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Silk scaffolds with gradient pore structure and improved cell infiltration performance

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ABSTRACT

Electrospun scaffold with three-dimensional (3D) geometry and appropriate pore structure is an important challenge to mimic natural tissues such as skin, cartilage, etc. In this work, 3D silk fibroin (SF) electrospun scaffolds with gradient pore size were prepared by combining multi-step electrospinning with low temperature (LTE) collecting. The LTE electrospun scaffolds achieved 3D macro-structure with large pore size. The effects of relative humidity (RH), collecting temperature on the morphology of the scaffolds were investigated by scanning electron microscopy and computed tomography. The pore size of the scaffolds was tailored by adjusting SF concentration, electric field, flow rate, needle gauge and collector temperature during electrospinning at 50% RH. L929 cell infiltration results of the scaffolds showed that conventional electrospun scaffolds with small pore size (average diameter 5.9 ± 1.4 μm) restrained cell proliferation and infiltration. On the contrary, LTE electrospun scaffolds with medium pore size (average diameter 11.6 ± 1.4 μm) improved cell proliferation obviously. Large pore size scaffolds (average diameter 37.2 ± 12.9 μm) was beneficial to cell infiltration depth in the thickness direction of the scaffolds. The scaffolds, which were integrated with layers of small, medium and large pores, are promising in the repair of tissue with gradient pore structures.

1. Introduction

Three-dimensional scaffolds with gradient pore structures play a very important role in tissue engineering such as skin [1], vessel [2], esophagus [3], bladder [4], and cartilage [5]. Multiscale scaffolds well mimic natural extracellular matrix (ECM), which has the obvious hierarchical organization from nanoscale to macroscale [6]. Thus it is crucial to control and modulate the scaffold architecture at the micro and nanoscale for the development of biomimetic tissue engineering scaffolds [7].

Pore geometry including pore size, porosity, tortuosity, and interconnectivity is frequently the focus for cell attachment, migration, growth, as well as nutrient transport in tissue scaffolds [8–11]. The optimal pore size for cell attachment, proliferation, and migration varies from 5 to 500 μm across cell types instead of a constant [12]. Lower porosity, as well as smaller pore size than cell's diameter may limit cellular ingrowth, nutrient diffusion and waste product removal. Too large pores provide small specific surface area and poor cell attachment [13]. It has been reported that two or more pore size distributions of a scaffold facilitate the culture of several cell types to produce multiple interfaces on a single scaffolds [14]. Furthermore, scaffolds with gradient pore size enhance the osteogenic differentiation of human mesenchymal stromal cells and optimize bone regeneration [14,15]. Structural gradients have been found in human body mainly at the interface between tissue such as osteochondral, and dermis [16,17]. Up to date, 3D gradient pore scaffolds have been fabricated via electrospinning [18], lyophilization [17,19], additive manufacturing [15], or combination of the methods [20].

Electrospun scaffolds are able to build blocks from 1D to 3D structure by assembling fibers with diameters ranging from nano- to micrometers as well as wires and tubes [21]. The electrospun fibers show an obvious advantage to some other tissue scaffolds because of the high

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surface-to-volume ratio, and the nanofibrous structure with interconnected pores, similar to ECM [22–24]. However, the relatively small pore and thin mat depth obstruct the cellular infiltration and new tissue ingrowth of electrospun scaffolds. To increase the average pore size of electrospun scaffolds, salt leaching [25], cryogenic electrospinning [26], electrospraying sacrificial PEO-microparticles [27], and pattern collectors [28] have been used, respectively. However, the natural hierarchical structure was mainly ignored in the pore-enlarged electrospun scaffolds. Silk fibroin, a natural structure protein possesses distinctive biological properties, has been used as scaffolds in various regeneration tissue such as skin [29], bone [30], cartilage [31], vascular [32], and urethra [33]. Electrospun mats from regenerated silk fibroin (RSF) aqueous solution have been considered as a promising candidate for mimicking ECM. Moreover, freeze-drying is a versatile technique to tune pore size of scaffolds [34–37] by controlling parameters of ice nucleation and growth [19,26,38].

The objective of this study is to develop RSF scaffolds with 3D gradient pore geometry by combining single-step electrospinning and low temperature collection. As RSF aqueous solution is used for electrospinning, the process is environmentally friendly. The pore size, porosity, and morphology of monolayer were characterized, while cell proliferation and infiltration in RSF scaffolds with different pore sizes were evaluated by in vitro cell culture. A gradient architectural structure was assembled in the scaffolds accordingly. This electrospun scaffolds with gradient pore size are promising for biomimetic human skin construction, full-thickness skin tissue repair, or urethral reconstruction.

2. Experimental

2.1. Materials

Bombyx mori cocoons were purchased from Tongxiang, China. Cellulose dialysis membranes (Molecular weight cutoff 14,000 ± 2000 D) were acquired from Yuanju Co., Ltd. (Shanghai, China). Mouse fibroblasts (L929) were obtained from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Trypsin, Dulbecco Modied Eagle's Medium (DMEM, Gibco Inc.), fetal bovine serum (FBS, HyClone Inc.) and penicillin-streptomycin (Gibco Inc.) were obtained from Jinuo Biomedical Technology co., Ltd. (Hangzhou, China). All other chemicals of analytical grade were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China).

