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Design and Engineering of Silk Fibroin Scaffolds with Biomimetic Hierarchical Structures

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Supporting Information

Materials and methods

1. Material preparation

Aqueous regenerated silk fibroin (RSF) solutions were prepared from *Bombyx mori* silkworm cocoons. Briefly, the degummed silk fibroin fiber was dissolved in a LiBr (9.3 M, Sigma-Aldrich, USA) solution for 1 hr at 40 °C. The resulting solution was dialyzed against deionized water with a cellulose dialysis membrane (MWCO 6000-8000 Da, Spectra/ Por, USA) at room temperature for 3 days to remove LiBr. The dialyzed silk fibroin solution was collected and stored at 4 °C. RSF solutions containing Ca^{2+} ions were prepared by mixing RSF solution (3.5% w/w) with $CaCl_2$ (1 M, Sigma-Aldrich, USA) aqueous bulk solutions to give defined Ca^{2+} ion concentrations (5, 10, 15 and 20 mM), referred to as "Ca5, Ca10, Ca15 and Ca20" respectively.

Porous silk scaffolds containing Ca^{2+} were fabricated by a freeze drying method. 1-butanol solution (10% v/v) was added gradually, with stirring, to a silk fibroin aqueous solution (3.5% w/w) with Ca^{2+} (10 mM) at the predetermined volume ratio ($V_{fibroin}$:$V_{butanol}$=2:1). The mixed solution was poured into a mold and frozen immediately at −20 °C for 12 hrs, then freeze dried at about −85 °C for 48 hrs to obtain the calcium induced hierarchically structured silk fibroin scaffolds (Ca/SFSs). The control pure silk scaffolds without Ca^{2+} ions were prepared by the same method and are referred to as "SFSs".

2. Material characterization

2.1 Aqueous regenerated silk fibroin (RSF) solution monitoring

To examine the influence of Ca^{2+} on RSF, the turbidity changes in silk fibroin solutions were monitored at 550 nm using a Microplate Reader System (ELx800, BioTek Instruments, USA). In addition, the particles sizes in the RSF solutions with different Ca^{2+} ion concentrations were measured by a High Performance Particle Sizer (HPPS) (model HPP5001, Malvern Instruments Ltd., UK). The zeta potential of the silk fibroin molecules was obtained by a Zetasizer Nano ZS Particle Characterization System (Malvern Instruments Ltd., UK).

Circular Dichroism (CD) spectrum of RSF was collected on a Jasco spectropolarimeter, model J-810, with a quartz cell of 2 mm path length. The protein solutions of RSF of different Ca^{2+} concentrations were diluted to 0.1 mg mL^{-1}. All samples were scanned at 25 °C with a 3-s accumulation time at the rate of 50 nm min^{-1} with a response time of 4 s, and the results were averaged from at least three repeated experiments. Two major time-dependent points were identified in the spectra at 199 and 217 nm, attributable to the change of the ratio between random coil and β-sheet conformations, respectively. Protein samples were incubated for 2 hrs at room temperature.

Fluorescence spectroscopy is widely used to interpret protein structure at atomic resolution. The aromatic amino acids, e.g., Trp, Tyr, and phenylalanine (Phe), offer intrinsic fluorescent probes of protein conformation, dynamics, and intermolecular interactions [1]. Accordingly, the fluorescence investigation on RSF presented here aims to describe the details of this large protein structure along with the mechanism of its conformational transition by using Tyr, Trp residues in fibroin. All fluorescence spectra were monitored at room temperature using a Cary Eclipse fluorescence spectrophotometer (Varian, USA). Protein concentrations were diluted to 0.2 and 2.5 mg/mL for Tyr and Trp, respectively. The fibroin samples were excited at 274 nm, and data were recorded between

290 and 400 nm for Tyr, while excitation at 295 nm and data collection between 300 and 450 nm was used for Trp, with a bandwidth of 5 nm for both the excitation and emission monochromators. When protein concentration dependence was determined, all samples were equilibrated at room temperature for 1 hr prior to measurement.

