Research Article

Biomimetic apatite layer formation on a novel citrate starch scaffold suitable for bone tissue engineering applications†

Jhamak Nourmohammadi¹,*, Sepideh Shahriarpanah¹, Negin Asadzadehzanjani¹, Shila khaleghpanah², Shafagh Heidari³

1- Department of Life Science Engineering, Faculty of New Sciences and Technologies, University of Tehran, Tehran, Iran.
2- Department of material science, Sharif University, Tehran, Iran.
3- National cell bank, Pasteur institute, Tehran, Iran.

*Corresponding author:
Jhamak Nourmohammadi; PhD.
Faculty of New Sciences and Technologies, University of Tehran, P.O.Box: 14395-1561, Tehran, Iran.
Tel: +98-21-66118560
Email: J_nourmohammadi@ut.ac.ir

†This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: [10.1002/star.201500216].

This article is protected by copyright. All rights reserved.
Received: August 10, 2015 / Revised: December 31, 2015 / Accepted: January 8, 2016
Abstract

The formation of biomimetic bone-like apatite layers throughout the biopolymer-based hydrogel scaffold is an attractive approach in bone tissue engineering. Here, the starch scaffold was prepared using a combination of particulate leaching and freeze-drying techniques. The fabricated structures were then modified by citric acid to investigate the formation of bone-like apatite layer on the porous citrate-based scaffold after soaking in simulated body fluid (SBF). The Fourier Transform Infrared (FTIR) spectra and X-ray diffraction (XRD) patterns revealed that the B-type carbonated apatite has successfully deposited on the scaffold after immersing in SBF for 28 days. Indeed, high chemical affinity of carboxyl group in citrate starch resulted in primary heterogeneous nucleation of apatitic calcium phosphate throughout the starch hydrogel. Moreover, the biological activity of MG63 osteoblast-like cells cultured on the scaffold was assessed using indirect MTT assay and cell attachment experiment. The results indicated that the cells show significant biocompatibility and cell attachment.

Keywords: Citrate starch; Apatite deposition; Bone tissue engineering; Scaffold; Biomimetic method.
1. Introduction

These days, starch-based materials have become popular in the field of biomedical engineering due to their low cost, availability, biocompatibility, biodegradability, and ease of processability. As a polysaccharide, starch consists of two biomacromolecules: 20% amylose (linear; $\alpha$-1, 4-glycosidic linkage) and 80% amylopectin (branched; $\alpha$-1, 4 and $\alpha$-1, 6-glycosidic linkage) [1]. The preparation of porous starch-based structures has been investigated in many reports. Nasri-Nasrabadi et.al. [2] prepared a porous starch/cellulose nanofibers composite that could potentially be used in cartilage tissue engineering. Moreover, as mentioned by Salgado et.al. [3], the scaffold fabricated by the blend of corn starch with ethylene-vinyl alcohol demonstrates appropriate properties for bone regeneration. Nevertheless, the lack of osteo-conductivity and osteo-inductivity of these materials limit their application as bone substitutes [3, 4].

To examine the bioactivity of such scaffolds, researchers incorporate hydroxyapatite, bioactive glass, and bioactive glass-ceramics into the starch matrix [5-7]. However, the lack of homogeneity during the distribution of ceramic fillers into polymer matrix and the weak bonding between ceramic and polymer matrix were observed in such reports. Fortunately, the intrinsic nature of apatite deposition on the surface of CaO-SiO$_2$ glasses has developed an effective approach in coating bone-like apatite layer on a variety of materials such as metals, ceramics, and polymers [8]. Reportedly, negatively charged functional groups such as carboxyl (COOH) are also considered as nucleation sites for apatite deposition [8-10]. K. Park et.al. [11] proposed that in-situ grafting of carboxyl group on polylactic acid (PLA) scaffold induces apatite nucleation after immersion in Simulated Body Fluid (SBF). Moreover, O. Petrauskaite et.al. [12] reported that apatite layers can be formed on carboxyl-functionalized cellulose matrix in the presence of SBF. Thus, introducing carboxyl groups into starch structures seems to be effective in the formation of apatite layer on its surface. Citric acid can be used as a
safe and cost-effective method to create carboxyl groups on polysaccharides such as cellulose and starch [13,14]. In addition, it enhances the mechanical properties as well as water sensitivity of starch hydrogels through cross-linking [14].