2.2. Silk fibroin solution preparation

B. mori cocoons were boiled twice in 0.5 wt% Na_2CO_3 solution for 30 min to remove sericin from SF. The degummed silk was then dissolved using 9.0 M LiBr aqueous solution (1:10 w/v) at 40 °C for 2 h. After diluted and centrifuged at 3500 rpm for 10 min, the supernatant silk solution was dialyzed against deionized water with the cellulose dialysis membrane for 3 days to remove LiBr. Then silk solutions for further electrospinning were concentrated to 31 wt%, 33 wt% and 37 wt%, respectively, using forced airflow at 5 °C.

2.3. Low temperature electrospinning (LTE) of RSF scaffolds

The low temperature electrospinning setup was shown in Fig. 1, where a stainless steel box with two chambers was used as the electrospun collector. In order to investigate the effects of post-treatment and relative humidity (RH) on the 3-D geometry and the morphology of LTE scaffolds, regular spinning conditions in our previous study were adopted [39]. Briefly, the RSF aqueous solution (33 wt%) was electrospun to fibers at a flow rate of 1.2 mL/h through 20G (0.6 mm) needle nozzle. The experiments were performed at a voltage of 20 kV and a needle tip-to-collector distance of 10 cm. The electrospun samples were collected at the RH of 40 ± 3%, 50 ± 3%, 60 ± 3%, respectively. The LTE setup was used (medium pore layer, MPL in Fig. 1) and the samples were named as MPL-Rgl-RH40 (medium pore layer, regular spinning conditions, RH 40%), MPL-Rgl-RH50 and MPL-Rgl-RH60, respectively. The temperature of the stainless steel collecting plate (thickness: 1 mm) was controlled by liquid nitrogen and measured by a digital display thermometer (Shengtong Instruments Co., Hebei, China).

To prepare scaffolds with different pore sizes, electrospinning parameters were adjusted as shown in Table 1. For small pore layer (SPL) scaffold, no liquid nitrogen was added into the stainless steel box, which was used as a collector of conventional electrospinning process [40]. To fabricate the MPL scaffolds with enlarged pore size and 3D geometry, the fiber diameters were enlarged by adjusting the correlative parameters such as RSF concentrations, flow rates, needle gauge and simultaneously depositing the fibers with ice crystal at about −80 °C. In this case, the upper chamber of the box was full of liquid nitrogen, while the bottom chamber of the box was kept empty to obtain the temperature near −80 °C of the collecting plate. To further increase the void of the scaffolds (large pore layer, LPL), the diameters of fibers were further enlarged by depositing the fibers at higher temperature (circa −50 °C). The temperature of the collector was adjusted by filling liquid nitrogen in the bottom chamber of the box. At the same time, the upper chamber was kept empty. Consequently, a scaffold with three layers whose voids increased from SPL to LPL was assembled at the end of the process.

2.4. Post-treatment of RSF scaffolds

The post-treatment of the scaffolds was performed according to literatures [41,42]. Briefly, the scaffolds were immersed in 90% (v/v) ethanol solution for 30 min, then lyophilized in a freeze-dryer for 12 h or annealed in water vapor at 37 °C and 90% RH for 36 h to ensure the stability in water of RSF scaffolds. MPL-Rgl-RH50 scaffold was used for the study of post-treatment, while all other scaffolds were post-treated by soaked in 90% ethanol solution for 30 min followed by lyophilization.

2.5. Morphology observation of RSF scaffolds

Scanning electron microscopy (SEM, JEOL JSM-5600LV, Japan) was used to observe the morphology of LTE RSF scaffolds at a voltage of 13 kV after sputter-coating with gold. The 3D geometry of LTE scaffolds was characterized with computed tomography (CT) carried out on BL13W1 beam line (energy 25 keV, resolution 9 μm) at Shanghai Synchrotron Radiation Facility. The average diameters of the fibers were measured using image analysis software (UTHSCSA Image Tool 2.0). For each sample, 100 fibers from SEM images were randomly counted.

2.6. Pore size and porosity analysis

The pore size of the scaffolds was obtained based on the SEM images [43]. Briefly, the area of irregular pores of the electrospun scaffolds were recorded and then converted to a circular area. The circular diameter was regarded as the pore diameter. The pore size was analyzed using Image Pro Plus software (IPP6.0). Three randomly selected regions were taken for each scaffolds.

The porosity, ε, was calculated according to the equation,

$$\varepsilon/\% = (1 - \rho_{scaffold}/\rho_{material}) \times 100$$

where $\rho_{scaffold}$ was determined by weighing the samples and measuring their dimensions. $\rho_{material}$ is the density of silk fibroin (1.30 g/cm^3) [44].

2.7. Mechanical properties

The rectangular specimens (35 × 5 mm^2) cut from the SPL, MPL,