



Enhanced cell affinity of PHBHHx composite scaffold with polylactide-graft-hydroxyapatite as compatibilizer

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ABSTRACT

Poor interfacial bonding and liability to aggregation were the major obstacles for designing of composite scaffolds. Herein, hydroxyapatite (HA) surface-grafted with poly (L-lactide) via ring-opening polymerization was prepared and introduced into PHBHHx-based complex scaffold serving as oligomer compatibilizer. The physico-chemical properties, including superficial roughness and wettability, mechanical strength, as well as *in vitro* bio-activity were systematically investigated. Improved cellular response was acquired owing to the favourable uniformity and interfacial compatibility, as well as the decreased contact angle and elevated roughness, through surface-modification strategy. Moreover, some of the osteogenic marker genes (COL I, Runx2, OCN and OPN) were up-regulated, thus made for the osteogenic differentiation. Our study extrapolated that the PHBHHx-based composite scaffold combined with surface-graft HA compatibilizer might be a potential candidate for bone repairing.

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1. Introduction

As a member of naturally occurring biodegradable poly-hydroxyalkanoates synthesized by bacteria, poly (3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx) has attracted much interest as biomaterials [1] for tissue regeneration due to its regulable physical properties, biodegradability and good biocompatibility. Nevertheless, it is necessary to improve the poor hydrophilicity and lack of cell recognition sites of PHBHHx, which lead to deficient cell affinity. Furthermore, the mechanical strength and toughness of PHBHHx are lower than those of natural cortical bones. Thus many effects have been made to ameliorate cell affinity [2] and mechanical performance [3]. One of the promising approaches to overcome these problems is introducing hydrophilic inorganic fillers into the aliphatic polyester.

Hydroxyapatite (HA, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$), the primary mineral constituent of natural bone and teeth, has been widely used in bone surgery, dentistry and athroplasty [4–6]. Particularly, HA particle is one of the most popular inorganic fillers for preparation of inorganic/organic composite biomaterials, endowing the osteoconduction, hydrophilicity, and mechanical strength especially in bone repairing. However, the liability to aggregation and poor interfacial bonding with polymer

matrix reduce its mechanical and biological properties. In order to resolve this problem, many methods have been reported in recent studies. Surface modification is an efficient way to improve the superficial activity, so as to achieve specific functionality [7]. A variety of investigations have been reported for methodologies such as the adsorption of carboxylic acid [8], isocyanate [9], and sulfonate [10] on surface of HA particle. Meanwhile, some researchers have also reported the surface modification of HA with alkyl phosphate [11], hexanoic and decanoic acids [12], and hexamethyldisilazane [13] for the purpose of obtaining better scattering as a filler in polymer matrix. Also, silane coupling agent [14], zirconyl salt [15], polyacid [16], and poly(ethylene glycol) [17] have also been applied in modification of HA. Recently, Chen and partners [18] modified the surface of HA particle by ring-opening polymerization of lactide. The results indicated that the modified HA particles dispersed more uniformly and exhibited better mechanical properties in polylactic acid (PLA) matrix compared with unmodified HA. In addition, rapid mineralization and osteoconduction were obtained in modified groups when it was used *in vivo* implantation to repair radius defect of rabbit [19,20]. Nevertheless, the mechanism of the cellular response to the surface-grafted composite scaffold has not been revealed clearly.

In this work, PLLA-graft-HA nanoparticles (denoted as g-HA), which was synthesized via ring-opening polymerization of L-lactic acid, was incorporated into PHBHHx (abbreviated as PHB) to fabricate PHB-based composite scaffold. Herein, g-HA was not only one of the bone-like components, but also acted as compatibilizer to bridge the polymeric matrix and inorganic nanoparticles. The physicochemical properties,

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in vitro bioactivity, as well as cell affinity were systematically investigated. Furthermore, the representative osteogenic genes expression were explored to decipher the effect of g-HA on specific cellular behaviors. It was assumed that the improved compatibility and modified surface properties of PHB-based composites, which originated from the incorporation of g-HA compatibilizer, can result in better physical properties, cell affinity and osteogenesis. Therefore, the introduction of surface-graft oligomer was an expectative strategy for promoting bone regeneration.

2. Materials and methods

2.1. Materials

PHBHHx containing 12% 3-hydroxyhexanoate (HHx) ($M_w = 535,000 \text{ g mol}^{-1}$) was purchased from Shantou Lianyi Biotech Company (Guangdong, China). HA was synthesized in our laboratory [21] with an acicular crystal of about 100 nm in length and 20–40 nm in width, and the Ca/P ratio of 1.67. Nanoparticles were conducted at 120 °C in vacuum desiccation for 24 h before use. L-lactide was obtained from Sigma-Aldrich (USA) and recrystallized twice from ethyl acetate before use. Xylene and ethyl acetate were dried over sodium and calcium hydride, respectively. Stannous octoate was purchased from Sigma-Aldrich (USA). Other reagents were of analytical grade and used as received.

