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

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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° ± 1.5° and 58° ± 1.7° for TCP50 and TCP30, respectively) than pure PCL (85° ± 1.3°), 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.

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 24 hours, the maximum force required to inject the paste was measured.
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:

\[ I = \frac{m_{\text{injected}}}{m_{\text{initial}}} \times 100\% \]  

where \( I \) is the injectability, \( m_{\text{injected}} \) and \( m_{\text{initial}} \) 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°/min. Raman measurements were performed using B&W Tek'siRaman 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 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°C/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 KHPO\(_4\), 0.305 g of MgCl\(_2\)-6H\(_2\)O, 0.2775 g of CaCl\(_2\), and 0.071 of g Na\(_2\)SO\(_4\) in 1000 mL of distilled H\(_2\)O and was buffered to a pH of 7.4 with hydrochloric acid (HCl) and trishydroxymethyl aminomethane (CH\(_2\)OH\(_3\)CNH\(_2\)). All chemicals used were of reagent grade. The solution in the well was then transferred to a new 96-well plate and read using Tecan Infinite 200 PRO microplate reader (Tecan, Man- nedorf, 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.

**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 (Scien-cell) 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, Mannedorf, Switzerland) at 620 nm, according to the manufacturer’s recommendation. The cells were detached using a trypsin-EDTA solution (Caisson Laboratories, North Logan, UT, USA) after being washed three times with cold PBS. Samples 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 cells 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\(_2\)SO\(_4\) 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 450 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.

**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 results were then transferred to new 96-well plates and read using Tecan Infinite 200 PRO microplate reader (Tecan, Mannedorf, 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.

**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 morphology

After the cells had been seeded for 1 day and 3 days, the PLCL/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 PLCL/β-TCP composites in the medium were analyzed using an inductively coupled plasma-atomic emission spectrometer (Perkin-Elmer OPTIMA 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 405 nm. All experiments were done in triplicate. The addition of 5N NaOH and quantified by absorbance at 405 nm. All experiments were done in triplicate. The dried specimens were then mounted on stubs, coated with gold, and viewed using scanning electron microscopy (JEOL JSM-7401F, Tokyo, Japan).

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 PLCL/β-TCP biocomposites

The setting time is one of most important factors in the clinical use of this compound. 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. 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. 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 changes in its crystallinity.

A Raman spectra of the PLCL/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. In addition, the other peaks at 1470–1415 cm⁻¹ (ν(OC₃H₉)), 1303–1281 cm⁻¹ (ν(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.

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

**Immersion studies of PCL/\(\beta\)-TCP biocomposites**

The degradation rates of the PCL/\(\beta\)-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, Mg0 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 \(\beta\)-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/\(\beta\)-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/\(\beta\)-TCP composites tend to have lower contact angles (47°/C14/C6 and 58°/C14/C6 for TCP50 and TCP30, respectively) than pure PCL (85°/C14/C6), which are generally more hydrophilic. Cell proliferation gradually increases along with the amount of \(\beta\)-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.
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/\( \beta \)-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.

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,
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

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**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.

**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.
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. 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. Setting time is a key factor, and a long setting time will lead to clinical problems under clinical conditions. Fernandez et al. 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 9](image_url) The morphology of human bone marrow mesenchymal stem cells cultured on polycaprolactone/β-tricalcium phosphate (TCP) specimens after different time-points.

![Figure 10](image_url) (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.
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. 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. 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. In view of the significant influence on hBMSCs adhesion, proliferation, and differentiation, surface wettability is a key parameter that should be considered.

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. Following initial adhesion, cells will secrete ECM into their environment, such as COL, FN, and vitronectin, which subsequently affect the cells' behavior. These proteins will adsorb onto the material's surface and supply a provisional matrix for cell adhesion. The dominant role of protein adsorption in the regulation of cell behavior has been identified. Differential ECM adsorption on the various material surfaces accounts for the observed variability in cell adhesion. In addition, ECM molecules express and synthesize COL and FN during the various stages of osteogenesis. In vivo, the adsorption to the bioma- terial surface of bioactive proteins from the serum and bodily fluids at the surgical site is known to influence cellular–material interactions.

Recent studies also show that β-TCP promotes hBMSC proliferation and differentiation. 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. 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. 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 bio-composites. 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 bio-composites 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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