Strong and Bioactive Tri-calcium Phosphate Scaffolds with Tube-like Macropores

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Abstract. Calcium phosphate ceramic scaffolds have been widely investigated for bone tissue engineering due to their excellent biocompatibility and biodegradation. Unfortunately, they have the shortcoming of low mechanical properties. In order to provide strong, bioactive, and biodegradable scaffolds, a new approach of infiltrating the macro-tube ABS (acrylonitrile butadiene styrene) templates with a hydroxyapatite/bioactive glass mixed slurry was developed to fabricate porous Si-doped TCP (tri-calcium phosphate) scaffolds. The porous Si-doped TCP ceramics with a high porosity (~65%) and with interconnected macro-tubes (~0.8mm in diameter) and micropores (5-100 µm) had a high compressive strength (up to 14.68±0.2MPa), which was comparable to that of a trabecular bone and was much higher than those of pure TCP scaffolds. Additional cell attachment study and MTT cytotoxicity assay proved the bioactivity and biocompatibility of the new scaffolds. Thus a potential bioceramic material and a new approach to make the potential scaffolds were developed for bone tissue engineering.

Introduction

For bone tissue engineering as an alternative for treating deteriorated bones, bioceramic scaffolds have been developed and used over the past two decades. A scaffold should ideally be biocompatible, biodegradable, osteoconductive, osteoinductive, and sufficiently strong to provide a structural support during the bone growth and remodelling. Calcium phosphate ceramics are extensively used in medical and dentistry industries due to their bone-like compositions. In particular, tri-calcium phosphate (TCP) is an important biodegradable ceramic material and has been subjected to intensive studies [1]. However, the low compressive strength of the TCP scaffolds has limited their applications in bone tissue engineering.

To improve the mechanical strength of ceramic scaffolds, many methods have been developed, and these methods can be classified into the following three types. Firstly, type one is for the methods of preventing or decreasing the microstructural defects of the struts of scaffolds and thus improving the mechanical strength of the scaffolds [2-5]. For example, scaffolds of a bioactive borosilicate glass had a sufficient viscous flow during the sintering, leading to dense struts and a compressive strength of as high as ~10 MPa for the overall porosity of ~70%[3]. Secondly, type two refers to those methods involving the use of naturally strong materials for the struts. For instance, a glass-ceramic scaffold had an interconnected macroporous structure with a porosity up to 50% and still showed an orthotropic mechanical behaviour and strength well above 20 MPa [6]. Thirdly, type three stands for the methods
of controlling the geometries of the porous structures, namely, creating bimodal pores\cite{7}, fabricating regular pore structures\cite{8}, aligning the pores \cite{9}, and introducing additional micro-ribs\cite{10}.

The aim of this study was to explore another method to fabricate bioactive and relatively strong Si-doped tricalcium phosphate scaffolds, which would be potential for bone tissue engineering. Specifically, the macropores of the scaffolds were fabricated using the ABS polymeric tempppates made by a technique of fused depositing modelling, and the thick struts were formed via reaction sintering of a hydroxyapatite-bioactive glass mixture at the temperature of 1400 °C.

Materials and methods
Preparation of scaffold templates. In this study, scaffold templates with macro-channels were prepared using a 3D printer, which was based on fused deposition modelling - a rapid prototyping technique. The material used for the templates was acrylonitrile butadiene styrene (ABS). Fig. 1 shows the scaffold templates of different sizes and shapes prepared by the 3D printer. The ABS scaffold templates were then dipped into a molten wax so that the struts of the templates were coated with a layer of wax. It was expected that the wax would create a gap between the ABS struts and the ceramic filler in a template, as the wax would be removed during heating earlier than the ABS struts. The gap could cater for the thermal expansion of the ABS struts during firing so that the ceramic filler, which would become a ceramic scaffold after burning the ABS, would not be over-stressed and broken into pieces.

