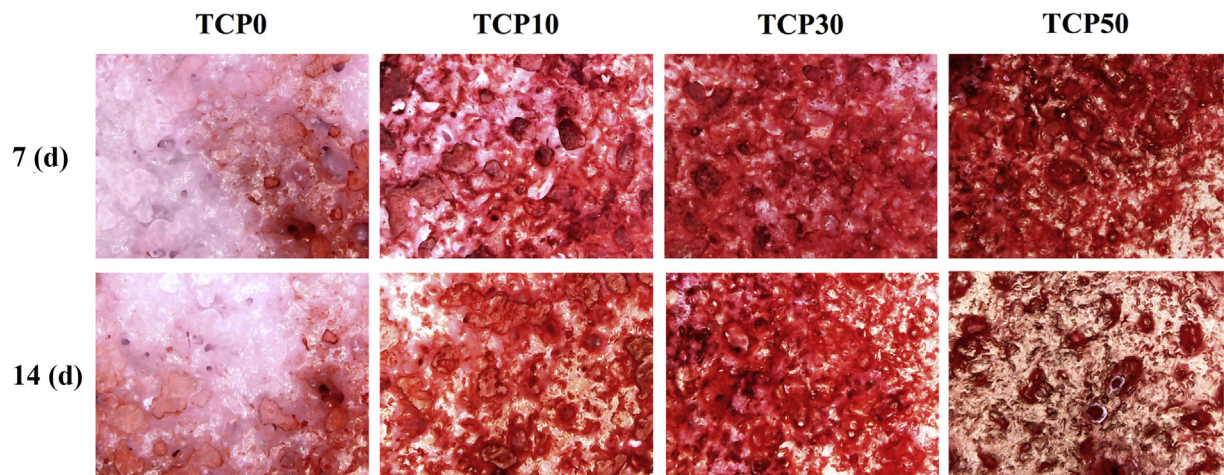


Figure 11 The photograph of calcium mineral deposits by Alizarin Red S assay of human bone marrow mesenchymal stem cells cultured on various specimens for 7 days and 14 days. TCP = tricalcium phosphate.

kyphoplasty.²⁷ The injectable cement can mold to the shape of a bone and hardens when injected *in situ*, thus shortening the surgical operation time and reducing post-operative pain.²⁸

As the β -TCP content is increased, the dissolution rate also increases steadily. Previous studies have shown that β -TCP bioceramics degrade relatively quickly and promote new bone formation *in vivo*.²⁹ The weight loss of the composites may mainly be due to the degradation of the β -TCP. Biodegradation is critical when synthesizing a new material: the degradation rate must help hasten and ease the process of tissue regeneration.³⁰ In addition, the hydrophilic properties of biomaterials for bone substitute are important for induction of early cell attachment and growth because osteoblasts favor hydrophilic surfaces. This has also been observed in biocomposites where a reduction in contact angle results in composites with heightened cell adhesion.³¹ In view of the significant influence on hBMSCs adhesion, proliferation, and differentiation, surface wettability is a key parameter that should be considered.³²

Cell adhesion requires an appropriate proteinaceous substrate to which cell adhesion receptors, such as integrins, can attach and form cell-anchoring points. The dominant role of protein adsorption on various composites and the regulation of cell adhesion has been identified.³³ Following initial adhesion, cells will secrete ECM into their environment, such as COL, FN, and vitronectin, which subsequently affect the cells' behavior.³⁴ These proteins will adsorb onto the material's surface and supply a provisional matrix for cell adhesion.³⁵ The dominant role of protein adsorption in the regulation of cell behavior has been identified.³⁴ Differential ECM adsorption on the various material surfaces accounts for the observed variability in cell adhesion.²⁷ In addition, ECM molecules express and synthesize COL and FN during the various stages of osteogenesis.^{36,37} *In vivo*, the adsorption to the biomaterial surface of bioactive proteins from the serum and bodily fluids at the surgical site is known to influence cellular-material interactions.³⁸

