A study on the in vitro degradation properties of poly(l-lactic acid)/β-tricalcium phosphate (PLLA/β-TCP) scaffold under dynamic loading

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1. Introduction

The aims of bone tissue engineering are to repair and restore damaged bone tissue function through employing three fundamental "tools", namely seed cells, growth factors and scaffolds [1]. The scaffold is one of the critical factors among these three components. It should provide adequate mechanical properties and three-dimensional interconnected pores for cell attachment, migration and proliferation [2]. Furthermore, the scaffold should be osteoinductive and degradable. The degradation rate of scaffolds must be appropriately tuned with the growth rate of the new tissue so that an early stress shield of the injured site can be avoided, and the injury site has regenerated at the point of scaffold degradation [3,4].

The in vivo osteoinductivity and degradability of scaffolds are commonly simulated in vitro by immerging them into a simulated body fluid (SBF). Through the studies of the apatite formation and the in vitro degradation properties, the in vivo osteoinductivity and degradation properties of scaffolds can be predicted [5–9]. Currently, these in vitro simulations are typically performed only in non-moving (i.e. "static") simulated body fluid that contains ions similar to those of blood plasma [5]. However, when considering the extent of flow in the physiological environment, a dynamic SBF simulation is probably more representative [10,11]. Agrawal [12] studied the effects of fluid flow on the degradation kinetics of PLA–PGA at a flow rate of 250 μl/min for 6 weeks. In addition, as bone is always under continual stress in vivo in a time dependent external stress environment, it is also very important to study the effects of mechanical loading on the degradation of scaffolds. Therefore, it is necessary to investigate the degradable properties and osteoinductivity of scaffolds under dynamic loading with SBF flow in order to simulate the situation in vivo at the early stage of implantation.

Therefore, we first investigated the effects of dynamic loading on the in vitro degradation characteristics of porous scaffolds under SBF flow, which could be used to evaluate the performance of the scaffolds under the in vivo cyclic stress generated under physiological loading [13].

Poly(l-lactic acid) (PLLA) and β-tricalcium phosphate (β-TCP) are widely used in tissue engineering for the regeneration of bone tissue, due to their biocompatibility and biodegradability [14,15]. However, the brittle nature of β-TCP confines clinical application to non-load-bearing repair and substitution [16]. The acid degradation products of PLLA could result in aseptic inflammation, and their hydrophobicity can significantly affect cell penetration into the scaffolds [17]. To improve the bioactivity and mechanical properties so as to meet the basic requirement for the repair of bone, one potentially promising approach is to design a composite scaffold.
scaffold combining the advantages of these two biomaterials and attempting to avoid the limitations of each.

In this paper, we describe the preparation of the composite PLLA/β-TCP scaffolds produced using a mold-pressing method. The effects of dynamic loading, under SBF flow, on the apatite formation ability and degradation behaviour of the composite scaffolds were systematically investigated. To this end, we fixed the stress at 0.1 MPa and the frequency at 0.6 Hz [18–21]. The experiments were performed for 6 weeks in a customized SBF dynamic system.

2. Materials and methods

2.1. Materials

β-TCP particles were fabricated by precipitation reaction. Known amounts of calcium chloride and sodium carbonate were separately dissolved in de-ionized water in a three-neck flask to obtain a saturated solution, and the solutions were filtered to remove impurities. Then, the sodium carbonate solution was added to the calcium chloride solution under continuous stirring under room temperature to form precipitates. The precipitate mixture was filtered and washed in de-ionized water four times and then in ethanol twice. After drying in a vacuum oven at 80 °C for 48 h, calcium carbonate powder was obtained. Next, the as-prepared calcium carbonate (CaCO3) was dissolved in aqueous solution to form slurry to which phosphoric acid solution was added, according to the ratio of Ca to P (Ca:P = 1.5). The solution was stirred ultrasonically for 2 h, then the precipitation mixture was filtered and again washed in de-ionized water four times and then in ethanol twice. The obtained precursor was dried in a vacuum oven at 80 °C for 48 h and calcined at 950 °C for 2 h and then cooled. A uniform and ultra-fine β-TCP powder was obtained. The mean particle size measured by a laser particle size analyzer was 2.41 μm. PLLA (Mn = 735 kDa, M = 580 kDa, dispersity = 1.27) was provided by Chengdu Organic Chemicals Co. Ltd. (more information about PLLA is provided in Tables 1 and S.1 of Supplementary supporting information). The other chemical agents were purchased from Chengdu Changzheng Chemical Co. Ltd., China.