2.2 Silk fibroin scaffolds characterization

The pore microstructure of the prepared Ca/SFSs and SFSs was investigated by a scanning electron microscope (SEM, JSM-6700F, JEOL, Japan). The pore distribution of prepared scaffolds was analyzed from SEM images by using image J software. Before test, the samples were all sputtered with platinum. Fourier transform infrared spectra (FTIR) in attenuated total internal reflection mode (ATR) was performed by using an FTIR spectrometer (Nicolet 380, Thermo Scientific, USA) with a single-reflection diamond crystal. The spectra were recorded with an accumulation of 64 scans and resolution of 4 cm^{-1}.

The compressive strength and modulus of the scaffolds (10 mm × 10 mm × 10 mm) were measured using a universal testing machine (Instron Microtester 5848, USA) at a crosshead speed of 0.5 mm min^{-1} at room temperature. Four samples were used for each type of scaffolds.

The dissolution of calcium ions from the prepared Ca/SFSs into the culture medium is also assessed, which was evaluated by atomic adsorption spectroscopy (AAS). The prepared Ca/SFSs and SFSs were cut into 8 × 8 × 1 mm pieces and placed in 24-well plastic culture plates filled with 1 mL low glucose culture medium containing Dulbecco's modified Eagle's medium (DMEM; Gibco, Invitrogen, CA), fetal bovine serum (10%, HyClone, Logan, UT), penicillin and streptomycin (Invitrogen). The silk scaffolds were incubated in a 5% CO_2 incubator at 37 $^{\circ}C$ for 6 days, and the medium was replaced every 3 days. After soaking for 1, 3, 4 and 6 days, the culture medium was collected. The calcium ion concentrations in the culture medium were measured by an emission flame photometer (Model PFP7, JenWay, England). Liquefied petroleum gas and air were supplied as the source of flame. The flow rate of fuel was adjusted to get the maximal sensitivity. The samples were filtered by a cellulose membrane filter (0.22 μm, Whatman) and diluted with nitric acid (1% w/v) prior to measurements.

3. In vitro culture and evaluation

3.1 Cell seeding and culture on scaffolds

Human bone marrow-derived mesenchymal stem cells (HMSCs) were expanded in low glucose culture medium containing Dulbecco's modified Eagle's medium (DMEM; Gibco, Invitrogen, CA), fetal bovine serum (10%, HyClone, Logan, UT), penicillin and streptomycin (Invitrogen) at 37 °C under 5% CO_2 atmosphere. Medium was changed every 3 days. When HMSCs became near confluent, they were harvested for seeding on scaffolds.

Sterilized scaffolds were cut into 8 × 8 × 1 mm pieces and transferred into 24-well plastic culture plates. A total of 1×10^5 HMSCs suspended in a culture medium (70 μL) were seeded at the centre of each scaffold, and incubated at 37 °C under 5% CO_2 atmosphere for 2 hrs. Then culture medium (1 mL) was added into each well. The HMSC-seeded silk scaffolds were grown *in vitro* in a 5% CO_2 incubator at 37 °C, with the medium being replaced every 3 days until being harvested.

3.2. Cell morphology and proliferation on scaffolds

At the designated time points, the HMSC-seeded scaffolds were washed with PBS twice, fixed with Glutaraldehyde solution (2.5 %) for 2 hrs at 4 °C, and washed by PBS. Then samples were dehydrated in ethanol solutions with gradually increased ethanol concentration. The dried HMSC-seeded scaffolds were coated with Pt and observed by SEM.

Cell viability and proliferation were measured by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT; Sigma, USA) staining. The HMSC-seeded scaffolds, at desired time points, were incubated in MTT solution (5 mg mL^{-1} MTT in cell culture medium) in a 5% CO_2 incubator at 37 °C for 4 hrs. The intense purple colored formazan derivative formed via cell metabolism was eluted and dissolved in dimethylsulfoxide (400 μL $well^{-1}$, DMSO; Merck, Germany). The absorbance was measured at 550nm. Cell number was correlated to optical density (OD).