Fig. 1 ABS scaffold templates: (a) a photograph; (b) dimensions shown in the top view; (c) dimensions shown in the side view.
Preparation of TCP Ceramic Scaffolds. In this project, a bioactive glass was added into a hydroxyapatite (HA) ceramic to form a biodegradable tricalcium phosphate (TCP) ceramic. The bioactive glass was firstly prepared by dissolving 33.5 grams of tetraethyl orthosilicate (TEOS, 98%), 7 grams of Ca(NO$_3$_2)·4H$_2$O, 3.65 grams of triethyl phosphate (TEP, 99.8%) and 5 grams of 37% HCl into 300 grams of ethanol to maintain a molar ratio of Si:Ca:P = 80:15:5. The mixture was then stirred for 24 hrs at the room temperature. After drying and crushing, the mixture was calcined at 700 °C for 5 hrs to obtain the required bioactive glass powder.

An aqueous slurry of 80g of HA powder and 20g of bioactive glass powder was prepared by ball milling for 2 hrs. A binder of polyvinyl alcohol (PVA) (0.25 wt% on solid basis) and 3 drops of a dispersant were added into the slurry to maintain a suitable rheological property. Then the ABS scaffolds were infiltrated with the ceramic slurry. The ceramic-filled scaffold templates were dried in a fume hood for at least 24 hrs and reaction sintered at 1400°C for 3 hrs. After cooling down from the sintering temperature, porous scaffolds of the TCP phase doped with the Si element were obtained.

Sample characterization.

Porosity. The total porosity of the sintered ceramic scaffolds was determined using the following procedures [11]: firstly the bulk density ($\rho_B$) was obtained by dividing the weight of the scaffold by the volume of the scaffold, then the theoretical density of the TCP ($\rho_0$) was assumed to be 3.10 g/cm$^3$, and finally the relative density (R.D.) was obtained by calculating ($\rho_B/\rho_0$)x100%, leading to the total porosity = 100% - R.D.

X-ray diffraction (XRD). XRD was used to identify the crystallographic phases of the sintered scaffolds. For the XRD analysis, the samples were ground into fine powders and each powder was mounted in a specimen holder fitting the diffractometer (6000 Shimadzu). Scanning with the Cu K$_\alpha$ ray ($\lambda$=1.5406 Å) was conducted using a 20 angle of from 20° to 45°. The scan rate and the step size were kept at 2.0° min$^{-1}$ and 0.02°, respectively.

Scanning electron microscopy (SEM)/ energy dispersive spectroscopy (EDS). The topographical images of the sample surfaces or the fracture surfaces were examined under a FEI QUANTA 200 SEM machine. In addition, the elements present in the samples, especially in the top layers, were analyzed using EDS.

Mechanical testing of the TCP scaffolds. The compressive strengths of the scaffolds were measured using an Instron tester (Model 5567). The cross-head loading speed was set at 0.5 mm/min. Seven to nine identical specimens for each sample group were tested.

Apatite-formation ability in the SBF. The SBF solution was prepared according to the procedure described by Kokubo [12]. The TCP scaffolds were then soaked in the SBF at 37°C for up to 21 days with the SBF being refreshed every week, and the ratio of the sample weight to the SBF volume (mg/ml) being set at 3:5. After soaking, the samples were removed from the SBF, gently washed with deionized water, dried at the room temperature, and then characterized under SEM.

Cell seeding and culture. Human bone marrow was sourced from patients in the Orthopaedic Department of Prince Charles Hospital with informed consent and ethics approval from the Ethics Committee of Queensland University of Technology. The human bone marrow stromal cells (BMSCs) for this study were then isolated by density gradient centrifugation. The BMSCs were seeded into culture flasks using Dulbecco’s Modified Eagle Medium (DMEM, Invitrogen Pty Ltd) containing 10% fetal calf serum (FCS; Invitrogen) and 1% penicillin/streptomycin (Invitrogen) at 37°C in 5% CO$_2$. The medium was changed twice weekly to wash out all non-adherent cells. After the cells reached 80% confluence, the cells were trypsinized and re-suspended in DMEM. About 1×10$^5$ cells were seeded onto each scaffold (~ 1 cm$^3$) and cultured for one week in DMEM at 37°C in 5% CO$_2$.