2.2. Preparation of PLLA/β-TCP porous scaffold

The composite porous scaffolds were fabricated using a solvent self-proliferating/model compressing/particulate leaching technique. Briefly, a known amount of PLLA was dissolved in chloroform to obtain 4% (wt/v) PLLA solution and then the β-TCP and ammonium bicarbonate powders (NH4HCO3) (200 ≤ particle size ≤ 400 μm), i.e. the porogen, were added into the PLLA solution. The mass ratio of β-TCP to PLLA was 65:35, similar to the ratio of inorganic to organic composition in nature bone [22,5]. The mass percentage of porogen was 60% (i.e. the mass ratio of NH4HCO3 to β-TCP and PLLA was 1.5:1). The polymer solution was mixed under continuous stirring. Next, the suspension solution was dropped slowly into the mixture solvent of acetone and ethanol while stirring. The amount of the mixture solvent was enough to expand the chloroform solvent. PLLA does not dissolve in the mixture solvent of acetone and ethanol, and this generates high supersaturation ratio of the solute, resulting in fast nucleation of PLLA and creating small composite particles. After this was finished, the deposited compound PLLA/β-TCP precipitates were filtrated and washed in absolute ethanol to obtain PLLA/β-TCP particles. These mixture particles were loaded into a stainless steel mold with 5 mm diameter and pressed at 7 MPa for 5 min under room temperature to obtain a cylindrical specimen of about 10 mm height. The specimens were then immersed in de-ionized water for 48 h to leach the porogen NH4HCO3 and then dried in a vacuum oven.

Fig. 1. Schematic graph shows the apparatus for studying the degradation of biodegradable scaffolds in flow conditions using a peristaltic pump and with dynamic loading using minor motor.

2.3. In vitro degradation of the PLLA/β-TCP scaffold

31 PLLA/β-TCP scaffolds were divided into 3 groups: the original group (7 specimens), the SBF flow group (12 specimens) and the dynamic loading with SBF flow group (12 specimens). A litre of simulated body fluid (SBF) was prepared by dissolving NaCl 7.995 g, NaHCO3 0.353 g, KCl 0.224 g, KH2PO4·3H2O 0.228 g, MgCl2·6H2O 0.305 g, CaCl2 0.227 g, and Na2SO4 0.071 g into distilled water. The solution was buffered at pH 7.4 by adjusting the volume amount of Tris (tris-hydroxymethylaminomethane) and HCl at 36.5 °C. The in vitro degradation studies were performed by immersing the scaffolds in a customized dynamic flow chamber (Fig. 1) at 37 °C in a cyclic water bath. In the SBF flow system, fresh SBF was introduced into the chamber at the same rate as it is removed, keeping the volume constant during the experiment. A flow rate of 2 ml/100 ml min, close to the flow rate of bone fluid in bone tissue [23,24], was produced by using one channel of a peristaltic pump. In the dynamic loading group with SBF flow, the scaffolds were subjected to dynamic loading with 0.6 Hz and 0.1 MPa [18–21] for 6 weeks. After soaking for different time periods, a set of scaffolds of two groups were taken out from the SBF solution, rinsed gently with de-ionized pure water and dried in a vacuum oven for further characterization.

2.3.1. Morphology

Scanning electron microscopy (SEM) (JSM-5900LV, Japan) was used to characterize the internal and external morphologies of the scaffolds before and after immersion.

2.3.2. Mass changes

The mass change was determined by the equation: wt% = 100 × (Wf − Wo)/Wo, where W0 was original weight, and Wf was dried weight.