This study introduces a new bioactive citrate starch scaffold for bone tissue engineering applications. Therefore, a three-dimensional (3D) porous starch scaffold was prepared via a combination of particulate leaching and freeze-drying methods. The carboxyl groups were then incorporated into the scaffold using citric acid. Finally, the possibility of forming bone-like apatite layer on the surface of the structures was determined through immersing in SBF. Structural characterization and the biological responses of MG63 osteoblast-like cells seeded on the fabricated scaffolds were also evaluated.

2. Experimental

2.1. Materials

The soluble starch from potato was supplied by Sigma (St. Louis, USA). Besides, citric acid (100241, ≥ 99.5%, Merck, Germany), NaCl (106400, ≥ 99.5%, Merck, Germany), NaHCO₃ (106329, ≥ 99.7%, Merck, Germany), KCl (104936, ≥ 99.5%, Merck, Germany), K₂HPO₄·3H₂O (P3786, ≥ 98%, Sigma, USA), MgCl₂·6H₂O (172571, ≥ 99%, Merck, Germany), Na₂SO₄ (106649, ≥ 99%, Merck, Germany), CaCl₂ (102378, ≥ 98%, Merck, Germany), Tris (Hydroxy-methyl-amino-methane) ((CH₂OH)₃CNH₂; 108387, ≥ 99.8%, Merck, Germany), hydrochloric acid fuming (HCl 37%; 100317, Merck, Germany), absolute ethanol (107017, ≥ 99.5 %, Merck, Germany), and table sugar (Sucrose; C₁₂H₂₂OH, Bartar, Iran) were purchased.

2.2. Fabrication of citrate starch scaffold

The citrate starch porous scaffold was fabricated through particulate leaching and freeze-drying methods. Potato starch (5gr) was added to 50 mL of distilled water and heated with continuous stirring at 90°C. Prior to gelatinization, progen (70 wt% table sugar (150-350 µm)) was added to
the paste, stirred carefully, and poured into 48-well tissue culture polystyrene plates. The samples were kept in 4 °C overnight and then frozen at -20 °C (Emersun refrigerator; model HR1560D, Iran) for 48 h before freeze-drying (Operon, South Korea). In order to leach out the sugar, the freeze-dried scaffolds were soaked in ethanol (70%) for 3 days. The samples were then immersed in 30% (w/v) citric acid-ethanol solution at room temperature. Afterwards, the specimens were dehydrated in a forced-air oven (Behdad, Iran) at 60 °C. After 24 h, the temperature increased to 120 °C for 6 h to obtain citrate starch. In the end, the scaffolds were washed thoroughly with deionized water to remove the unreacted citric acid.

2.3. Bioactivity evaluation of citrate starch scaffold

To evaluate the apatite formation on the citrate starch scaffold, the cylindrical samples (11×10 mm²) were immersed in 30 ml of SBF and stored in a 37°C incubator (Memmert, Germany) for different time periods up to 28 days, with the solution refreshed every 24 h. The SBF solution was prepared according to our previous study [15]. Briefly, appropriate amounts of NaCl (7.996 g), NaHCO₃ (0.350 g), KCl (0.224 g), K₂HPO₄·3H₂O (0.228 g), MgCl₂·6H₂O (0.305 g), Na₂SO₄ (0.071 g), and CaCl₂ (0.278 g) were dissolved in deionized water, and buffered with Tris (Hydroxy-methyl-amino-methane) and hydrochloric acid to obtain pH 7.4 at 37°C. At the end of immersion period, the samples were removed from the SBF, washed gently with deionized water and dried at room temperature.

3. Characterization

3.1. Microstructure evaluation

Surface morphologies of the citrate starch scaffold and SBF-treated scaffold were observed by scanning electron microscopy (SEM; Mira 3-XMU, Czeck Republic) using gold coating. The changes in the composition of the specimens before and after immersing in SBF were evaluated by energy dispersive spectroscopy (EDS). The image J software (National institutes of Health,
Bethesda, Maryland, USA) was used to calculate the size and distribution of the pores. Approximately 100 random pores from a number of SEM images (secondary electron mode) were analyzed. The porosity percentage of the scaffold was calculated as follows [16]:

\[
\text{Porosity (\%)} = \frac{A_P}{A_T} \quad \text{Eq. (1)}
\]

\(A_P\) is the total area of the pores in each cross-section and \(A_T\) is the total area of each cross-section. Image J was used for \(A_P\) and \(A_T\) measurements from different cross-sections of SEM images (n=5).

