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Silk scaffolds with tunable mechanical capability for cell differentiation

Shumeng Bai [a,b], Hongyan Han [c,1], Xiaowei Huang [a,b], Weian Xu [c], David L. Kaplan [a,d], Hesun Zhu [e], Qiang Lu [a,b,*]

[a] National Engineering Laboratory for Modern Silk & Collaborative Innovation Center of Suzhou Nano Science and Technology, Soochow University, Suzhou 215123, People's Republic of China
[b] College of Textile and Clothing Engineering, Soochow University, Suzhou 215123, People's Republic of China
[c] School of Biology and Basic Medical Sciences, Soochow University, Suzhou 215123, People's Republic of China
[d] Department of Biomedical Engineering, Tufts University, Medford, MA 02155, USA
[e] Research Center of Materials Science, Beijing Institute of Technology, Beijing 100081, People's Republic of China

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ABSTRACT

Bombyx mori silk fibroin is a promising biomaterial for tissue regeneration and is usually considered an "inert" material with respect to actively regulating cell differentiation due to few specific cell signaling peptide domains in the primary sequence and the generally stiffer mechanical properties due to crystalline content formed in processing. In the present study, silk fibroin porous 3D scaffolds with nanostructures and tunable stiffness were generated via a silk fibroin nanofiber-assisted lyophilization process. The silk fibroin nanofibers with high β-sheet content were added into the silk fibroin solutions to modulate the self-assembly, and to directly induce water-insoluble scaffold formation after lyophilization. Unlike previously reported silk fibroin scaffold formation processes, these new scaffolds had lower overall β-sheet content and softer mechanical properties for improved cell compatibility. The scaffold stiffness could be further tuned to match soft tissue mechanical properties, which resulted in different differentiation outcomes with rat bone marrow-derived mesenchymal stem cells toward myogenic and endothelial cells, respectively. Therefore, these silk fibroin scaffolds regulate cell differentiation outcomes due to their mechanical features.

1. Introduction

Tissue engineering has been stimulating the development of biomaterial scaffolds with tuned properties to satisfy the requirements of specific tissue regeneration, and to actively regulate cell behaviors [1–5]. Aside from specific peptide epitopes, scaffold morphology and topology, crystallinity and stiffness are among the parameters that influence cell behavior, extracellular matrix production and functional tissue reconstruction [6–10]. Recently, silk fibroin, a fibrillar protein, has attracted attention for support matrices for stem cells, nerve cells, fibroblasts, osteoblasts and other cell types, as well as scaffolds for many different types of tissues for engineering, including skin, nerve, bone, cartilage and blood vessels [11–15]. These studies were prompted in part due to the impressive biocompatibility, unique mechanical properties, tunable biodegradability and minimal inflammatory reactions with silk fibroin [16–20].

Biomimetic nanostructures are an emerging strategy in designing scaffolds with better biocompatibility [21–25]. Electrospinning, an effective way to prepare biomimetic nanofibrous structures was used to generate silk fibroin sheets and tubes [26–29], but is a difficult approach to generate scaffolds with microporous structures (pore size > 100 μm) and sufficient thickness for bone, cartilage, liver or muscle regeneration as examples [30,31]. Other processing options, such as salt leaching and freeze-drying, could be used to design complex three dimensional (3-D) microporous silk fibroin scaffolds [32–34], but do not generate biomimetic nanostructures.

Different approaches and post-treatment processes have been developed to control the crystallinity of silk fibroin scaffolds [35–37]. Although some improvements were achieved, softening the water-insoluble scaffolds to match the requirements of different soft tissues is difficult since the high crystallization, a prerequisite for water-insolubility of silk fibroin scaffolds, usually results in higher stiffness [35,36]. Therefore, silk fibroin scaffolds are once considered "inert" matrices with respect to directing cell differentiations [8]. Recently, a self-assembly mechanism to

* Corresponding author at: National Engineering Laboratory for Modern Silk & Collaborative Innovation Center of Suzhou Nano Science and Technology, Soochow University, Suzhou 215123, People's Republic of China. Tel.: +86 512 67061649.
E-mail address: Lvqiang78@suda.edu.cn (Q. Lu).
[1] The author has same contribution with the first author.

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control silk fibroin nanofiber formation in aqueous solution was reported [38,39] and 3-D porous silk fibroin scaffolds composed of nanofibers were successfully prepared using a lyophilization process [40–42]. However, the nanofibrous architectures are usually occluded, thus failing to directly interact with cells seeded on these matrices. Post-treatment processes such as methanol annealing were required to achieve water insolubility of the scaffolds, causing an increase in stiffness [41,42]. Silk fibroin nanofibers with high beta-sheet structure were prepared in our recent study [43]. Unlike previous water-insoluble silk fibroin materials with high beta-sheet content [33,41], these nanofibers could be homogeneously dispersed in water to form stable solutions, thus serving an inducer of silk fibroin nanostructures with control of conformational transformations.

Therefore, in the present study, silk fibroin nanofibers were used to induce nanofiber growth and conformational transformations in fresh silk fibroin solutions to generate biomimetic nanofibrous/microporous silk fibroin scaffolds with tunable stiffness. The insoluble regenerated silk fibroin scaffolds were prepared directly from aqueous solution without the use of any additives or post-treatments, avoiding higher stiffness and higher crystallinity formation. Scaffold stiffness and nano-topography could be tuned by changing the nanofiber content in the fresh solution before lyophilization, and then provide stimulating cues to actively influence stem cell growth and differentiation so that silk fibroin is a useful option for soft tissue studies.