Recent studies also show that β -TCP promotes hBMSC proliferation and differentiation.³⁹ The injectable PCL/ β -

TCP composites tested in this study have been shown to be able to release both calcium ions, perhaps because the surface-mediated Ca ion exchange affects cell behavior. The local changes in extracellular Ca ions may regulate cell activity, and have a significant effect on the proliferation and differentiation of cells.⁴⁰ Extracellular Ca ions have been documented as being a major proponent in transmitting messages which affect cell behavior, thus having a significant effect on the regulation of the proliferation and differentiation of osteoblasts.⁴⁰ Several studies have shown that the microenvironment in close contact with the materials surface, which is dependent upon the evolution of Ca concentration of the culture medium, can affect the expression of the osteogenesis-related proteins of the cells and the formation of calcium nodules.^{41,42} There is genetic control of the cellular response of primary cells to the bone substitutes. Osteoblast differentiation is generally accompanied by ALP and OC secretion.

In this study, degradable PCL-based composite cement containing PCL and β -TCP in different ratios was prepared and analyzed. Our research reveals that the mechanical properties of biocomposites increase steadily with a decrease in the β -TCP portion in the ratio of various biocomposites. The dissolution rate of PCL/ β -TCP is strongly dependent on the β -TCP content, and PCL/ β -TCP composites with a lower PCL content show higher dissolution rates. The results obtained in this study may be useful for designing PCL/ β -TCP biocomposites with optimal biological and degradation properties. Our results suggest that the incorporation of β -TCP into PCL is a useful approach for obtaining composites with improved properties in regards to setting time, degradation, and osteogenesis behavior. Thus, we suggest 50% weight PCL-containing β -TCP biocomposites may be the best choice for hard tissue repair applications.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

