Polymer 98 (2016) 172–181

Contents lists available at ScienceDirect

Polymer

journal homepage: www.elsevier.com/locate/polymer

Macroporous poly(lactic acid) construct supporting the osteoinductive porous chitosan-based hydrogel for bone tissue engineering

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ARTICLE INFO

Article history:
Received 1 March 2016
Received in revised form
10 June 2016
Accepted 11 June 2016
Available online 13 June 2016

Keywords:
Poly(lactic acid)-chitosan-hydroxyapatite
Compressive strength
hMSCs

ABSTRACT

Poor mechanical performance of porous chitosan-hydroxyapatite systems is the main limitation in bone tissue engineering. If we merge good mechanical performance of poly(lactic acid) construct with osteoinductive and bioresorbable properties of chitosan-hydroxyapatite porous hydrogel, we can obtain a material that meets necessary requirement for bone tissue substituent. With this in mind, we propose the combination of 3D printing technique and the thermally-induced phase separation method for simultaneous modification of biological properties of poly(lactic acid) and load-bearing properties of chitosan-hydroxyapatite porous hydrogel. 3D printed poly(lactic acid), PLA, construct has been used as a mechanical support with very large pore diameter of 960 ± 50 μm allowing enough free space (~60% of porosity) to form porous composite hydrogel by freeze gelation. *In situ* formation of hydroxyapatite within chitosan hydrogel has ensured higher human mesenchymal stem cell osteogenesis during 21 days of culture. Positive modification of poly(lactic acid) has been simultaneously utilized to improve the compressive strength of composite hydrogel which has been confirmed by Young's modulus ranging from lower values reported for cancellous bone in dry state. Considering positive osteogenic signal accompanied with suitable mechanical properties, our scaffolds have shown good potential as bone tissue substituent.

1. Introduction

Chitosan-hydroxyapatite based composites have been widely used in bone defects reconstruction and have proved to exhibit great biocompatibility, osteoconductivity, minimal foreign body reaction, good wettability and suitable degradation rate [1–3]. Likewise, chitosan-based materials possess ability to be processed in various geometrical shapes with different processing techniques. Even though chitosan-hydroxyapatite scaffolds show favourable characteristics for bone tissue engineering, biomechanical properties of those systems cannot meet applications *in vivo* [4,5]. To overcome the mechanical drawback of natural polymers, additional synthetic polymers have been used. In particular, poly(lactic acid), PLA, is a currently used biodegradable synthetic polymer that has been approved by FDA for various biomedical applications [6–9]. So far, there are many studies focused on reinforcing the structure of natural polymer-apatite composites by physical blending with poly(lactic acid) solution [10–12]. Certain methods have been applied for preparing composite scaffolds based on poly(lactic acid), chitosan and calcium phosphate with different pore range. Layer-by layer self-assembly approach was applied to achieve better uniformity in mineralization of calcium phosphate crystals [13]. It was shown that the pre-coating of poly(lactic acid) fibres with chitosan prior to SBF mineralization can ensure nucleation sites for apatite formation and more biologically active composite. Homogeneous nanocomposite hydroxyapatite/poly(lactic-acid)-chitosan system can be obtained by *in situ* precipitation method inducing multi-order template effect, as previously done by Cai

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http://dx.doi.org/10.1016/j.polymer.2016.06.030
0032-3861/

et al. [14]. They have indicated that poly(lactic acid) plays a very important role in the control of the special structure and the enhancement of the mechanical properties of the composite scaffold.

Macroporous scaffolds are of great importance for hard tissue regeneration. Thermoplastic properties of poly(lactic acid) allow formation of three-dimensional structures by 3D printing technologies with defined pore size and shape for suitable cell environment [15]. Such printed structures show significantly better compressive strength with respect to those produced by techniques such as solvent-casting and particle leaching, freeze-extraction or gas foaming [16]. However, poly(lactic acid) is well known by its long-term degradation and inflammation after 12 months of implantation in *vivo* [17]. Incorporation of calcium phosphates can inhibit inflammation at the first stage of regeneration, but postponed reaction is still observed. Combination of amino polysaccharide could be an alternative for attenuating negative poly(lactic acid) behaviour *in vivo*.

The aim of this research is to produce a three-component system with suitable microstructure, hydrophilicity, biocompatibility, bioactivity and mechanical properties for bone tissue defect repair combining freeze-gelation and 3D printing technology. Our previous studies [18,19] have indicated highly porous chitosan-based scaffolds with 30% weight ratio of *in situ* formed hydroxyapatite as a suitable environment for differentiation of MC3T3-E1 preosteoblasts during 14 days of culture. *In situ* synthesis has provided good dispersion of formed HA particles and formation of non-stoichiometric more bioresorbable hydroxyapatite. High porosity was obtained using freeze-gelation as method for scaffolds production. The following study highlights improvement of mechanical properties of chitosan-based structure applying 3D printed poly(lactic acid) scaffold as a biodegradable construct. To authors' knowledge, this is the first time that vacuum impregnation and freeze-gelation techniques are combined to prepare highly porous chitosan-hydroxyapatite scaffold within a poly(lactic acid) lattice suitable for proliferation and differentiation of human mesenchymal stem cells. Likewise, 3D printing technology was used to fabricate a poly(lactic acid) construct with the largest possible pore diameter for porous hydrogel filling without disrupting the lattice. This way, good biomechanical performance of a potential bone substituent can be accomplished. Moreover, impregnating the poly(lactic acid) lattice with osteoinductive chitosan-based hydrogel will fulfil better biocompatibility and necessary fluid retention, while high interconnected hydrogel porosity will allow easier cell adhesion and migration, and unhindered transport of nutrients and metabolic waste.