3.3. Reserve transcription and real-time quantitative RT-PCR analysis

The effect of Ca/SFSs on HMSCs osteogenic differentiation was assessed by reverse transcription-polymerase chain reaction (RT-PCR) to measure the mRNA expression of alkaline phosphatase (ALP), type I collagen (COL1) and osteocalcin (OCN) in all treatment groups. Scaffolds were cut into 8 × 8 × 1 mm pieces and transferred into 24-well plastic culture plates and a total of 1×10^5 HMSCs were placed onto each scaffold. The medium was changed after 24 hrs to osteogenic differentiation medium (high glucose DMEM containing 10% FBS, 50 mM ascorbic acid 2-phosphate, 10 mM β-glycerol phosphate and 100 nM dexamethasone (Sigma Aldrich)). HMSCs cultured on the

scaffolds were harvested after 14 days in culture. The scaffolds seeded with cells were washed with PBS, digested with collagenase (0.25%, Invitrogen) for 3 hrs. The cells were collected by centrifugation, and total RNA was extracted from cells using an RNeasy Mini Kit (Qiagen, USA) following the supplier's instructions. Total RNA is measured using the NanoDrop (Nanodrop Technologies, Wilmington, DE, USA). The RNA samples were reverse transcribed into cDNA according to the manufacturer's protocol (iScript cDNA synthesis Kit, BioRad). RT-PCR was performed in a RT-PCR machine using the QuantiTect RT-PCR Kit (Qiagen, USA). The primer sequences of selected genes for RT-PCR were summarized in Table 1. At least three replicates were performed on each sample. In each run of PCR, human housekeeping gene GAPDH was used as the reference transcript. All cDNA samples were analyzed for the transcript of interest and the housekeeping gene in independent reactions. The Ct value for each sample was defined as the cycle number at which the fluorescence intensity reached a certain threshold where amplification of each target gene was within the linear region of the reaction amplification curves. Relative expression level for each gene of interest was normalized by the Ct value of human housekeeping gene GAPDH using an identical procedure.

4. Statistical Analysis

All data were expressed as means ± standard deviations (SD). The statistical significance of differences among each group was examined by the t-test. Significance was set at $p < 0.05$ level.

References

[1] Y. H. Yang, Z. Z. Shao, X. Chen, P. Zhou, Biomacromolecules, 2004, **5**, 773.

Table 1 Primer pairs used in real-time PCR analysis

Gene	Forward primer	Reverse Primer
ALP	5' TCAGAAGCTCAACACCAACG 3'	5' TTGTACGTCTTGGAGGGC 3'
Col I	5' CTTTGGAGCCAGCTTGGA 3'	5' GTGGGCTTCCTGGTGA 3'
OCN	5' GCAAAGGTGCAGCCTTTGTG 3'	5' GGCTCCCAGCCATTGATACAG 3'
GAPDH	5' GGGCTGCTTTTAACTCTGGT 3'	5' GCAGGTTTTTCTAGACGG 3'

Supplementary Figures and Equations

Equations

$$J = B\exp\left[-\frac{16\pi\gamma_{cf}^{3}\Omega^{2}}{3kT\left[kT\ln(1+\sigma)\right]^{2}}\right] \quad (1)$$

with $$\sigma = (C_i - C_i^{eq})/C_i^{eq} \quad (2)$$

where γ_{cf} is the interfacial free energy between the ambient phase and highly dispersed phase, Ω is the volume per structural unit, k is the Boltzmann constant, B is a constant for a given system, C_i^{eq} and C_i are the equilibrium and actual concentrations of silk fibroin, respectively.

