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Silk porous scaffolds with nanofibrous microstructures and tunable properties

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ABSTRACT

Scaffold biomaterials derived from silk fibroin have been widely used in tissue engineering. However, mimicking the nanofibrous structures of the extracellular matrix (ECM) for achieving better biocompatibility remains a challenge. Here, we design a mild self-assembly approach to prepare nanofibrous scaffolds from silk fibroin solution. Silk nanofibers were self-assembled by slowly concentrating process in aqueous solution without any cross-linker or toxic solvent and then were further fabricated into porous scaffolds with pore size of about 200–250 μm through lyophilization, mimicking nano and micro structures of ECM. Gradient water/methanol annealing treatments were used to control the secondary structures, mechanical properties, and degradation behaviors of the scaffolds, which would be critical for different tissue regeneration applications. With salt-leached silk scaffold as control, the ECM-mimetic scaffolds with different secondary structures were used to culture the amniotic fluid-derived stem cells *in vitro* to confirm their biocompatibility. All the ECM-mimetic scaffolds with different secondary structures represented better cell growth and proliferation compared to the salt-leached scaffold, confirming the critical influence of ECM-mimetic structure on biocompatibility. Although further studies such as cell differentiation behaviours are still necessary for clarifying the influence of microstructures and secondary conformational compositions, our study provides promising scaffold candidate that is suitable for different tissue regenerations.

1. Introduction

Biomimetic materials are booming areas in biomaterials and tissue engineering, in which mimicking microstructural features of extracellular matrix (ECM) is a feasible way to improve the biocompatibility of different materials [1–3]. In these studies, porous 3-D nanofibrous scaffold fabrication is becoming important since growing studies have confirmed that the nanofibrous structure of ECM is a critical factor in providing suitable micro-environments for cell growth and proliferation [4–8].

Because of their impressive biocompatibility, biodegradability, minimal inflammatory reactions and excellent mechanical properties, silk-based materials have been used in different tissue regenerations such as bone, cartilage, blood vessel, skin and nerve [9–16]. Although silk-based scaffolds hold promise for tissue repairs, a challenge for scaffold fabrication remains to further improve silk biocompatibility, feasibility and inductivity for different tissue regeneration needs [17–21]. The design of silk-based scaffolds with nanofibrous structures is considered as suitable choice for further improving their biocompatibility. Electrospinning is a versatile technique for producing nanofiber-based biomaterials, and has been used to prepare silk fibroin fibrous matrices with diameters from a few micrometers down to the tens of nanometers [22]. Unfortunately, it is still difficult for electrospinning to prepare complex 3-D porous structures that are suitable for tissue regenerations. Lyophilization and salt-leaching are other methods to prepare silk scaffolds, which is powerful for porous structure fabrication but weak for nanofiber assembly [23–29]. In our previous study, collagen was used to induce the self-assembly of silk fibroin to form nanofibers. The porous scaffolds were subsequently achieved through lyophilization [4].

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Then, through controlling the self-assembly process of silk to form nanofibers in aqueous solution, pure silk fibroin scaffolds composed of nanofibers were also prepared by freeze–drying the nanofiber solution [30] and showed better biocompatibility than silk scaffolds without nanofiber structure. However, further studies are still necessary to regulate the mechanical properties and degradation behaviors of the nanofiber scaffolds in order to adapt different tissue regenerations.

Therefore, in our present study, the silk nanofiber scaffolds were treated by improved gradient water–methanol annealing processes to induce silk I and silk II formation. Compared to the salt-leached silk scaffolds, the nanofiber scaffolds containing different silk I and silk II contents showed tunable degradation behaviors, various mechanical properties, and better cell growth *in vitro*, implying better feasibility for different tissue regenerations.

2. Materials and methods

2.1. Preparation of silk solutions

Bombyx mori silk fibroin solutions were prepared according to our previous published procedures [31]. Cocoons were boiled for 20 min in an aqueous solution of 0.02 M Na_2CO_3 and then rinsed thoroughly with distilled water to extract the sericin proteins. After drying the extracted silk fibroin was dissolved in 9.3 M LiBr solution at 60 °C for 4 h, yielding a 20% (w/v) solution. This solution was dialyzed against distilled water using dialysis tube (MWCO 3500) for 72 h to remove the salt. Then the solution was centrifuged at 9000 r min^{-1} for 20 min at 4 °C to remove silk aggregations formed during the process. The final concentration of silk in water was about 6%, determined by weighing the remaining solid after drying.

