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Contributed Paper

Self-setting Calcium Phosphate Enhanced with Osteoconduction and Bioactivity for Bone Cement

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ABSTRACT

Calcium phosphate cements (CPC) have been widely applied as bone repair materials, biodegradable biomaterials or tissue engineering scaffolds. CPC can set and harden in the body at low temperature, but their use has been restricted due to their low biodegradable rate and osteoconductivity. The aim of this work was to study the effects of β-tricalcium phosphate (β-TCP; β-$Ca_3(PO_4)_2$) and chitosan content on the properties of the CPC. β-TCP was mixed into the apatite-based cement with concentrations up to 40 % (w/w) to form the so-called composite biphasic calcium phosphate cements (BCPC). The compressive strength, bioactivity, degradation and cytotoxicity of the samples were evaluated after soaking in a simulated body fluid (SBF) at 37 °C for 7 days. The degradation rate was found to be higher in cement containing increasing β-TCP concentrations. Apatite formation with oriented plate-like morphology was denser on the BCPC surface after soaking in SBF for 7 days. This BCPC could be considered as a highly biodegradable and bioactive bone cement compared to the apatite CPC. This new self-setting calcium phosphate cement containing 20 % (w/w) β-TCP formed a mixture of HA:β-TCP at a ratio of 60:20 after setting and was found to be a good candidate for bone cement applications.

Keywords: bone cement, apatite, biphasic calcium phosphate, tricalcium phosphate, biodegradation

1. INTRODUCTION

Calcium phosphate cements (CPC) have been widely applied as bone repair materials, biodegradable biomaterials and tissue engineering scaffolds. Therefore, the biodegradation rate of calcium phosphate cements should be adjustable to meet the needs of their varied applications (i.e., filler, coating, drug carrier, bone cement, tissue regeneration, scaffolding, etc.). However, the biodegradation rate of a single calcium phosphate phase cannot be varied or modulated [1-3].

Calcium phosphates, composed of β-tricalcium phosphate (β-TCP; β-$Ca_3(PO_4)_2$) and Hydroxyapatite (HA; $Ca_5(PO_4)_3(OH)$), are also known as biphasic calcium phosphates. They have been intensively investigated, because their biodegradation rate, bioactivity and osteoconductivity can be adjusted by changing the ratios between β-TCP and HA phases. β-TCP appears to be the most soluble phase, while HA is the most inert phase at pH 7.0 in water. Increases in the degradation rate of biphasic formulations correlates with the increasing ratio of β-TCP/HA. Thus the *in vivo* bioresorbability of such formulations can be modulated fine tuning its phase composition. One of the main advantages of biphasic calcium phosphate is that it has high biodegradability as compared with apatite CPC [1-2, 4-5].

In this study, apatite CPCs containing 0-40 % (w/w) β-TCP were investigated. The effect of β-TCP content on bone cement properties such as the biodegradation, compressive strength, *in vitro* bioactivity and cytotoxicity were assessed and compared with those of apatite-based bone cement. The phase composition and bioactivity of set cements containing different concentrations of β-TCP were also determined after immersion in SBF solution.

