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Fabrication and biocompatibility of poly(L-lactic acid) and chitosan composite scaffolds with hierarchical microstructures

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ABSTRACT

The scaffold microstructure is crucial to reconstruct tissue normal functions. In this article, poly(L-lactic acid) and chitosan fiber (PLLA/CTSF) composite scaffolds with hierarchical microstructures both in fiber and pore sizes were successfully fabricated by combining thermal induced phase separation and salt leaching techniques. The composite scaffolds consisted of a nanofibrous PLLA matrix with diameter of 50–500 nm, and chitosan fibers with diameter of about 20 μm were homogenously distributed in the PLLA matrix as a microsized reinforcer. The composite scaffolds also had high porosity (>94%) and hierarchical pore size, which were consisted of both micropores (50 nm–10 μm) and macropores (50–300 μm). By tailoring the microstructure and chemical composition, the mechanical property, pH buffer and protein adsorption capacity of the composite scaffold were improved significantly compared with those of PLLA scaffold. Cell culture results also revealed that the PLLA/CTSF composite scaffolds supported MG-63 osteoblast proliferation and penetration.

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1. Introduction

Scaffolds play an important role in tissue engineering as extracellular matrix to enhance cell attachment, proliferation, and migration, leading to reconstruct tissue normal functions [1–3]. To this end, many natural and synthetic polymers were used to fabricate scaffold. Among them, poly(L-lactic acid) (PLLA), poly(caprolactone), collagen, chitosan, alginate have been widely studied and proven to be promising candidates for scaffold materials [4–8]. For instance, Papenburg et al. fabricated a nanofibrous PLLA scaffold with fiber diameter in the range of 50–500 nm by phase inversion method, which provided good cell adhesion and proliferation for culturing of C2C12 pre-myoblasts [9]. However, pure PLLA nanofibrous scaffold has its limitations on lack of biologically active moieties [10], acidic degradation products [11], and relatively low mechanical properties [12,13]. Jiao et al. reported that coating chitosan on a PLLA fibrous scaffold could improve its mechanical strength and biocompatibility [14]. Zhang et al. braided PLLA and chitosan fibers made from wet-spinning into a fabric slice, and found that the mixed fabrics could slow down the degradation rate and provide excellent adhesion for osteoblast [15]. These reports indicated that PLLA and chitosan composite scaffolds in different combinations might be potential candidates for tissue engineering.

Besides the materials, the scaffold morphology and microstructure, including fiber size, porosity, and pore size, are important for the function of scaffold [13,16–19]. Previous studies confirmed that scaffolds with nanoscale architecture and high surface area facilitated cell normal functions and nutrient transportation [17,20–25]. However, the nanosized scaffolds are limited by low mechanical properties and poor cellular penetration due to the pore size normally smaller than several microns [11, 13,16,26]. These limitations could be overcome with a novel hierarchical structure. On the one hand, introducing the microsized fibers into nanosized scaffolds would significantly enhance the scaffold [27,28]. On the other hand, the nanosized scaffolds with micropores could co-exist with macropores with a pore diameter around several hundreds of micrometers, which are suitable for cell penetration, neovascularization and nutrient transportation [13,29–31]. So, fabrication of porous scaffolds with hierarchical microstructures is a promising challenge [32,33].

In this study, PLLA/chitosan fiber (PLLA/CTSF) composite scaffolds with hierarchical fiber and pore sizes were fabricated by combining thermally induced phase separation (TIPS) and salt leaching (SL) techniques. We hypothesized that the composite scaffolds with tailoring chemical composition and hierarchical microstructures would provide excellent biocompatibility, enhanced mechanical properties, and improved cell proliferation and penetration.

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2. Experimental section

2.1. Materials

PLLA with inherent viscosity of 1.6 dL/g was supplied by Shandong Medical Appliance Company, China. According to supplier's specification, the molecular weight of PLLA is 56,000. Chitosan fibers with diameter of 20 μm (95% deacetylated), prepared by solvent spin method, were provided by Shanghai Tianqing Biomaterials Ltd. Sodium chloride and tetrahydrofuran (THF) were purchased from Qingdao Sanhe Chemicals and used as received.

2.2. PLLA/CTSF composite scaffold preparation

The PLLA/CTSF composite scaffold was prepared by combining TIPS and SL method. Briefly, A PLLA solution of 5% (w/v) was first made in THF at 50 °C in a seal flask. Chitosan fibers, cut into short fiber with length of 2 mm, were gradually added into the PLLA solution, while being stirred by magnetic stir bar. The mixed solution was poured into a glass tube (diameter = 12 mm, length = 75 mm). Pre-sieved sodium chloride particles (50–300 μm) were slowly added into glass tube until the same level of PLLA/CTSF mixed solution. The glass tube was transferred into a freezer and quenched at −18 °C for at least 6 h. The casted sample was taken out from the glass tube and deionized water was used for salt leaching and solvent exchange at 4 °C for 5 days. The samples were refrozen at −18 °C, lyophilized in a freeze-dryer (FD-1C-50, China), and cut into discs with thickness of 2.0 mm after fully dried. Pure PLLA scaffolds without chitosan fibers were fabricated with the same procedures mentioned above as controlled samples.