2. Experimental section

2.1. Preparation of aqueous silk fibroin solutions

Silk fibroin solution was prepared according to our previously described methods [38]. *Bombyx mori* 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. The degummed silk was dissolved in 9.3 M LiBr solution at 60 °C, yielding a 20% (w/v) solution. This solution was dialyzed against distilled water, using a dialysis tube (MWCO 3500, Pierce, Rockford, IL, USA) for 72 h to remove the salt. The solution was optically clear after dialysis and was centrifuged at 9000 rpm for 20 min at 4 °C to remove silk fibroin aggregates formed during the process. The final concentration of aqueous regenerated silk fibroin solution was about 6 wt.%, determined by weighing the remaining solid after drying.

2.2. Preparation of silk fibroin nanofiber solutions

The silk fibroin nanofiber was assembled as reported in our recent study [43]. Fresh silk fibroin solution was treated by a concentration-dilution process. The solution (6 wt.%) was slowly concentrated to about 20 wt.% over 24 h at 60 °C to form metastable nanoparticles, and then diluted to 0.5 wt.% with distilled water. The diluted silk fibroin solution was incubated for above 24 h at 60 °C to induce the nanofiber formation.

2.3. Preparation of silk fibroin scaffolds

Silk fibroin scaffolds were prepared via a modified lyophilization method. The fresh silk fibroin solution was blended with the silk fibroin nanofiber solution at dry weight ratios (silk: silk nanofiber) of 100: 0, 98: 2, 93.7: 6.3, 88.2: 11.8, and 66.7: 33.3, respectively. The blend silk fibroin solutions were diluted to 2 wt.% with distilled water, and then poured into cylindrically-shaped containers. The containers were placed at −20 °C for 24 h to freeze the samples, which were then lyophilized for about 72 h. After lyophilization the scaffolds were prepared and termed SS-0, SS-2, SS-6.3, SS-11.8, and SS-33.3, respectively. Since the soluble ingredient could be removed within 12 h based on the residual mass (%) results (Fig. 1), the regenerated silk fibroin scaffolds were immersed in distilled water at 37 °C for 12 h to remove any residual soluble silk fibroin to achieve final scaffolds with biomimetic nanostructures and tunable stiffness.

2.4. Morphological structure of the scaffolds

The morphology of the scaffolds was observed using a scanning electron microscopy (SEM, Hitachi S-4800, Hitachi, Tokyo, Japan) at 3 kV. Samples were mounted on a copper plate and sputter-coated with gold prior to imaging. The pore diameters of scaffolds were measured using Image J software. Above 300 different sites for every sample were measured and then the average values were achieved.

2.5. Structural analysis of the scaffolds

The structure of the different scaffolds was analyzed by Fourier transform infrared spectroscopy (FTIR) on a Nicolet FTIR 5700 spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). For each measurement, 64 scans were coded with a resolution of 4 cm^{-1}, with the wavenumber ranging from 400 to 4000 cm^{-1}. Fourier self-deconvolution (FSD) of the infrared spectra covering the amide I region (1595–1705 cm^{-1}) was performed by Peakfit 4.12 software to identify silk secondary structures [44]. FSD spectra were curve-fitted to measure the relative areas of the amide I region components. The crystal structure of the scaffolds was confirmed with X-ray diffraction (XRD, X′ Pert-Pro MPD, PANalytical BV, Almelo, Netherlands) using monochromated CuKa radiation (30 mA, 40 kV) with a scanning speed of 0.6°/min.

2.6. Mechanical properties

The compressive properties of the samples in wet state were measured using an Instron 3366 testing frame (Instron, Norwood, MA, USA) with a 10 N capacity load cell [33,41]. The cylinder-shaped scaffolds (10 mm in diameter and 20 mm in height) were hydrated in water for 2 h and then measured with a cross head speed of 2 mm min^{-1} at 25 °C and 65% RH. The load was applied until the cylinder was compressed by more than 30% of its original length. The compressive modulus was calculated as the slope of the linear region of the stress–strain curve. All samples were measured in triplicate.

2.7. In vitro biocompatibility of the scaffolds

Bone marrow mesenchymal stem cells (BMSCs) derived from Sprague–Dawley (SD) rats were used to evaluate the *in vitro* biocompatibility of the scaffolds via DNA content assay, fluorescence staining and SEM, respectively [40]. The scaffolds were punched into small disks (diameter of 5 mm and height of 2 mm) for 96-well plates and sterilized with ^{60}Co γ-irradiation at the dose of 25 kGy. BMSCs were cultured in Dulbecco's modified Eagle medium (DMEM, low glucose) supplemented with 10% fetal bovine serum (FBS), and 1% IU ml^{-1} streptomycin-penicillin (Invitrogen, Carlsbad, CA, USA). The medium was replaced every 3 days, and the cultures were maintained in a humidified incubator at 37 °C and 5% CO_2. After reaching 90% confluence, cells were detached from Petri dish and seeded into the scaffolds at a density of 1.0×10^5 per well.

The cell morphology on the scaffolds was examined by confocal microscopy. After culture for 1, 6, and 12 days, the cell-seeded scaffolds were washed three times with PBS and fixed in 4%