2. MATERIALS AND METHODS

2.1 Materials and Method

The apatite-based cement powder consisted of α-tricalcium phosphate (α-TCP, α-$Ca_3(PO_4)_2$), dicalcium phosphate anhydrous (DCPA; $CaHPO_4$, assay ≥ 97.0% from FLUKA, Germany), calcium carbonate ($CaCO_3$, assay ≥ 99.0% from CARLO ERBA, Italy) and precipitated Hydroxyapatite (PHA) as described previously [6]. α-TCP was synthesized from DCPA and calcium carbonate by solid state reaction, following the method of Camire [7]. The mixture was calcined at 1350 °C for 4 h before quenching to room temperature. Then, the powder was ground and sieved through 325 mesh to obtain α-TCP particles with a mean particle size of 15.62 mm. PHA with a mean particle size of 30.42 ± 11.39 nm was prepared by the co-precipitation method [8]. For BCPC, β-tricalcium phosphate (β-TCP; β-$Ca_3(PO_4)_2$), assay ≥ 96.0% from FLUKA, Germany) powder was added into the cement powder at 0-40 % (w/w) and then mixed together using dry ball milling for 45 min. The liquid phase was the mixture of 1 M disodium hydrogen phosphate (Na_2HPO_4, assay ≥ 99.0% from MERCK, Germany) and 1 M sodium dihydrogen phosphate (NaH_2PO_4, assay min. 99.0% from GREC, New Zealand) to obtain a solution at pH 7.4. The cement powder and liquid solution were mixed in a mortar with the ratio of liquid and powder phase (L/P) at 0.50 ml/g.

2.2 The Bone Cement Properties Testing

2.2.1 *Compressive strength testing*

The compressive strength of the bone cement (6 mm diameter and 12 mm height) after soaking in simulated body fluid (SBF) solution at 37 °C for 7 days was determined using the Shimadzu Universal Tensile Testing

Machine (UTM; UH-100A) at a loading rate of 1 mm/min reported as the mean ± standard deviation. The SBF medium consisted of ion concentrations of Na^+= 142.0, K^+= 5.0, Ca^{2+}= 2.5, Mg^{2+} = 1.5, Cl^- = 148.8, HCO_3^- = 4.2, HPO_4^{2-} = 1.0; and SO_4^{2-} = 0.5 mM [9] in 1 L distilled water and obtained an initial pH 7.4 at 36 °C.

2.2.2 *In vitro* bioactivity

For *in vitro* bioactivity testing, the bone cements were immersed in the SBF at pH 7.4 in a 37 °C incubator for 7 days with a surface area to volume ratio of 0.1 cm^{-1}. The surface morphology of the bone cement was observed by scanning electron microscope (SEM; JCM-5000, JEOL) at 10 kV.

After curing at 37 °C, 100% humidity and soaking in simulated body fluid (SBF) for 7 days, the phase composition of bone cement was characterized by X-ray diffraction (XRD; Bruker/D2-PHASER) with the range 2θ of 20° to 40° and step sizes was 0.02.

After setting of the bone cement, the progress of conversion of the starting cement powders to the final setting phases was evaluated using the Rietveld method [10]. Rietveld refinements were carried out in triplicate in order to quantify the phases present. For this purpose, the Inorganic Crystal Structure Database (ICSD) was used, including structural models for HA (ICSD No.87668), β-TCP (ICSD No. 6191), DCPA (ICSD No. 917), CaCO3 (ICSD No. 73446) and α-TCP (ICSD No. 923) [11]. The Rietveld refinements were performed using the software X'Pert High Score Plus. XRD spectra analyzed with Rietveld refinement were also used to quantitative analysis of the main phase of each the bone cement.

2.2.3 *In vitro* biodegradation

To evaluate the *in vitro* degradation rate of the BCPC paste, the set cement disks were soaked in SBF at 37 °C, pH 7.4 for 7 days with a surface area-to-volume ratio of 0.1 cm^{-1} [12]. The solution was replaced every 3 days. After each immersion period, the disks were dried at room temperature for 24 h and the final weight of each sample was carefully measured. The degradation was then calculated using equation (1) and reported as the mean ± standard deviation.

$$\text{Degradation (\%)} = \frac{\textit{Initital weight - weight after soaking}}{\textit{Initital weight}} \times 100 \quad (1)$$

2.2.4 *In vitro* cell proliferation and *in vitro* cytotoxicity test

The osteoblast-like cell line, MC3T3-E1, was cultured and maintained in 6-well plates containing complete medium 88% Dulbecco's Modified Eagle Medium (DMEM), 10% Fetal Bovine Serum (FBS), 1% Penicillin streptomycin and 1% L-glutamine). The cell culture atmosphere was 5% CO_2 at 37 °C. The medium was changed every 48-72 hours [13-17].