2.3.3. Porosity

Porosity was measured by using a conventional Archimedes immersion technique. Each scaffold with dry mass (Gd) was placed in the sample chamber of a porosity analyzer (DXR, Xiangtan apparatus plant, Hunan, China), and then after negative pressure was
applied, water was pumped into the chamber from the inner channel under vacuum. The water thus penetrated into the pores of the scaffolds. Next, the scaffold was removed from the water. The water on the surface of scaffold was gently wiped away and the scaffold was weighed to determine its saturated mass \( G_2 \) in air. The porosity was then calculated as follows:

\[
\text{Porosity} = \frac{(G_2 - G_1)}{\rho \pi R^2 h} \times 100\% ,
\]

where \( \rho \) was density of water, \( R \) was the radius of the scaffold i.e. 2.5 mm, and \( h \) was the length of scaffold which was measured using a vernier caliper. The average value of three samples was taken as representative of the porosity of the scaffolds.

### 2.3.4. Compressive strength

The compressive strengths before and after SBF immersion were determined using a universal mechanical testing apparatus (Shimadzu, AG-IS20KN, Japan) at ambient temperature. Samples were compressed between platens with a constant deformation rate of 1 mm/min. All results were the averages of three measurements.

### 2.3.5. Molecular weight

The molecular weight of PLLA before and after degradation was measured at \( 35^\circ C \) by Gel Permeation Chromatography (GPC, Agilent 1100) on a Waters 2410 instrument. The composite scaffold was dissolved in tetrahydrofuran (THF) and filtered with a 0.22 \( \mu m \) filter to remove the \( \beta \)-TCP particles, thus producing a THF solution of PLLA. The flow rate of THF was 1.0 ml/min, polystyrene standard (Polyscience Co.) was chosen as calibration and Waters Millennium 32 as the data-processing software. The weight-averaged molecular weights \( (M_w) \) of the scaffolds were determined.

### 2.3.6. Statistical analysis

All the experiments were performed three times, and three samples were employed at every test point. All data were arranged as mean \( \pm \) standard deviation (S.D.). Significant differences were determined by using Student’s \( t \)-test and a value of \( p < 0.05 \) was considered to be statistically significant.

### 3. Results and discussion

#### 3.1. Characterizations of PLLA/\( \beta \)-TCP scaffolds

Fig. 2 shows the SEM morphologies of porous PLLA/\( \beta \)-TCP composite scaffolds that have three-dimensional irregular porous structures together with good interconnections between the pores. The pore size ranged from 100 \( \mu m \) to 250 \( \mu m \) (Fig. 2a). The pore size was little smaller than that of the added porogen. The reason might be that some of the porogens were crushed in the press shaping process. The measured porosity of the scaffolds was 57\% (S.D.: 1.2). In this study, the amount of the added porogen was 60 wt\%. The porosity and pore size can be controlled by the mass and particle size of porogen. The scaffold has an ideal porous structure with adequate pore size and porosity, which may be helpful to cell attachment and nutrient delivery. In addition, PLLA and \( \beta \)-TCP particles are homogeneously distributed and dispersed (Fig. 2b), implying that the synthesis method made it possible to obtain scaffolds with high mechanical properties. The compressive strength of the composite scaffolds was 4.76 MPa (S.D.: 0.19). This result is satisfactory, compared with that of natural bone (2–10 MPa)[25]. It also suggests that the composite scaffold can support new bone tissue regeneration at the site of implantation [26].