3.2. IR spectroscopy

Structural changes of the scaffolds before and after 28 days of soaking in SBF were analyzed with a FTIR spectrometer (Bruker Vector 33, Germany) within 400–4000 cm\(^{-1}\) region. Pellets were prepared from a mixture of dried sample (1 mg) and KBr powder (300 mg). All measurements were done at a 4 cm\(^{-1}\) resolution.

3.3. XRD analysis

The phase composition of the scaffold before and after immersion in SBF solution was characterized by X’Pert Pro MPD X-ray diffractometer using Cu\(\alpha\) radiation at 40 kV and 30 mA. 2\(\theta\) angles ranged from 20\(^\circ\) to 40\(^\circ\) using 0.02\(^\circ\) step and 20s counting time.

3.4. Evaluation of pH value

The changes in pH value of SBF were measured at different time intervals using a pH meter (\(\Omega\)Metrohm 827, Switzerland). The measurements were carried out for three specimens (n=3).

3.5. The amount of the deposited apatite layer

The amount of the deposited apatite layer throughout the scaffold was determined by Eq. (2) at different soaking times [17].

\[
\text{Formed apatite (mg)} = W_1 \cdot \left(\frac{W_F}{148R}\right) \quad \text{Eq. (2)}
\]
W₁ and W₂ are the weight of dried and wet samples after soaking in SBF, respectively. **SR is the** swelling ratio of each sample, which was calculated by gravimetric method [18]. Briefly, the scaffold with the initial weight of (w₀) was soaked in deionized water (T=37 °C) for about 24 h. Afterwards, the excess water was wiped up with filter paper and then weighted (Ws). The SR (%) was measured based on the following equation. All measurements were performed for five samples (n=5).

$$SR \, (\%) = \left( \frac{W_s - W_0}{W_0} \right) \times 100$$

**Eq. (3)**

3.6. Mechanical properties

The Universal testing machine (Zwick/Roell Z050, 0.1 KN load cell) with a cross-head speed of 1 mm/min was used to determine the compressive yield strength and modulus of each scaffold. Five cylinder-shaped samples (d= 6 mm, h=12 mm) from each composition were analyzed. The compressive modulus was measured by the slope of the initial linear section of the stress-strain curve. Moreover, the compressive yield strength was calculated at the yield point of the stress-strain curve.

3.7. Biological activity analysis

The human osteoblast-like cells (MG63; National Cell Bank of Iran, Pasteur Institute) were cultured in Dulbecco’s Modification of Eagles Medium (DMEM; GIBCO, Scotland) containing 10% Fetal Bovine Serum (FBS; Gibco, Renfrewshire, Scotland), 100 U/mL penicillin (Sigma, Saint Louis, USA), and 100 μg/mL streptomycin (Sigma, Saint Louis, USA). For cell attachment assay, the MG63 cells were cultured on the sterilized samples (5x 10⁴ cells/ cm²) and then kept in a humidified incubator with 5% CO₂. After 3 days of cell culture, the cells were fixed with 4% (v/v) glutaraldehyde solution in PBS, washed thoroughly with deionized water and dehydrated by graded alcohol solution (10% ethanol increments; each step 10 minutes). In the end, the dried scaffolds were sputter-coated with gold and viewed by SEM.
Cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT, Sigma, Saint Louis, USA) assay based on extraction method [19]. Here, the culture medium was chosen to be the control group. In order to obtain samples’ extraction, 200 µl of the culture medium was added to each sample and kept in humidified incubator with 5% CO₂ for 3 and 7 days. Meanwhile, the MG63 cells (1x10⁴ cells/well) were cultured in different sterilized plates. After 24 h, the culture medium was taken out and replaced by 100 µl of 3 and 7-day extracts. In the next 24 h, the medium was removed, 100 µl of MTT solution (0.5 mg/ml) in PBS was added to each well and kept at 37 °C for 4 h. After that, the formed purple formazan crystals were solubilized with 50 µl DMSO (Sigma, St. Louis, USA) for 10 min and then the absorbance was read at 545 nm using an ELISA Reader (Stat Fax-2100; GMI, Inc., Miami, FL, USA).