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References

- Chen YW, Yeh CH, Shie MY. Stimulatory effects of the fast setting and degradable Ca–Si–Mg cement on both cementogenesis and angiogenesis differentiation of human periodontal ligament cells. *J Mater Chem B* 2015;3:7099–108.
- Ji L, Wang W, Jin D, Zhou S, Song X. *In vitro* bioactivity and mechanical properties of bioactive glass nanoparticles/polycaprolactone composites. *Mater Sci Eng C Mater Biol Appl* 2015;46:1–9.
- Gan Y, Dai K, Zhang P, Tang T, Zhu Z, Lu J. The clinical use of enriched bone marrow stem cells combined with porous beta-tricalcium phosphate in posterior spinal fusion. *Biomaterials* 2008;29:3973–82.
- Wang S, Zhang Z, Zhao J, et al. Vertical alveolar ridge augmentation with β -tricalcium phosphate and autologous osteoblasts in canine mandible. *Biomaterials* 2009;30:2489–98.
- Chen WL, Chen CK, Lee JW, Lee YL, Ju CP, Lin JHC. Structure, properties and animal study of a calcium phosphate/calcium sulfate composite cement. *Mater Sci Eng C Mater Biol Appl* 2014;37:60–7.
- Kung FC, Lin CC, Lai WFT. Osteogenesis of human adipose-derived stem cells on hydroxyapatite-mineralized poly(lactic acid) nanofiber sheets. *Mater Sci Eng C Mater Biol Appl* 2014;45:578–88.
- Shim JH, Yoon MC, Jeong CM, et al. Efficacy of rhBMP-2 loaded PCL/PLGA/ β -TCP guided bone regeneration membrane fabricated by 3D printing technology for reconstruction of calvaria defects in rabbit. *Biomed Mater* 2014;9:065006.
- Zhang E, Zhu C, Yang J, et al. Electrospun PDLLA/PLGA composite membranes for potential application in guided tissue regeneration. *Mater Sci Eng C Mater Biol Appl* 2016;58:278–85.
- Hsu SH, Hsieh CT, Sun YM. Synthesis and characterization of waterborne polyurethane containing poly(3-hydroxybutyrate) as new biodegradable elastomers. *J Mater Chem B* 2015;3:9089–97.
- Cooke SL, Whittington AR. Influence of therapeutic radiation on polycaprolactone and polyurethane biomaterials. *Mater Sci Eng C Mater Biol Appl* 2016;60:78–83.
- Sun H, Mei L, Song C, Cui X, Wang P. The in vivo degradation, absorption and excretion of PCL-based implant. *Biomaterials* 2006;27:1735–40.
- Chen CH, Lee MY, Shyu VBH, Chen YC, Chen CT, Chen JP. Surface modification of polycaprolactone scaffolds fabricated via selective laser sintering for cartilage tissue engineering. *Mater Sci Eng C Mater Biol Appl* 2014;40:389–97.
- Gao X, Song J, Ji P, et al. Polydopamine-templated hydroxyapatite reinforced polycaprolactone composite nanofibers with enhanced cytocompatibility and osteogenesis for bone tissue engineering. *ACS Appl Mater Interfaces* 2016;8:3499–515.
- Goonasekera CS, Jack KS, Cooper-White JJ, Grøndahl L. Dispersion of hydroxyapatite nanoparticles in solution and in polycaprolactone composite scaffolds. *J Mater Chem B* 2016;4:409–21.
- Yazdimaghani M, Razavi M, Vashaei D, Tayebi L. Surface modification of biodegradable porous Mg bone scaffold using polycaprolactone/bioactive glass composite. *Mater Sci Eng C Mater Biol Appl* 2015;49:436–44.
- Chang NJ, Chen YW, Shieh DE, Fang HY, Shie MY. The effects of injectable calcium silicate-based composites with the Chinese herb on an osteogenic accelerator *in vitro*. *Biomed Mater* 2015;10:055004.
- Chen YW, Ho CC, Huang TH, Hsu TT, Shie MY. The ionic products from mineral trioxide aggregate induced odontogenic differentiation of dental pulp cells via activation of the Wnt/ β -catenin signaling pathway. *J Endod* 2016;42:1062–9.
- Huang MH, Kao CT, Chen YW, et al. The synergistic effects of Chinese herb and injectable calcium silicate/b-tricalcium phosphate composite on an osteogenic accelerator *in vitro*. *J Mater Sci: Mater Med* 2015;26:161.
- Flores-Cedillo ML, Alvarado-Estrada KN, Pozos-Guillén AJ, et al. Multiwall carbon nanotubes/polycaprolactone scaffolds seeded with human dental pulp stem cells for bone tissue regeneration. *J Mater Sci Mater Med* 2016;27:35.
- Kao CT, Huang TH, Chen YJ, Hung CJ, Lin CC, Shie MY. Using calcium silicate to regulate the physicochemical and biological properties when using β -tricalcium phosphate as bone cement. *Mater Sci Eng C Mater Biol Appl* 2014;43:126–34.
- Gupta KK, Kundan A, Mishra PK, et al. Polycaprolactone composites with TiO₂ for potential nanobiomaterials: tunable properties using different phases. *Phys Chem Chem Phys* 2012;14:12844–53.
- De Aza PN, Santos C, Pazo A, De Aza S, Cusco R, Artus L. Vibrational properties of calcium phosphate compounds. 1. Raman spectrum of β -tricalcium phosphate. *Chem Mater* 1997;9:912–5.
- Shie MY, Chang HC, Ding SJ. Effects of altering the Si/Ca molar ratio of a calcium silicate cement on *in vitro* cell attachment. *Int Endod J* 2012;45:337–45.
- Shie MY, Chen DCH, Wang CY, Chiang TY, Ding SJ. Immersion behavior of gelatin-containing calcium phosphate cement. *Acta Biomater* 2008;4:646–55.
- Khan AF, Saleem M, Afzal A, Ali A, Khan AR. Bioactive behavior of silicon substituted calcium phosphate based bioceramics for bone regeneration. *Mater Sci Eng C Mater Biol Appl* 2014;35:245–52.
- Fernández E, Ginebra M, Boltong M, et al. Kinetic study of the setting reaction of a calcium phosphate bone cement. *J Biomed Mater Res* 1996;32:367–74.
- Su YF, Lin CC, Huang TH, Chou MY, Yang JJ, Shie MY. Osteogenesis and angiogenesis properties of dental pulp cell on novel injectable tricalcium phosphate cement by silica doped. *Mater Sci Eng C Mater Biol Appl* 2014;42:672–80.
- Liu H, Li H, Cheng W, Yang Y, Zhu M, Zhou C. Novel injectable calcium phosphate/chitosan composites for bone substitute materials. *Acta Biomater* 2006;2:557–65.
- Liu S, Jin F, Lin KJ, et al. The effect of calcium silicate on *in vitro* physicochemical properties and *in vivo* osteogenesis, degradability and bioactivity of porous β -tricalcium phosphate bioceramics. *Biomed Mater* 2013;8:025008.
- Chen YW, Hsu TT, Wang K, Shie MY. Preparation of the fast setting and degrading Ca–Si–Mg cement with both odontogenesis and angiogenesis differentiation of human periodontal ligament cells. *Mater Sci Eng C Mater Biol Appl* 2016;60:374–83.
- Yeh CH, Chen YW, Shie MY, Fang HY. Poly(dopamine)-assisted immobilization of *Xu Duan* on 3D printed poly(lactic acid) scaffolds to up-regulate osteogenic and angiogenic markers of bone marrow stem cells. *Materials* 2015;8:4299–315.
- Kao CT, Lin CC, Chen YW, Yeh CH, Fang HY, Shie MY. Poly(dopamine) coating of 3D printed poly(lactic acid) scaffolds for bone tissue engineering. *Mater Sci Eng C Mater Biol Appl* 2015;56:165–73.
- Shie MY, Chang HC, Ding SJ. Composition-dependent protein secretion and integrin level of osteoblastic cell on calcium silicate cements. *J Biomed Mater Res Part A* 2014;102:769–80.