#### 3.2. Apatite formation and growth

After the scaffolds were immersed in the SBF flow, typical apatite layer precipitates could be observed. The formation of bioactive calcium phosphate is typically used to predict the \emph{in vivo} bone-bonding ability of biomaterials, as proposed by Kokubo [5] and extensively used to evaluate the osteoinductivity of scaffolds. There are also many studies reporting that the apatite deposition ability on the surface of scaffolds in SBF is well correlated with their \emph{in vivo} bone bioactivities (e.g. [27–30]). Fig. 3a and b indicate that the surface of the scaffolds was covered with a thick apatite layer after 4 weeks immersion in the static SBF (for comparison). Additionally, new apatite granules were continuously deposited on the surfaces of the formed apatite layer (Fig. 3b). The apatites were gradually deposited from the surface to the inner pores. After 4 weeks, there were many small apatite granules on the walls of the inner pores (SEM inserted in Fig. 3a). However, after immersion in the SBF flow under dynamic loading conditions for 4 weeks, apatite deposits were apparent only on the deep walls of the inner pores (Fig. 3c and d), while the surface was not covered with an apatite layer. The formation of apatite is dependent on the threshold of nucleation. Under SBF flow conditions, the Ca\(^{2+}\), PO\(^{4-}\) can diffuse easily into solution and be taken out of the sample chamber by the cyclic flow of SBF. Therefore, on the surface of specimens, the threshold concentration for nucleation is hard to achieve. However, in the inner pores, high Ca\(^{2+}\), PO\(^{4-}\) concentration is easier to accumulate thus attaining the nucleation threshold. Therefore, one might predict that it is not easy for scaffolds to form an apatite layer \emph{in vivo} at the early stage following implantation, because there is a flow of body fluid and also mechanical stress. Fig. 3e and f show the morphologies of formed apatite on the surface of the scaffold in the SBF flow group under no loading and dynamic loading conditions for 6 weeks. Under dynamic loading conditions, the apatites are not layers but granular spheres (Fig. 3f). Due to the similarity of apatite formed in static SBF, in flow SBF or under dynamic loading conditions, the characterizations of apatite by XRD, FTIR are not
Fig. 3. SEM micrographs of apatite formation after immersion for: 4 weeks in static SBF (a, b); 4 weeks in the flow SBF with dynamic loading (c, d); and 6 weeks in the flow SBF without dynamic loading (e) and with dynamic loading (f).

provided here. More information can be available in our previous paper [31].

3.3. Porosity and mass changes

It can be seen from Fig. 4 that the porosity of the PLLA/β-TCP scaffolds under dynamic loading or no dynamic loading conditions firstly decreased and then steadily tended to increase up to the degradation time. However, the increased rate of porosity under dynamic loading conditions was higher than that under no dynamic loading conditions. At the early stage of degradation, apatite formation was faster than the degradation of the scaffold. Therefore, the formed apatite compensated for the increase in porosity. After 2 weeks, the porosity of scaffold decreased under the two conditions. With increasing degradation time, the degradation of PLLA and the dissolution of β-TCP accelerated the increase in porosity. The porosity of the scaffolds under dynamic loading conditions increased faster than that of the scaffolds in the SBF flow group without dynamic loading and there was a significant difference after 4 weeks (p < 0.05). Fig. 5 indicates that all the PLLA/β-TCP scaffolds after in vitro degradation under dynamic

Fig. 4. Changes in porosity of scaffold as a function of degradation time under the two conditions (n = 3).
loading or no dynamic loading conditions tend to increase their masses with increasing time, but the amount of mass increase under the dynamic loading conditions was little lower than that under no dynamic loading conditions. Both the degradation of the PLLA/β-TCP scaffold and the formation of apatite contributed to the mass changes of the composite scaffolds. The resultant mass results from the difference between the increased mass of the deposited apatite and the mass loss of the scaffolds. In general, the mass of the scaffolds increased quicker at the beginning and then slowed down thereafter. The reason is that the apatite deposition is faster than the degradation of PLLA and the dissolution of β-TCP at the early stage of degradation. Under dynamic loading conditions, dynamic loading accelerated the degradation of PLLA and the dissolution of β-TCP particles and also decreased the deposition of apatite. All of these led to a slower mass increase rate for the scaffolds under dynamic loading conditions. Furthermore, after about 6 weeks, the mass change of the scaffolds began to attain equilibrium. The reason may be that although apatite was formed, the degradation of scaffold began to increase under dynamic loading conditions, indicating that dynamic loading promotes the degradation of the scaffolds. This is consistent with the change of porosity. After 4 and 6 weeks, the resultant mass changes attained equilibrium, which would indicate important significance, because the ideal degradation rate of the scaffolds should match the formation rate of new bone when the scaffolds are implanted in vivo [32].