2.2. Preparation of porous composite scaffolds

As previously reported [18], g-HA was synthesized *via* ring-opening polymerization, which was initiated by hydroxyl groups on the surface of HA nanoparticles and L-lactide was surface grafted on HA. Briefly, 2 g L-lactide was dissolved in 10 mL xylene at 120 °C under inert argon. 2 g HA was suspended in 20 mL dried xylene together with 0.002–0.02 mL $\text{Sn}(\text{Oct})_2$ catalyst in a *schlenk* tube. The suspension was heated to 90 °C and dropped into the L-lactide solution under nitrogen protection with stirring. After the reaction continued at 120 °C for 18 h, mixture was cooled down to room temperature. The g-HA particles were separated by centrifugation at 20,000 rpm and washed with excessive chloroform for five times to completely remove the free poly (L-lactide) that did not graft on the surface of HA particles. Finally, the separated sediment was vacuum dried at 50 °C overnight. According to the thermal gravimetric analysis (TGA), the amount of surface-grafted polymer was about 5.0 wt%.

The composite porous scaffolds, whose composition was shown in Table 1, were fabricated with the solvent casting/particulate leaching method. To commence, various proportion of nanoparticles were pre-suspended in a certain volume of chloroform and mixed into a 10% PHB/chloroform (w/v) solution with stirring overnight and ultrasonic treatment for 30 min. The trisodium citrate particles with 100–350 μm in diameter were mixed into PHB solution with 1:9 mass ratio of PHB composites/trisodium citrate (w/w). The mixture was cast in glass disks and dried in the air for 3 days. After solvent evaporation in air at room temperature, the scaffolds were removed to dry under vacuum

for 48 h to totally evaporate the solvent. Trisodium citrate particles were subsequently removed from the composites by leaching method. Briefly, scaffolds were soaked in distilled water for 3 days with water changed every 12 h and then dried under room temperature.

The same procedure was conducted for the composite films, which were used in contact angle and surface roughness measurements. In short, the suspending mixture composed of PHB and various proportions of HA or g-HA nanoparticles in chloroform solution (1 wt%) was casted into a Petri dish. After solvent evaporation in air at room temperature, the film was removed to dry under vacuum for 48 h.

Obtained composite scaffolds were cut into small bars ($2 \times 1 \times 0.3 \text{ cm}^3$), and the films were then cut into a certain shape to fit 24-well plate. All of samples were sterilized by irradiation with ^{60}Co of 25 kGy before use.

2.3. Characterization of composite scaffolds

2.3.1. Morphological observation and porosity determination

The morphology of porous composite scaffolds and the distribution of particles in PHB matrix were studied under a scanning electron microscope (SEM). Cross-sections of the composite scaffolds were obtained by immersion in liquid nitrogen for 10 min and subsequent transverse rupture of the samples. After sputter-coating with Au/Pd for 40 s, samples were observed by a Philips XL30 ESEM FEG (Japan) Instrument.

The porosity values of the scaffolds were measured by *Archimedes principle* according to a published method [22,23]. Briefly, the sample was immersed in a graduated cylinder containing a known volume (V_1) of ethanol, and a series of brief evacuation–repressurization cycles were conducted to force the ethanol into pores of the sample. Cycles were continued until no air bubbles were observed emerging from the sample. The total volume of ethanol and the ethanol-impregnated scaffold was then recorded as V_2 . The ethanol-impregnated sample was removed from the cylinder carefully and the residual ethanol volume was recorded as V_3 . The volume of the ethanol held in the foam was $V_1 - V_3$, which was determined as the void volume of the foam. The total volume of the foam was $V_2 - V_3$. Thus the open porosity of the sample was obtained by:

$$\varepsilon = (V_1 - V_3) / (V_2 - V_3) \times 100\% \quad (1)$$

2.3.2. Contact angle and surface roughness

Water contact angle of the films was measured at 25 °C with a contact angle meter (SL600, Solon Information Technology Co. Ltd., Shanghai, China) [24]. 10 μL distilled water was gently dropped onto the surface of a film and the measurement time was <10 s. At least three measurements on different film locations were averaged for data analysis.

Surface roughness of the films was investigated by tapping mode imaging with $50 \times 50 \mu\text{m}^2$ on atomic force microscopy (AFM, SPA-300HV, SIINT, Japan). A cantilever was oscillated at its resonant frequency and scanned across the sample surface. The data were obtained and the probed peak height offered the atomic sensitivity.

2.3.3. Mechanical properties

Dumbbell-shaped samples with effective dimensions of $50 \times 4 \times 1 \text{ mm}^3$ were prepared by press-molding under 10 MPa pressure at room temperature. Tensile test for these samples was carried out after 24 h, aiming at eliminating residual stress. Measurements were conducted on an Instron 1121 machine at a crosshead speed of 1 mm/min. The average tensile strength and modulu data were both obtained by averaging over five samples.

Compressive test was implemented on a Lloyd tensile instrument (Model M30 K) at the crosshead speed of 1 mm/min. The dimension of the scaffolds was 10 mm in diameter and 10 mm in thickness.

Table 1
The composition of composite porous scaffolds.

Group	Composition		
	PHBHHx	HA	Graft-HA
PHB	100%		
5 HA/P	95%	5%	
10 HA/P	90%	10%	
5 g-HA/P	95%		5%
10 g-HA/P	90%		10%
20 g-HA/P	80%		20%

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