Figures

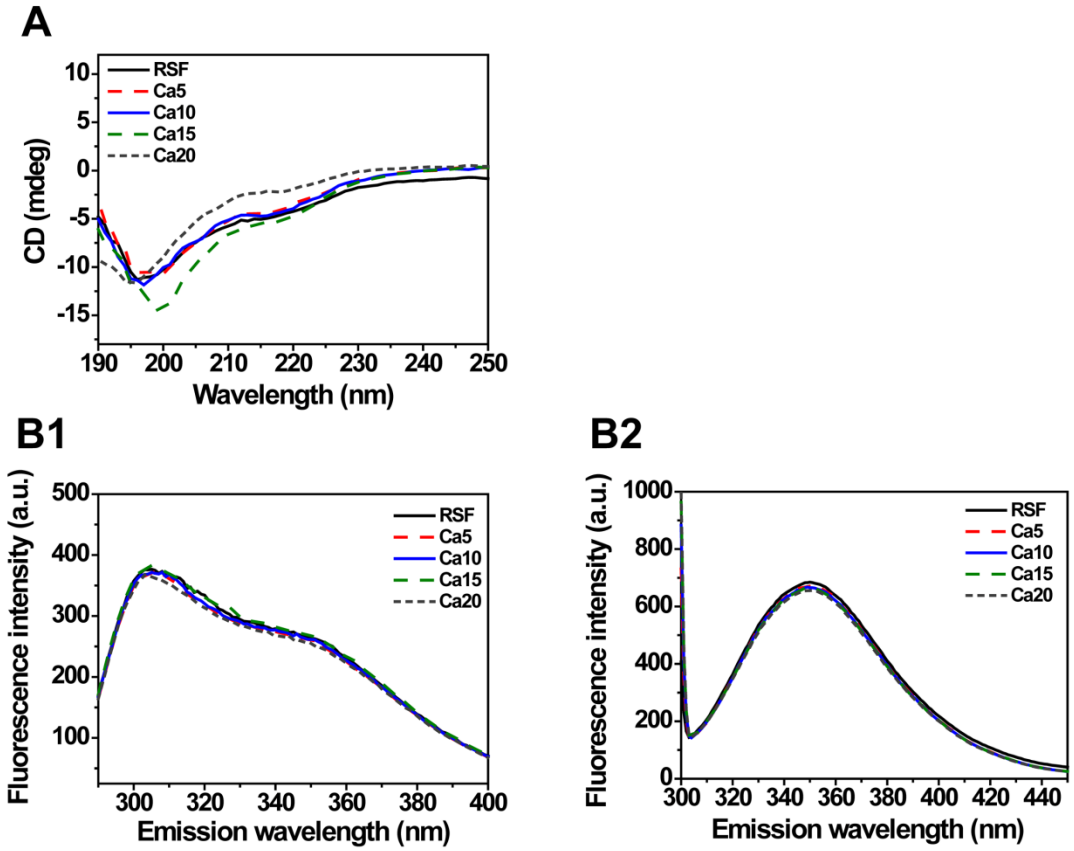


Fig. S1 (A) CD spectra of silk fibroin solution with and without different Ca^{2+} ion concentrations: 0, 5,

10, 15, 20 mmol L^{-1}. (B) Fluorescence emission spectra excited at 274 nm and eliminated the influence of Tyr (B1), at 295 nm and eliminated the influence of Trp (B2).

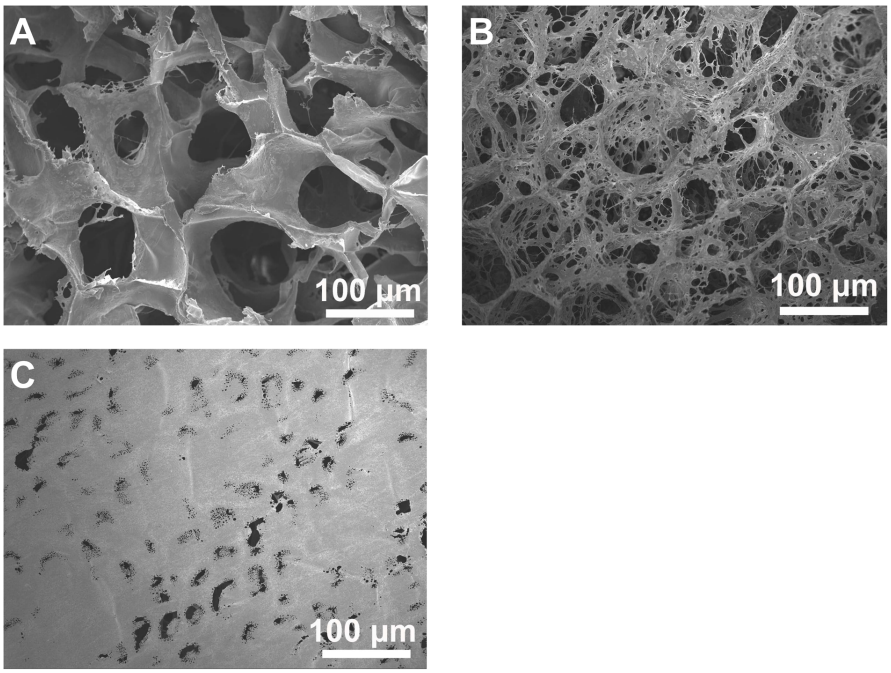
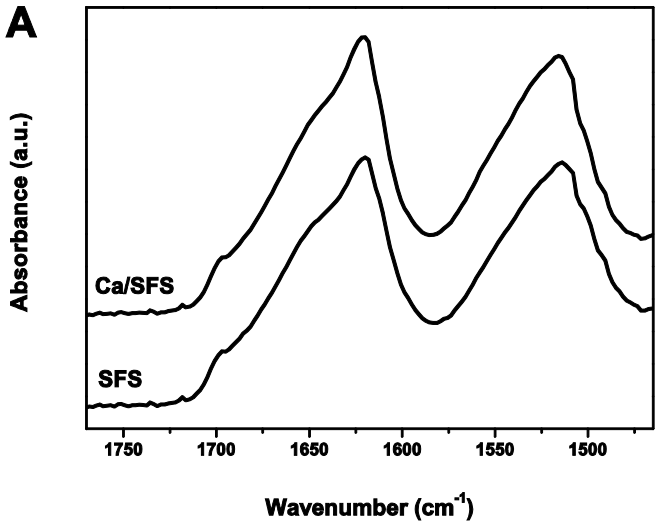