Observation of cell attachment on scaffolds using SEM. The cell culture medium in each well was pipetted out, and immediately replaced with phosphate buffer saline (PBS). The rinsing was repeated three times for each sample, and then the scaffolds were fixed with a 3% glutaraldehyde solution. The scaffolds were then processed twice using the cacodylate buffer for 20 min each. Then
they were soaked in an osmium tetroxide solution for 1 hour, and dehydrated through a series of ethanol solutions with graded concentrations, followed by two changes of 100% amyl acetate for 15 min each. The scaffolds were then dried using a supercritical point dryer before observation under SEM.

**Cytotoxicity test by the MTT assay.** To evaluate the proliferation of BMSCs on different materials, BMSCs were seeded at a density of 1×10^4 cells/well into a 24-well plate and incubated for 4 hours. Then 20 mg of the Si-doped TCP powder was added to the culture plate. Cells were then incubated at 37°C in 5% CO₂ for 1, 3, 6 and 9 days. Then, 40 μL of 0.5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) solution (Sigma, Aldrich) was added in each well and incubated for 4 hours at 37°C. The reaction was terminated by the addition of 100 μL dimethyl sulfoxide. The absorbance of the formazan was read at 495 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader (Bio-Rad Laboratories, Pty. Ltd, Gladesville, New South Wales, Australia). The MTT assay was to assess the cell viability and the cell growth rate based upon the conversion of MTT formazan. For comparison, BMSCs' proliferation in the normal cell culture medium was evaluated by the same procedure.

**Results and discussion**

**Confirmation of the TCP phase.** Phases formed in the scaffolds during the sintering process depended on two external factors, i.e. the sintering time and the sintering temperature. With the sintering temperature changing from 850°C to 1200°C, the decomposition of the HA phase was initiated, possibly according to the following equations:

\[
\begin{align*}
\text{Ca}_{10} \text{(PO}_4\text{)}_6\text{(OH)}_2 & \rightarrow \text{Ca}_{10} \text{(PO}_4\text{)}_6\text{(OH)}_2 \cdot 2\text{H}_2\text{O} + x\text{H}_2\text{O}_{\text{gas}} \\
\text{Ca}_{10} \text{(PO}_4\text{)}_6\text{(OH)}_2 & \rightarrow 2\text{Ca}_3 \text{(PO}_4\text{)}_2 + \text{Ca}_4 \text{P}_2 \text{O}_9 + \text{H}_2\text{O}_{\text{gas}}
\end{align*}
\]

After the sintering temperature was decreased to the room temperature, most of the α-TCP was transformed to β-TCP (stable at low temperatures). Miao et. al. [13] suggested that some α-TCP phase may be retained due to the elastic strain constraint from the surrounding matrix. In addition, according to the HA decomposition equations shown above, there were a number of minor phases detected after the sintering process. It was then decided to add the bioactive glass powder into the HA powder in order to adjust the Ca/P ratio, and produce required TCP phases. As a result, the phases achieved in the sample should contain both α-TCP and β-TCP (mainly β-TCP). This was confirmed by the XRD analysis (Fig. 2). Thus, it was believed that Si-doped TCP scaffolds were obtained from the HA/bioactive glass mixture.