For *in vitro* cell proliferation and cytotoxicity testing, the proliferation rate and the number of viable MC3T3-E1, osteoblast-like cells growing on the bone cement samples was measured using the dimethylthiazol diphenyl tetrazolium bromide (MTT) assay [16]. The optical density (OD) was recorded in a microplate reader at 590 nm. The experiment was repeated three times. In brief, after incubating at 37 °C and 5% CO_2 for 1 day, 100 µl of 0.5 mg/ml MTT solution was added to extract/cell constructs and cultured for 4 h at 37 °C. 100 µl dimethyl sulfoxide then was added to each well. The plate was incubated for 5 min, and the optical density (OD) at 590 nm was measured with an enzyme-linked immunoadsorbent assay microplate reader.

Three samples per group were tested in the experiment. The results were reported as the percentage of cell viability and expressed as the mean ± standard deviation. The percentage of the viable cells was calculated from the optical density (O.D.) values using Eq. (2) and (3), respectively [18].

$$\%\text{Cell Inhibition} = \frac{O.D.\ value\ (control\ cells) - O.D.\ value\ (treated\ cells)}{O.D.\ value\ (control\ cells)} \times 100 \quad (2)$$

$$\%\text{Cell viability} = 100 - \%\text{Cell Inhibition} \quad (3)$$

Control cells were cells cultured without sample and treated cells were cells cultured with sample.

3. RESULTS AND DISCUSSION

Calcium phosphate based cements are widely used to serve many purposes. They can act as fillers, coatings, drug carriers, bone cements, and as scaffoldings for tissue regeneration. However, the setting rate and biodegradation limits their usability. Here, this work aimed to improve upon hydroxyapatite cements by the addition of β-tricalcium phosphate. The properties of the new cements were evaluated and compared with those of control calcium phosphate based cement.

3.1 Compressive Strength Testing

Compressive strength was determined by Universal Testing machine. Figure 1 plots the relationship between the compressive strength of the cement samples and the percentage of β-TCP content after immersion in SBF at 37 °C for 7 days. The compressive strength of the bone cement samples decreased from 21 MPa to 13 MPa by increasing the β-TCP content up to 40 % (w/w).

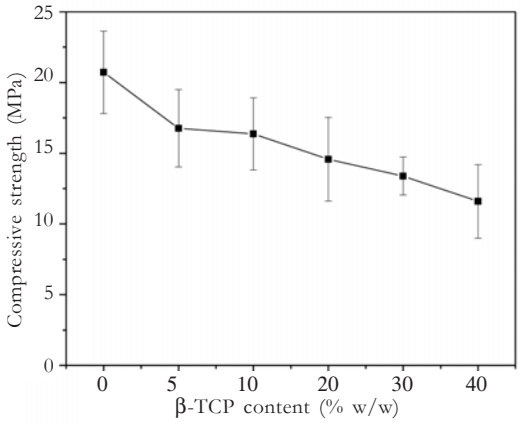

Figure 1. Compressive strength of the bone cements with various contents of β-TCP after soaking in SBF solution at 37 °C for 7 days.

3.2 *In vitro* Bioactivity

To determine the bioactivity of bone cement samples, phase analysis of the set cemet was analyzed by XRD technique. The XRD patterns of the bone cement with 0-40 % (w/w) β-TCP after soaking in SBF for 7 days are shown in Figure 2. The XRD patterns of the apatite-based cement exhibited the characteristic peaks around 2θ = 31.774°, 32.197° and 32.902° attributed to the presence of calcium-deficient hydroxyapatite (CD-HA, JCPDS card No. 00-009-0432). This result indicates that approximately 80 % of the apatite-based bone cement was converted to CD-HA after setting in SBF for 7 days. Unreacted DCPA (JCPDS card No. 00-070-0359) was also present as indicated by peaks at 2θ = 26.547° and 30.241°. Phase analysis of CPC containing β-TCP demonstrated that some α-TCP (JCPDS card No. 00-009-0348), $CaCO_3$ (JCPDS card No. 00-047-1743) and β-TCP (JCPDS card No. 01-070-2065) phases presented after setting as shown in Figure 2. Apatite formation was found that it decreased in the set cement with increasing β-TCP content. Futhermore, apatite formation

resulting from calcium and phosphate ions released from α-TCP, DCPA, $CaCO_3$ and PHA.