### 3.4. Changes in molecular weight of PLLA

Changes in the molecular weight of the PLLA component in the scaffolds at the 4th and 6th week degradation under dynamic loading or no dynamic loading conditions are shown in Table 1 (data relating to the molecular weight of PLLA is provided in the Supplementary Information). It can be seen from the results that the average-weight molecular weight of PLLA in the scaffolds under dynamic loading conditions reduced faster than that under no dynamic loading. This result is consistent with the trend of mass loss, which indicates that the investigated mechanical loading increased the degradation of the scaffold. The decrease in molecular weight resulted from hydrolysis and macromolecular scission of PLLA. The acidic degradation products of PLLA would lead to the decrease of pH value that may lead to cell morphological changes, and eventually to cell death. In vivo an inflammatory response could be induced by the release of low-molecular weight chains [33]. Consequently, complex interpenetrating polymeric networks filled with alkalescent β-TCP particles could be optimized to avoid, as much as possible, the acid situation. In our experiment, the pH value did not, however, significantly decrease due to the addition of alkalescent β-TCP. A part of the acidity of the degradation products could be neutralized by alkaline β-TCP in the scaffolds. Faster degradation of the scaffolds under dynamic loading conditions produced a higher reduction in molecular weight and dissolution of β-TCP.

### 3.5. Changes in mechanical strength

Fig. 6 shows the changes in the compressive strength of the porous PLLA/β-TCP scaffolds with immersion time under the two conditions. It can be seen from Fig. 6 that the compressive strength of the composite scaffolds under dynamic loading conditions was lower than that for no dynamic loading conditions. The mechanical loading at 0.6 Hz increased the degradation of the scaffold ($p < 0.05$). However, the dynamic loading did not produce a deterioration in the scaffold performance after 6 weeks. The compressive strength remained at 3.45 MPa. These results might be explained by the Biot’s theory of poroelastic solids [34], which described the behavior of fluids in porous materials and provided the physical basis for load-induced fluid flow in bone. According to this theory, compression deforms the solid matrix of a porous material, instantaneously increasing the pressure in the fluid within the pores. Disparate pressures between the interior and exterior of a porous solid cause a net flow of fluid, which seems to be relevant to the situation of the SBF flow group. As a result, changes in mechanical strength may not become significantly worse than that of groups in only flow SBF.

Additionally, 6 weeks of degradation might be too short a time period to predict the change in mechanical property of the scaffolds implanted in vivo for long time. In the next step, this experiment should be performed in a range of frequency of dynamic loading for longer degradation time [35].

<table>
<thead>
<tr>
<th>Time</th>
<th>Groups</th>
<th>$M_w$ (kDa)</th>
<th>$M_n$ (kDa)</th>
<th>$M_d$ (kDa)</th>
<th>Dispersity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 week</td>
<td>Flow SBF</td>
<td>735</td>
<td>580</td>
<td>857</td>
<td>1.27</td>
</tr>
<tr>
<td></td>
<td>Dynamic loading</td>
<td>443</td>
<td>318</td>
<td>575</td>
<td>1.39</td>
</tr>
<tr>
<td>4 weeks</td>
<td>Flow SBF</td>
<td>403</td>
<td>255</td>
<td>549</td>
<td>1.58</td>
</tr>
<tr>
<td></td>
<td>Dynamic loading</td>
<td>412</td>
<td>283</td>
<td>539</td>
<td>1.46</td>
</tr>
<tr>
<td>6 weeks</td>
<td>Flow SBF</td>
<td>338</td>
<td>206</td>
<td>475</td>
<td>1.64</td>
</tr>
<tr>
<td></td>
<td>Dynamic loading</td>
<td>266</td>
<td>180</td>
<td>345</td>
<td>1.45</td>
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**Fig. 5.** Changes of mass as a function of degradation time under the two conditions ($n = 3$).

**Fig. 6.** Changes in compressive strength as a function of degradation time under the two conditions ($n = 3$).
4. Conclusions

The degradation behaviour of porous PLLA/β-TCP composite scaffolds was investigated under dynamic loading and no dynamic loading conditions in vitro under SBF flow conditions at 37 °C for 6 weeks. Results show increases in mass and porosity and reductions in average molecular weight of PLLA. Reduction in the compressive strength of the scaffolds under the dynamic loading conditions were more marked than those without dynamic loading. Results also indicate that the scaffolds possess good apatite formation ability under the two conditions. Mechanical loading at the physiological level can promote the degradation of the scaffold, but the dynamic loading did not remarkably deteriorate the mechanical performance of the scaffolds. All of these results indicated that dynamic loading under flow SBF conditions can accelerate the degradation of the PLLA/β-TCP composite scaffolds compared to the effect of SBF flow conditions without applied stress.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.medengphy.2008.11.014.

Conflict of interest statement

The authors do not have any possible conflicts of interest.

References