2.2. Preparation of nanofibrous silk scaffolds

Based on our silk self-assembly study [4,32], silk fibroin nanofibers were assembled by slowly concentrating the fresh solution to 25–30 wt% for 4 days at room temperature in fume hood. The nanofiber solution was diluted to 2.5 wt% with distilled water, and then poured into cylindrically-shaped container. The container was placed at −20 °C for 24 h to freeze the samples and lyophilized for about 72 h to achieve silk porous scaffolds. Modulated water/methanol annealing processes were applied to induce gradual transformations from random to silk I/silk II structures. The scaffolds were placed in desiccators filled with methanol/water blend solutions with a 25 in. Hg vacuum for 6 h to produce water-insoluble scaffolds. Methanol contents in these blend solutions were 0%, 10%, 30% and 50%, respectively. Traditional methanol annealing process also used to increase the β-sheet content through immersing the scaffolds in 80% (v/v) methanol solution for about 30 min [24,25,33].

2.3. Morphology analysis of the scaffold

The nanostructural transition of silk fibroin in aqueous solution was observed by AFM (Veeco, Nanoscope V, NY, America) in air. A 225 μm long silicon cantilever with a spring constant of 3 Nm^{-1} was used in tapping mode at 1.5 Hz scan rate. To prepare the samples for AFM imaging, different SF solutions were diluted to below 0.0001 wt% with deionized water to avoid masking the original morphology by multilayers of silk [34]. Once diluted, 2 μl of the diluted SF solution was quickly dropped onto freshly cleaved $4 \times 4\ mm^2$ mica surfaces and dried under a nitrogen gas. The morphology of the scaffolds was observed using a SEM (Hitachi S-4800, Hitachi, Tokyo, Japan). The specimens were fractured in liquid nitrogen using a razor blade and then sputter-coated with gold prior to imaging. Then the morphologies of the scaffolds were examined using a S-4800 SEM at 3 kV. Since previous study has indicated that the nanoscale architectures of silk fibroin are usually covered by compact surface of the pore walls [4], different scaffolds were incubated at 37 °C in 40 ml phosphate saline (PBS) containing 5 U ml^{-1} protease XIV for 6 h to degrade the compact surface. Then the nano-structural morphologies of the different scaffolds after degradation were examined using a Hitachi S-4800 SEM (Model S-4800, Hitachi, Tokyo, Japan).

2.4. Structural analysis of the scaffolds

The structure of the various scaffolds was analyzed by FTIR on a NICOLET FTIR 5700 spectrometer (Thermo Scientific, FL, USA) equipped with a MIRacle™ attenuated total reflection (ATR) Ge crystal cell in reflection mode. For each measurement 64 scans were coded at a resolution of 4 cm^{-1}, with the wave number ranging from 400 to 4000 cm^{-1} [31,35]. X-ray diffraction (XRD) was also used to determine crystal structure of the scaffolds. The experiments were performed with an X-ray diffractometer (X′Pert-Pro MPD, PANalytical B.V., Holland) using Cu *K*a radiation at 40 kV. Irradiation conditions were at 40 mA and a scanning rate of 0.6° min^{-1} [36].

2.5. Differential scanning calorimetry (DSC)

The thermal properties of the scaffolds were measured in a TA Instrument Q100 DSC (TA Instruments, New Castle, DE) under a dry nitrogen gas flow of 50 ml min^{-1}. The samples were heated at 2 °C min^{-1} from −30 °C to 350 °C [33].

2.6. In vitro enzymatic degradation

Different silk fibroin scaffolds were incubated at 37 °C in 40 ml of phosphate-buffered saline (PBS) containing protease XIV (5 U ml^{-1}). Each solution contained an approximately equivalent mass (40 ± 5 mg) of scaffolds. Solutions were replenished with enzyme and samples were collected daily. At designated time points the samples were rinsed with distilled water and prepared for mass balance assessment [37].

2.7. Mechanical properties

The compression properties of specimens (d = 10 mm, h = 11 mm) were measured with a cross head speed of 2 mm min^{-1} at 25 °C using an Instron 3366 testing frame (Instron, Norwood, MA) with a 10 N capacity load cell. The mechanical properties of the scaffolds were determined in wet conditions. For the wet conditions, the scaffolds were first hydrated in water for 2 h and then measured at 25 °C with a cross head speed of 2 mm min^{-1}. All samples were measured in triplicates [10].

2.8. Cell culture

Considering amniotic fluid-derived stem cells with a capacity to differentiate into multiple cells have been used in different tissue regeneration applications, and could avoid ethical concerns [38–41], amniotic fluid-derived stem cells were used to evaluate the cytocompatibility of the scaffolds. Samples of amniotic fluid were obtained from the First Affiliated Hospital of Soochow University (Suzhou, Jiangsu, China) following routine amniocentesis carried out on pregnant women at 15 to 35 weeks of gestation. All the procedures were performed following the guidelines established by the First Affiliated Hospital of Soochow University and the First Affiliated Hospital of Soochow University Ethics Committee; a written consent was obtained from each woman to use the amniotic fluid for research purposes. The isolation of