34. Hung CJ, Hsu HI, Lin CC, et al. The role of integrin α v in proliferation and differentiation of human dental pulp cell response to calcium silicate cement. *J Endod* 2014;40:1802–9.
35. Shie MY, Ding SJ. Integrin binding and MAPK signal pathways in primary cell responses to surface chemistry of calcium silicate cements. *Biomaterials* 2013;34:6589–606.
36. Kang Y, Kim SH, Bishop J, Khademhosseini A, Yang YJ. The osteogenic differentiation of human bone marrow MSCs on HUVEC-derived ECM and β -TCP scaffold. *Biomaterials* 2012;33:6998–7007.
37. Huang MH, Shen YF, Hsu TT, Huang TH, Shie MY. Physical characteristics, antimicrobial and odontogenesis potentials of calcium silicate cement containing hinokitiol. *Mater Sci Eng C Mater Biol Appl* 2016;65:1–8.
38. Vohra S, Hennessy KM, Sawyer AA, Zhuo Y, Bellis SL. Comparison of mesenchymal stem cell and osteosarcoma cell adhesion to hydroxyapatite. *J Mater Sci: Mater Med* 2008;19:3567–74.
39. Siddiqui N, Pramanik K, Jabbari E. Osteogenic differentiation of human mesenchymal stem cells in freeze-gelled chitosan/nano β -tricalcium phosphate porous scaffolds cross-linked with genipin. *Mater Sci Eng C Mater Biol Appl* 2015;54:76–83.
40. Dvorak MM, Siddiqua A, Ward DT, et al. Physiological changes in extracellular calcium concentration directly control osteoblast function in the absence of calciotropic hormones. *Proc Natl Acad Sci U S A* 2004;101:5140–5.
41. Engel E, Del Valle S, Aparicio C, et al. Discerning the role of topography and ion exchange in cell response of bioactive tissue engineering scaffolds. *Tissue Eng Part A* 2008;14:1341–51.
42. Shie MY, Ding SJ, Chang HC. The role of silicon in osteoblast-like cell proliferation and apoptosis. *Acta Biomater* 2011;7:2604–14.