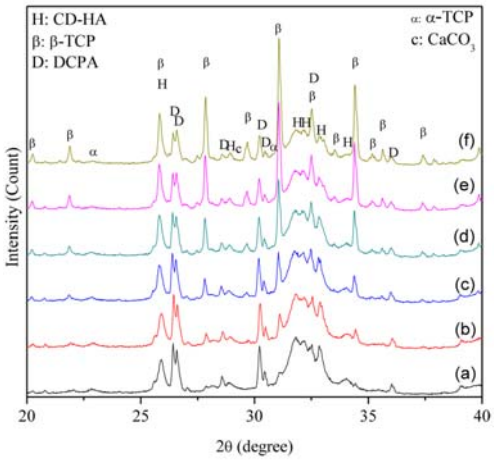

Figure 2. XRD patterns of the bone cements with various contents of β-TCP after soaking in SBF solution for 7 days. (a) 0 %w/w, (b) 5 %w/w, (c) 10 %w/w, (d) 20 %w/w, (e) 30 %w/w and (f) 40 %w/w β-TCP.

It was clear from Figure 3 that the phase fraction of CD-HA in the set cements decreased with increasing amount of β-TCP while the remaining DCPA and $CaCO_3$ were increased due to their less solubility at pH 7.4. From these patterns, it can be transformed into biphasic CPC between CD-HA and β-TCP.

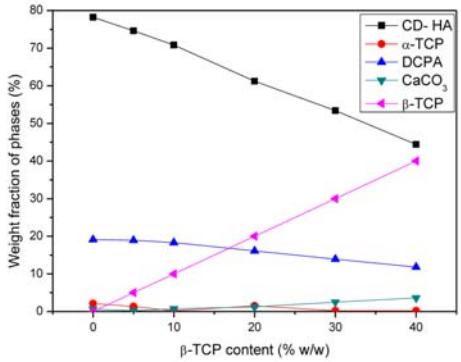

Figure 3. Weight fraction of phases containing in the set bone cements with various contents of β-TCP after soaking in SBF at 37 °C for 7 days (analyzed by Rievelt refinement).

The formation of apatite after soaking in a SBF solution at 37 °C for 7 days was observed on the surface of samples by SEM as shown in Figure 4. Fine apatite crystals were relatively homogenously distributed on the surface of the cement as shown in Figure 4(a). However, there was a rougher and much more irregularly shaped apatite layer on the bone cement with β-TCP addition [Figure 4(b) - 4(f)] after soaking. The apatite crystals were larger and became prominent and significantly denser on the surface of the bone cement containing 40 % (w/w) β-TCP [See Figure 4(f)]. The obtained porosity and crystal like-apatite formation on the surface tend to increase with increasing amounts of β-TCP. Thus, β-TCP addition enhanced the bioactivity of the bone cement associated with the formation of a apatite layer on the bone cement surface [2, 19]. The handing properties of biphasic calcium phosphate cements of different a/β-TCP ratio differed significantly from the conventional CPC. Injectibility, setting times increased considerably with increasing β-TCp content. It can be explained by the fact that β-TCP is not a setting reactant and does not hydrolyze into HA [20].

3.3 *In vitro* Biodegradation

To evaluate the biodegradability of our bone cement, the degradation rate was measured after soaking in SBF solution for 7 days at 37 °C as shown in Figure 5. The degradation of apatite-based cement was approximate to 17% due to the solubility of the remaining DCPA which was more soluble than α-TCP and β-TCP. With the addition of β-TCP comprising up to 40 % (w/w) of the apatite-based cement, the degradation under 7 days of the bone cement increased accordingly from 20 to 25%.