![Fig. 2 An XRD pattern of a sintered scaffold showing the main β-TCP phase and the minor α-TCP phase.](image)
**Macrostructure and microstructure.** It was required that the designed porous scaffolds would possess bone-like characteristics in some aspects such as material composition and pore morphology. Porous scaffolds have the advantage of promoting bone and soft tissues' development by introducing blood and nutrients into the scaffolds. Fig. 3(a) shows the macro-tube ceramic scaffold from the ABS template of a 13x13mm top area. Due to approximately 23% sintering shrinkage, the top area of the ceramic scaffold was decreased to 10x10mm. The width or diameter of the macro-tubes was about 0.8mm. Apart from the macro-tubes, the key design features of the scaffolds, some micro-pores were also presented in the scaffold material as shown in Fig. 3(b) and Fig. 3(c). This microporosity might be caused by the early stage of water vapour evaporation as shown in the HA decomposition equations, and due to the poor sinterability of the powder mixture involving high temperature reactions. Since the large pore size of a trabecular bone is about 1 mm in diameter [14], and the size of the interconnected pores for easy cell growth is approximately 50 μm[15], one can see that the hierarchic structure of the macro-tubes and the interconnecting micro-pores in Fig. 3 was suitable for osteoblasts to grow into the scaffold and for rapid vascularization. Fast formation of blood vessels would be required to maintain the growth of osteos in the large pores during bone ingrowth and bone remodelling [16].
Fig. 3 The porous structure of the Si-doped TCP scaffolds: (a) photo showing the macro-tubes, (b) SEM image showing the strut surface at a low magnification, and (c) SEM image showing the internal micropores of a strut at a high magnification.

**Porosity and mechanical strength.** The apparent sizes of the bulk scaffolds could influence their mechanical strength, their permeability and the extent of structural defects. Table 1 shows the mechanical strength and the porosity of the TCP scaffolds prepared using different ABS templates. In these kinds of scaffolds, macro-tubes were mainly responsible for the porosity, and the average porosity of the scaffolds was from 61.13±0.8% to 72.40±1.3%. Table 1 also indicates that as the surface area of the scaffolds was increasing, the strength was decreasing, which was due to more cracks and pores observed. The porosity and the strength results followed the well-known relationship, i.e., the compressive strength decreased rapidly with the increase of the porosity.

<table>
<thead>
<tr>
<th>Shapes of the scaffolds</th>
<th>Top area (mm²)</th>
<th>Average porosity (%)</th>
<th>Compressive Strength (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cube</td>
<td>26x26</td>
<td>72.40±1.3</td>
<td>2.52±1.05</td>
</tr>
<tr>
<td></td>
<td>13x13</td>
<td>63.34±1.5</td>
<td>9.98±1.40</td>
</tr>
<tr>
<td></td>
<td>7.5x7.5</td>
<td>61.65±0.5</td>
<td>14.25±0.3</td>
</tr>
<tr>
<td>Cylinder</td>
<td>3.14x6.5</td>
<td>64.26±0.7</td>
<td>11.13±1.2</td>
</tr>
<tr>
<td></td>
<td>3.14x3.7</td>
<td>61.13±0.8</td>
<td>14.68±0.2</td>
</tr>
</tbody>
</table>

For the decrease of the surface area from 26x26 to 7.5x7.5 mm², the compressive strength increased from 2.52±1.05 to 14.25±0.3 MPa, which was comparable to those values of a trabecular bone (2–20MPa). For comparison purpose, scaffolds were also made using a pure TCP powder and by using ABS templates of the top area of 13x13 mm². A higher porosity was observed in these pure TCP scaffolds (66.21±1.20%), resulting in a low compressive strength (2.8±1.3MPa). The difference in morphology between the Si-doped TCP scaffolds and the pure TCP scaffolds was due to the fact that
the bioactive glass was melt above 700°C, and the melted bioactive glass could heal some pores and cracks.

If the scaffold size was smaller than 7.5x7.5mm², the porosity would be less than 60% and the compressive strength would be higher. However, clinically speaking, this size might be too small and the porosity might be too low. If the size was bigger than 26x26mm², the compressive strength would be too low. Clearly, the Si-doped TCP scaffolds produced in this work had much improved mechanical properties compared to the pure TCP scaffolds.