2.3. Characterization of composite scaffold

The morphology of PLLA/CTSF composite scaffold was visualized using a JEOL JSM-6390LV scanning electron microscopy (SEM). Samples were sputter-coated with Au at 18 mA for 120 s before imaging.

The porosity of the composite scaffolds was measured by liquid displacement method. Detailed procedures were described in our previous paper [34]. In the experiment, 70% ethanol was used as displacement liquid because it penetrates easily into the composite scaffold.

The compressive modulus tests were determined by a universal electronic mechanical testing machine (DXLL-50,000, Denjie Instrument, Shanghai). The cross speed was set at 0.5 mm/min. The stress-strain were recorded until scaffolds were compressed to 50–60% of their original thickness. The compressive modulus of the scaffolds was calculated from the tangent slope of the stress–strain curves.

2.4. In vitro degradation

Scaffolds were sterilized with 70% ethanol and washed with normal saline 3 times before the degradation test. In vitro degradation of the PLLA/CTSF composite scaffolds was performed in normal saline with temperature controlled at 37 °C. The pH value was monitored at certain time interval.

2.5. Protein adsorption

Protein adsorption was determined by the method of Bradford [35]. Briefly, the weighted scaffolds were firstly immersed in ethanol for 30 min, and washed by phosphate buffered saline (PBS) thoroughly. Then each scaffold was incubated in a glass vial with 3 mL FBS/PBS solution (2.0% fetal bovine serum in 0.1 mol/L PBS, pH = 7.4) at 37 °C. The concentration of the protein in the FBS/PBS solution was measured with a commercial protein assay kit (Nanjing Jiancheng Biomed Ltd, China). The amount of proteins adsorbed was calculated by subtracting the amount of proteins left in the FBS/PBS solution after adsorption from the amount of proteins in control FBS/PBS solution (without sample) under the same incubation conditions.

2.6. Cell culture

Scaffolds were firstly sterilized with 70% ethanol and washed thoroughly with PBS before cell seeding. The selected scaffolds were initially seeded with 50,000 MG-63 (ATCC) in 50 μL and incubated for 2 h to allow cells to attach with the composite scaffolds, then the remaining 2 mL media was added to the well. The culture media used were DMEM with 10% fetal bovine serum (FBS, Invitrogen) and 1% penicillin–streptomycin (Invitrogen). The samples were cultured in a humidified incubator at 37 °C with 5% CO_2 atmosphere for 8 days with media change every two days.

2.7. Cell proliferation

MG-63 proliferation on the PLLA/CTSF composite scaffolds was determined by AlamarBlue colorimetric assay (Invitrogen). Briefly, at the end of the culture period, scaffolds were transferred to new well plates, washed gently with PBS, and incubated with 10% (v/v) AlamarBlue in DMEM with 10% FBS for 2 h at 37 °C. After incubation, fluorescence of AlamarBlue solution was determined by spectrophotometer (SpectraMax M2e, Molecular Devices) at 560 nm excitation and 590 nm emission in an opaque 96-well culture plate. The cell number was calculated from the determined fluorescence by a predetermined linear equation calibrated by the known cell numbers and their fluorescence.

2.8. Cell morphology

The cell morphology in the PLLA/CTSF composite scaffolds was analyzed by SEM imaging. Briefly, after 8 days of culture, samples were removed from media, rinsed with PBS, and fixed with Karnovsky's fixative for 12 h. Then fixed samples were rinsed with DI water and dehydrated using a water/ethanol gradient (water/ethanol ratio: 90:10, 75:25, 50:50, 25:75, 10:90, and 0:100) for 15 min at each concentration, and then critical point CO_2 dried using a Hitachi HCP-2 (Hitachi, Tokyo, Japan). Samples were vertically cross-sectioned into thin layer and sputter-coated with Au at 18 mA for 120 s, and imaged by SEM (JEOL JSM-6390LV).

2.9. Statistical analysis

The porosity, compressive modulus, and cell proliferation data were presented as the mean ± standard deviation. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Student's *t*-test. p values <0.05 were considered statistically significant.

3. Results and discussion

3.1. Scaffold morphology

The prepared PLLA/CTSF composite scaffolds had hierarchical microstructures both in the view of fiber and pore sizes. Fig. 1 showed the representative hierarchical microstructure of PLLA/CTSF composite scaffolds (100:20, w/w). As shown in the SEM images, the composite scaffolds were highly porous structure with macropores in the range of 50–300 μm (Fig. 1a) and micropores in the range of 50 nm–10 μm (Fig. 1c), which were formed by the SL and TIPS methods, respectively. All the macropores and micropores were highly interconnective, which benefit the cell migration and nutrient transportation. The PLLA nanofibers, diameter in the range of 50–500 nm, were the matrix of the composite scaffold (Fig. 1c), while the chitosan fibers, diameter around 20 μm, were homogenously dispersed in the PLLA matrix as reinforcer (Fig. 1b). The advantage of such hierarchical fiber size would not only

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