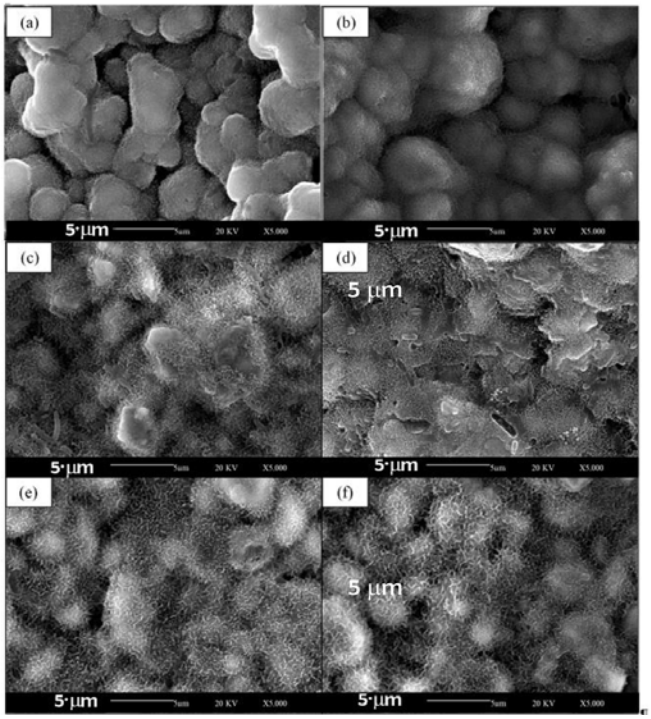

Figure 4. SEM micrographs of the cement surface with various contents of β-TCP;(a): 0 %w/w, (b): 5 %w/w, (c): 10 %w/w, (d): 20 %w/w, (e): 30 %w/w and (f): 40 %w/w β-TCP after soaking in SBF solution for 7 days.

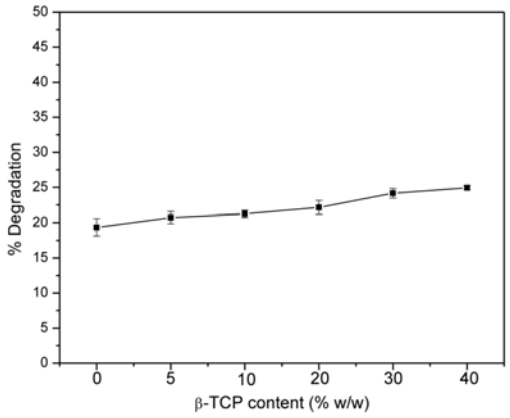

Figure 5. Degradation of the bone cements with various contents of β-TCP after soaking in SBF solution for 7 days at 37 °C.

It indicated that degradation of these bone cements resulted from the solution of DCPA and β-TCP phases as the cement sets. The degradation rate of calcium phosphate compounds can be predicted to be in the following order (at pH 7.4 in SBF solution): α-TCP>DCPAβ-TCPPHAHA. Thus, the *in vivo* bioresorbability of such formulations can be adjusted through the phase composition of the set cements [2, 4].

3.4 *In vitro* Cell Proliferation and Cytotoxicity Test

In order to test whether the bone cement can facilitate cell growth and not be toxic to the cells, *in vitro* cell proliferation and cytotoxicity tests were assessed using the MTT assay as shown in Figure 6 and Figure 7. The bone cement with 20 % (w/w) β-TCP was selected for this test due to the mixture ratio of HA:β-TCP at 60:20 after setting. Optical density (OD) and cell viability were evaluated for samples extracted from the medium at 1, 7, 14, 21 and 28 days. At concentrations of less than 25 mg/ml,

experimental samples presented statistically significant higher proliferation and cell viability levels as compared with the control sample. However, cell proliferation and viability did not significantly increase with increasing time; this might be due to an increase of calcium released from the bone cement. The apatite-based cement with 20 % (w/w) β-TCP was non-toxic to osteoblast cells which demonstrated good cell proliferation, comparable to controls. In addition, cell viability for the cement consisted of 20 % (w/w) β-TCP slightly increased after 14 days.