2. Materials and methodology

2.1. Materials

Commercial poly(lactic-acid) filaments (PLA) purchased from Plastic2Print®, (The Plastic Enterprise, Netherlands), chitosan (CHT, M_w = 100–300 kg/mol, DD = 0.95–0.98, Acros Organics), calcium carbonate ($CaCO_3$, calcite; TTT), urea phosphate ($(NH_2)_2CO{-}H_3PO_4$; Aldrich Chemistry), acetic acid (HAc; POCH), sodium hydroxide (NaOH, Carlo Erba), ethanol (EtOH, 96%, Kefo), lysozyme (LZ, from chicken egg white, 40,000 U/mg protein; Sigma) and sodium azide (NaN_3, BioUltra 99.5%; Sigma) were all of analytical grade.

2.2. Preparation of scaffolds

2.2.1. 3D printing of poly(lactic acid) scaffold

The poly(lactic acid) scaffold was printed by a fused deposition modelling system (FDM) in a 3D Touch Double Head printer (Bits from Bytes, 3D System). Layer resolution was 125 μm and die diameter was 400 μm.

Through a purpose-built program, by Ikasia Technologies, based on a linked lists structure, scaffolds were printed by a procedure of alternating circular layers with a twist of 90°, a variable displacement on the *z*-axis and no supporting material. The processing parameters were optimized in order to obtain the minimum filament diameter without discontinuities. The scaffold microstructure, particularly the distance between the filaments, were defined by the different variables used by the 3D modelling procedure. Three control parameters had a main importance to obtain the expected results: the hot-end temperature, the distance between points in the 3D model and the spindle speed of the printer.

The final printing procedure had duration of 10 min for each set of four scaffolds, the optimal hot-end temperature was 210 °C and the spindle speed was 55 rad/s. It must be noted that the hot-end temperature is significantly greater than the PLA melting point; however the time that PLA is submitted to this temperature is just a few seconds. Scaffolds with circular shape with 40 mm of diameter of and 5 mm of height were printed. The circular base was selected in order to enhance structural resistance and reducing printing time.

2.2.2. In situ synthesis of hydroxyapatite in chitosan solution

Chitosan-hydroxyapatite (CHT-HA) suspension with 30% weight ratio of hydroxyapatite was prepared by *in situ* precipitation reactions. Firstly, 1.2% of chitosan solution was prepared by dissolving chitosan powder into 0.36% of acetic acid solution. Then, specific amount of calcite was suspended in prepared chitosan solution to obtain 30% of final HA. Appropriate amount of urea-phosphate with respect to Ca/P ratio of 1.67 was then added. Reaction was performed for 4 days at 50 °C. As a control, 1.2% pure chitosan (CHT) solution was prepared with the same acetic acid concentration.

2.2.3. Preparation of poly(lactic acid)/chitosan-hydroxyapatite porous scaffold

Porous composite scaffolds were obtained by freeze-gelation technique. Previously printed poly(lactic acid) scaffolds with circular shape were cutted to defined dimensions (4–5 × 4–5 × 2–4 mm) and washed in 96% ethanol to remove possible surface impurities and dried at atmospheric conditions. Then, scaffolds were impregnated with CHT-HA suspension under vacuum (p = 0.11 bar) for 20 min by impregnation pump (CitoVac, Struers). After impregnation, scaffolds were remained in suspension for another 30 min. Impregnated scaffolds were frozen at −22 °C for 18 h and subsequently immersed into gelation medium consisted of 1 mol/dm^3 aqueous NaOH solution and ethanol (volume portions 1:1, pH = 12.9) at −22 °C for 12 h. After gelation, scaffolds were immersed into ethanol at −22 °C for 12 h and refreshed with new ethanol at room temperature for 24 h. Finally, scaffolds were dried at atmospheric conditions and cut from the matrix to obtain porous PLA/CHT-HA and PLA/CHT scaffolds, respectively (Fig. 1). The same protocol was also performed with CHT-HA suspension and CHT solution to obtain CHT-HA and CHT sponges as control materials for composition characterization.

2.3. Characterization of scaffolds

The Fourier transform infrared spectra (FTIR) of scaffolds were recorded by attenuated total reflectance (ATR) spectrometer for solids with a diamond crystal (Bruker Vertex 70) at 20 °C over the spectral range of 4000–400 cm^{-1}, with 16 scans and 4 cm^{-1} of resolution.

Mineralogical composition of prepared scaffolds was investigated by X-ray diffraction analysis (XRD) using a Shimadzu XRD-