Apatite-formation ability of the Si-doped TCP scaffolds in SBF. An SEM image of the scaffold after being soaked in the SBF solution for 21 days is shown in Fig. 4(a). It was noted that a rough deposit layer was formed on the TCP scaffold, and some crystal clusters were also found on the layer surface. To determine the chemical composition of the crystal deposits, the surface of the scaffold was further characterized by EDS, as shown in Fig. 4(b). The atomic ratio of Ca to P was about 1.65, which was close to that of a carbonated apatite. As reported elsewhere [17], under some conditions, HA-like materials, due to their compositional similarity to human bone tissue, are capable of stimulating the osteo-induction of stem cells in vivo, needless to say, the osteo-conductivity. The produced scaffolds would be potential for bone repair applications.

Fig. 4 (a) An SEM image of the scaffold after being soaked in the SBF for 21 days and (b) An EDS spectrum of the surface crystal deposits.

SEM images of cells grown on the scaffolds. The bone marrow stromal stem cells were seeded into the scaffolds by adding drops of the cell suspension. As a result, the cells were homogeneously seeded across the surface of the scaffolds. The penetration of the cells into the scaffolds was evaluated using the cross-sections of the scaffolds. SEM images (Fig. 5) revealed that the cells spread and adhered
well on the surfaces. Interestingly, it was noted that a significant amount of apatite crystal clusters was formed on the scaffold surface and distributed among the cells, which was advantageous as far as bioactivity was concerned. Generally speaking, apatite granules can be formed when a bioactive material is immersed in a simulated body fluid (SBF) solution for a length of time (Fig. 4(A)). However, in this experiment, the apatite granules were also formed when the Si-doped TCP scaffold was immersed into the DMEM solution. There were two possible reasons for the formation of the apatite granules: the first one being the DMEM solution, which possessed some ions; the second one being that some $\text{P}^{5+}$ and $\text{Ca}^{2+}$ ions could be slowly released into the DMEM solution from the TCP material, resulting in a modified medium solution similar to the SBF solution. Furthermore, the formation of the apatite granules was independent on the cells present in the medium, as apatite granules were also formed on the surface of the scaffold after 9 days of immersion in the cell-free medium, as shown in Fig. 6.

![SEM micrographs showing the attachment of the cells on the Si-doped TCP ceramic strut surface at two magnifications: (A) 800 x and (B) 2000x](image-url)
**Cell proliferation.** The MTT assay is one of important methods to evaluate the cytotoxicity of scaffolds and their slowly released species in an aqueous environment. Fig. 7 shows the result of the MTT assay, i.e. the proliferation status of the BMSCs in the negative control group (without the TCP scaffold) and in the experimental group (with the TCP scaffold) and cultured under the same conditions for 11 days. Obviously, the cells were proliferating with the culture time for both the groups, but the cell growth rate of the experimental group was basically higher than the negative control group. Thus, the Si-doped TCP scaffolds were proven to be non-cytotoxic and have a good biocompatibility *in vitro*.

![MTT assay results](image)

**Fig. 7** The MTT assay results showing the proliferation of BMSCs without and with the presence of the TCP scaffolds at different incubation periods and under the same culture condition. Error bars represent means ± standard deviation for n = 3.

**Summary**
A new approach of infiltrating the macro-tube ABS templates with a hydroxyapatite/bioactive glass
mixed slurry was developed to fabricate porous Si-doped TCP scaffolds containing mainly the beta-TCP phase. For the Si-doped TCP scaffolds of a high porosity around 61%, a high compressive strength of 14.68MPa was achieved, comparable to those values of a trabecular bone. The Si-doped TCP scaffolds had both interconnected macropores and micropores with pore sizes ranging from 5 μm to 100 μm. The Si-doped TCP scaffolds were also proved to be bioactive and biocompatible. While the newly developed scaffolds are potential for bone tissue engineering, their biodegradation rates and their possible osteo-inductivity need to be investigated through further animal in vivo tests.

Acknowledgement
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References