The cytotoxicity of the new bone cement containing 20 % (w/w) β-TCP was evaluated for osteoblast cells using the MTT test. The influence of immersion time in SBF on cell proliferation is shown in Figure 6. There was a significant reduction in osteoblast cell proliferation with increasing concentrations of bone cement with 20% (w/w) β-TCP from 50 mg/ml to 200 mg/ml at 7, 14 and 21 days of immersion.

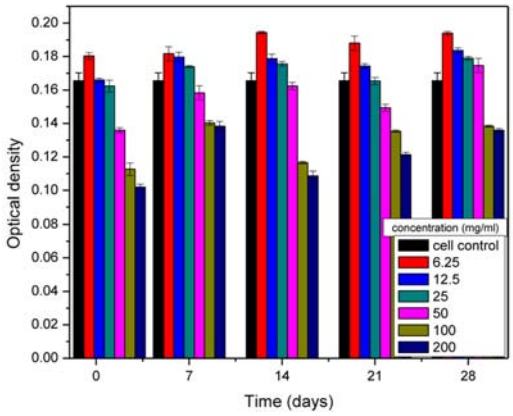

Figure 6. Cell proliferation of the bone cement with 20 %w/w β-TCP addition on MC3T3-E1 cells with various concentrations evaluated by MTT assay for 1, 7, 14, 21 and 28 days.

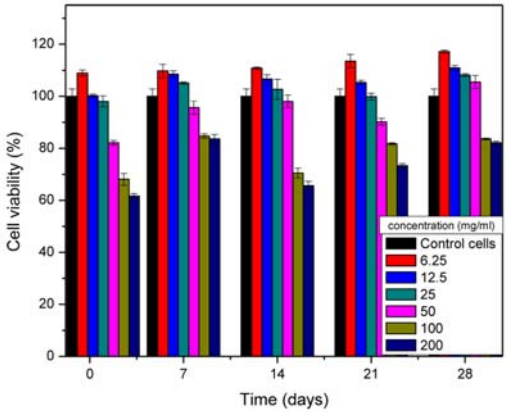

Figure 7. Cell viability of apatite-based cement with 20 % w/w β-TCP composites on MC3T3-E1 cells with various concentrations after soaking in medium for 1, 7, 14, 21 and 28 days as measured using MTT assay.

However, cell viability was found higher when concentrations was less than 50 mg/ml after soaking in medium. Low concentrations of cement with 20 % (w/w) β-TCP (6.25, 12.5 and 25 mg/ml) did not adversely affect osteoblast cell viabilities at all-time points. Here, it is interesting to note that at the lowest concentrations (6.25 mg/ml), there was a tendency towards higher cell viabilities (>110 %) than controls as can be seen in Figure 7, indicating a positive cell proliferative effect of the new bone cement. However, cell viability (60-70%) was monitored at higher concentrations (100-200 mg/ml) indicating that they had slightly cytotoxic effects above a concentration threshold. Thus, uncontrolled released of bone cement at these substitution sites should be carefully monitored.

4. CONCLUSION

This work reported the properties of new self-setting calcium phosphate bone

cement containing different concentrations of β-TCP. The addition of β-TCP into the apatite-based cement can improve the biodegradation rate and bioactivity of the bone cement. The degradation rate increased while the compressive strength decreased with increasing β-TCP. This new self-setting calcium phosphate cement containing 20 % (w/w) β-TCP formed a mixture of CD-HA:β-TCP at a ratio of 60:20 after setting. From this result suggested that it was suitable to serve as a bone cement with enhancing osteoconduction and bioactivity.

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