Fig. S2 SEM micrographs of the inner microstructures of SFSs (A), Ca/SFSs (B), and bottom surface of scaffolds (C).



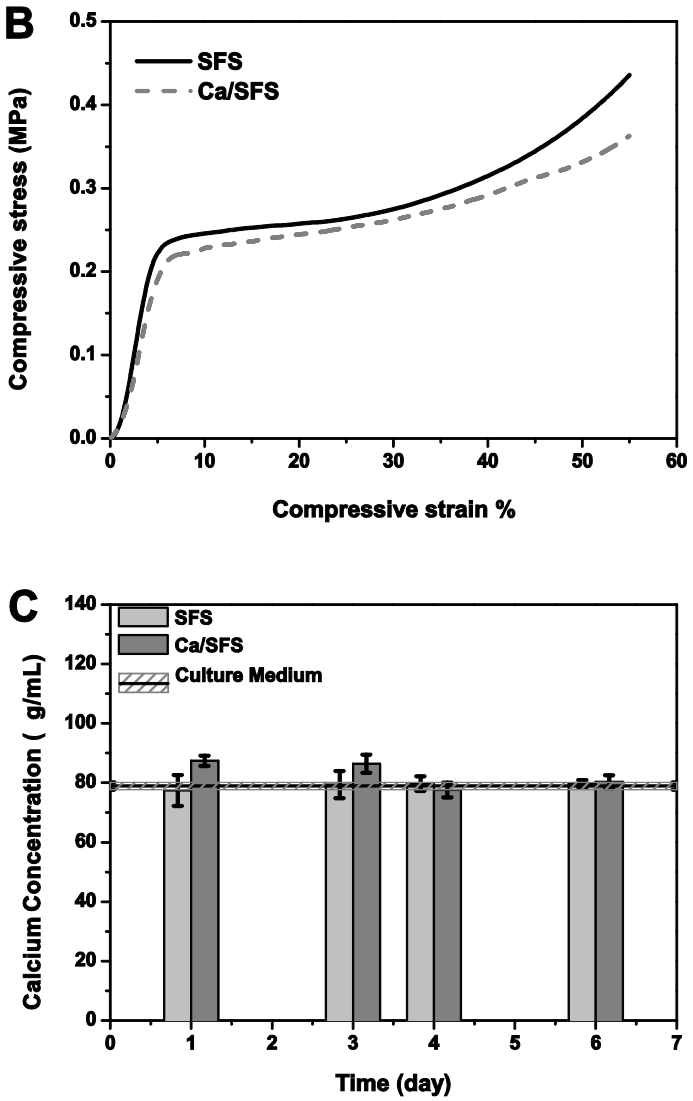


Fig. S3 (A) FTIR absorbance spectra of SFSs and Ca/SFSs in the amide I and amide II region. (B) Compressive stress-strain curves of SFSs and Ca/SFSs. (C) Calcium concentration in culture medium after SFSs and Ca/SFSs soaking for 1, 3, 4 and 6 days detected by AAS.