4. Results and discussion

Previous reports indicated that the porous structure plays a crucial role in the development of bone tissue engineering scaffolds. The pores are appropriate for increasing oxygen permeability and nutrient supply to deeper regions [20-22]. As shown in Fig. 1 the cross-section morphology, pore size distribution, and the EDS spectrum of citrate starch scaffold before soaking in SBF. The SEM images displayed interconnected porous structure with the pore size between 53.33 ± 4.03 µm and 239.8.1±10.27 µm (Fig.1). The average size of the pores was measured as 130.497±11.19 µm. Previous studies confirmed that the minimum pore size of 100-135 µm is required for bone tissue ingrowth [21, 22]. In addition to pore size, porosity is another key factor in designing suitable scaffold for BTE applications [20, 23]. Herein, the porosity percentage of the fabricated citrate starch scaffold was calculated as 65.5 ± 6.7 %, suggesting that the percentage is in the range of cancellous or trabecular bone [24]. The porosity and pore size results demonstrate that the scaffold can potentially support osteoblast cell attachment, cell growth, and neo-bone formation. Moreover, the elements detected by EDS spectrum were mainly carbon and oxygen
The compressive strength is another key factor in designing bone tissue engineering scaffolds. As shown in Fig. S1 (supporting information), the calculated amounts of compressive modulus and yield strength were 11.1 ± 0.57 MPa and 3.07 MPa, respectively. The mechanical properties of the fabricated scaffold (Fig. S1) were in the lower range of for human trabecular bone [24].

The SEM images and the EDS analyses of the scaffold after 14 and 28 days of soaking in SBF solution are shown in Fig. 2. It can be seen that the layers have deposited throughout the scaffold and fully covered the solid parts of the samples. On days 14 and 28 (Fig. 2d and 2h), new peaks related to the calcium and phosphorous appeared. Moreover, both Ca and P were uniformly distributed in the fabricated scaffolds, as confirmed by EDS elemental mapping of Ca and P (Fig. 2). The Ca/P atomic ratio of the deposited layers was 1.31 and 1.66 after 14 and 28 days, respectively (Table 1). This suggests that apatitic calcium phosphate deposited throughout the citrate starch scaffold at the end of the soaking period, which is in agreement with previous reports [25].

The amount of the formed apatite layer (Table 1) was calculated as 0.136 ± 0.014 (mg) in samples soaked for 14 days and it increased to 0.59 ± 0.03 (mg) after 28 days. As shown in Fig. 3, the pH of SBF decreased swiftly during initial soaking periods and then increased before reaching the pH of 7.56. The reduction in pH is probably due to the ionization of carboxyl groups in starch structure. This is in consistent with previous studies [9-11] in which carboxyl groups are negatively charged at neutral pH. Here, the calcium ions of SBF attached to these negatively charged carboxyl groups and formed complexes over time. Consequently, pH value rises when phosphate ions bond with calcium complexes, resulting in apatite nuclei formation. After nucleation, the calcium and phosphate ions of the SBF will continuously be absorbed, helping the formation of a stable bone-like apatite layer on the scaffold.
The IR Spectra of the samples before and after exposure to SBF are shown in Fig. 4. As can be seen, the spectrum of the citrate starch is characterized by well-resolved peak at 1745 cm\(^{-1}\) corresponding to carboxyl and ester carbonyl bands [14, 26]. The presence of carbonyl peak confirms the chemical linkage between citric acid and starch. In addition, there are several discernible peaks at 1156 and 1083 cm\(^{-1}\), which are attributed to C–O bond stretching in starch structure. The peaks at 916, 889 and 765 cm\(^{-1}\) are because of the entire anhydroglucose ring stretching vibrations. The band at 1658 cm\(^{-1}\) is assigned to O–H groups in absorbed water molecules. The band at 2935 cm\(^{-1}\) is due to the asymmetric and symmetric stretching of C-H and the bands at 1421 cm\(^{-1}\) and 1357 cm\(^{-1}\) are related to the angular deformation of C-H [27, 28].

After 28 days, the appearance of new peaks at 550-650 cm\(^{-1}\) and 950-1100 cm\(^{-1}\) is attributed to the stretching vibration of PO\(_4^{3-}\). Furthermore, the band at 1453 cm\(^{-1}\) comes from vibration of carbonate and is a characteristic of carbonated apatite [15, 26]. The IR spectra of the samples point to the formation of B-type carbonate apatite on the scaffold. This means that carbonate ions (CO\(_3^{2-}\)) occupied the trivalent anionic (PO\(_4^{3-}\)) sites in apatite structure [29].

The XRD patterns of the scaffold before and after 28 days of soaking in SBF are depicted in Fig. 5. The XRD spectrum of the pristine scaffold shows a broad peak, indicating the presence of amorphous phase. However, a sharp diffraction at 26.4° and a doublet peak at around 32° appeared after 28 days, which relate to the (0 0 2) and (2 1 1) planes in hydroxyapatite (JCPDS card No. 84-1998). This indicates that apatitic calcium phosphate formed on the fabricated scaffold after 28 days. The XRD results support the EDS and FTIR data.

Fig. 6 illustrates the viability of MG63 cells exposed to the scaffold’s extract after 3 and 7 days. The results show that the viability is more than 90%, while this amount slightly increased over time. This suggests that the scaffold has no cytotoxic effect on MG63 cells. The results are in agreement with previous reports in which the addition of citric acid to polysaccharide structure
did not exhibit any adverse effects [14, 30]. Moreover, as shown in Fig. 7, the cells adhered to the surface of the specimens, with cells connected to the adjacent cells in some regions. Both SEM images and MTT assay indicated that the citrate starch scaffold is compatible with osteoblast-like cells’ adhesion and proliferation.

5. Conclusions

In this study, a 3-D porous scaffold was achieved by freeze-drying method and particulate leaching technique and then modified with citric acid to obtain citrate starch scaffold. The porous structure was then immersed in SBF solution up to 28 days in order to assess the formation of bone-like apatite layer on the surface of the scaffold. The SEM images reveal that the citrate starch hydrogel has an average pore size of 130.057± 11.19 μm, which makes it a promising candidate for the purpose of bone tissue engineering. The EDS, FTIR and XRD analyses indicate the formation of carbonated-apatite layer throughout the scaffold. Finally, the images of MG63 osteoblast-like cells cultivated on the scaffolds represent suitable cell attachment and biocompatibility.

References:


**Figure Captions**

Fig.1. (a) SEM image, (b) pore size distribution, and (c) EDS spectrum of the pristine citrate starch scaffold (Secondary electron mode, × 250).

Fig. 2. (a) SEM image, (b) Ca mapping, (c) P mapping, and (d) EDS spectrum of 14-day soaked scaffold. (e) SEM image, (f) Ca mapping, (g) P mapping, and (h) EDS spectrum 28-day soaked scaffold (Secondary electron mode, × 500).

Fig. 3. Changes in pH value of SBF solution after various soaking periods

Fig. 4. Changes in the IR spectra of the scaffold before and after 28 days of soaking in SBF.
Fig. 5. The XRD patterns of pristine and 28-day soaked scaffold.

Fig. 6. The indirect MTT results of the scaffold after different incubation periods.

Fig. 7. The morphology of MG63 cells after 3 days culturing on the fabricated scaffold.

### Table

Table 1. The amounts and Ca/P atomic ratios of the formed apatite layer throughout the scaffold on days 0, 14, and 28.

<table>
<thead>
<tr>
<th>Soaking times in SBF</th>
<th>Apatite formed (mg)</th>
<th>Ca/P (at%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 day</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>14 days</td>
<td>0.136 ± 0.014</td>
<td>1.31</td>
</tr>
<tr>
<td>28 days</td>
<td>0.59 ± 0.03</td>
<td>1.66</td>
</tr>
</tbody>
</table>
Supporting Information:

Fig. S1: The stress-strain curve for citrate starch scaffold
Fig. 1

(a) Image of a material surface

(b) Bar chart showing distribution of pore sizes with average pore size indicated

(c) Graph showing another set of data with labels AXM and ADN
Fig. 2
Fig. 3
Fig. 6

Fig. 7