Supporting Information

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SI Materials and Methods

Silk-Fiber and Solution Preparation. Silk solution was prepared using *Bombyx mori* silkworm cocoons supplied by Tajima Shoji Co. according to protocols described in our previous studies (1). Briefly, cut pieces of cocoons were degummed in boiling 0.02 M sodium carbonate solution for 20 min followed by thorough washing in deionized water and air drying. After air drying, the silk fibers were divided into two batches, where one batch was used for alkali hydrolysis using sodium hydroxide. The second batch of degummed silk fibers was dissolved in 9.3 M LiBr solution at 60 °C yielding a 20% (wt/vol) solution. This solution was subsequently dialyzed against water using Slide a-Lyzer dialysis cassettes (Pierce, molecular weight cut off 3,500) for three days with frequent change of water. The final concentration of the aqueous silk fibroin solution was about 8% (wt/vol). Part of the silk solution was frozen at −80 °C and then lyophilized. The lyophilized silk sponge was added to hexafluoroisopropanol (HFIP) to prepare 25% (wt/vol) solvent-based silk solution.

Hydrolysis of Degummed Silk into Micron Range Fibers. The microfiber preparation process (Fig. 1*A*) can be divided into three stages: (*i*) preparation of degummed silk fibers from cocoons; (*ii*) hydrolysis of degummed silk fibers into micron-sized fibers; and (*iii*) washing/neutralization of the fibers and lyophilization. For degumming of silk fibers we used a similar preparation as described above for silk-fiber/solution preparation. For hydrolysis of fibers we used sodium hydroxide pellets (NaOH) weighing 3.5 gm (to obtain 17.5 M solution) were added to 5 mL of distilled water. When approximately 70% of the NaOH pellets are dissolved with an exothermic reaction, the dried degummed silk fibers weighing 0.35 gm were added and stirred with a spatula. To stop hydrolysis, 45 mL of water is added to the reaction mixture and centrifuged at 2,794 × g for 5 min. The supernatant is discarded and the fibers are resuspended in 50 mL of water, stirred, and centrifuged. This step is repeated from five to eight times to remove excess remaining alkali. The pH of the solution is measured and the pH is adjusted to 7.0 using hydrochloric acid. The neutralized fiber solution is again centrifuged at 3,500 rpm for 5 min and resuspended in water (repeated three to five times). Finally, the fibers are suspended in PBS and lyophilized to generate a silk-fiber powder. To obtain large (400–500-μm long) and medium (150–200-μm long) silk microfibers, the hydrolysis reaction was carried for 30 and 180 s, respectively. To obtain very fine/smaller (10–20-μm long) silk fibers, the reaction mixture was set up in a boiling water bath for 60 s to aid rapid hydrolysis.

Fabrication of Reinforced Fiber-HFIP Scaffolds. Silk-fiber reinforced-HFIP scaffolds were prepared by modifying our earlier methods for HFIP-based silk scaffolds (2). Two different ratios of 1:1 and 1:3 (wt/wt %) of HFIP-silk:silk-fiber composite scaffolds were fabricated comprised of 25% (wt/vol) HFIP-silk solution and silk fibers of larger (400–500 μm), medium (150–200 μm), and smaller (10–20 μm) diameters obtained by the hydrolysis method described. Briefly, 4 g of NaCl (particle size 800 μm) were sieved for each scaffold. Based on the microfiber ratio used for reinforcement, each silk microfiber type was weighed [e.g., for 1:1 ratio, weigh 0.25 g silk fibers for 1 mL of 25% (wt/vol) HFIP-silk]. For scaffold fabrication, silk microfibers were hydrated in water and then excess water was removed followed by the addition of 4 g of NaCl with gentle mixing. The fiber-salt mixture was then poured into a glass tube 10 mm diameter and the mixture was allowed to settle to the bottom with gentle tapping. Water was removed from the fiber-salt mixture by lyophilization. The height of the dry fiber-salt composite was measured and 1 mL of silk/HFIP solution (25 wt %) per centimeter height was added and then covered. The system was centrifuged at 3,649 × g for 5 min. Repeated centrifugation was used if required to completely distribute the HFIP-silk solution to all parts of the dry fiber-salt mixture. The materials were allowed to settle for 1 h and the cover was then removed to leave the tubes for 3–4 d in a fume hood to allow the HFIP to evaporate. Finally, 70% methanol was added to the tubes and then covered for 2 d. To perform salt leaching, the covers were removed and the scaffolds were placed in a beaker of water (2–3 L) with gentle stirring for 3–4 d until all of the salt was removed. To remove the salt, the scaffolds were removed from the glass tubes with a spatula and placed in a beaker with water (2–3 L) with slow stirring until all remaining salt was dissolved. Once the salt was removed, the scaffolds were transferred to 70% ethanol and stored. For control scaffolds, 25% (wt/vol) HFIP-silk solution was poured into 4 g of salt in a glass tube without silk microfibers.

Scanning Electron Microscopy. Fractured sections of the silk scaffolds were obtained in liquid nitrogen using a razor blade. The fracture surfaces were sputter-coated with Pt/Pd and morphology was examined with a field emission scanning electron microscope (FESEM) Zeiss Ultra55 or Supra55VP (Carl Zeiss AG). Pore size and wall thickness of silk scaffolds were analyzed with ImageJ 1.40 (Wayne Rasband).

Porosity Measurement by Liquid Displacement. Porosity of the fiber reinforced-HFIP scaffolds was determined via liquid displacement with hexane, as previously reported (2). After fabrication, the scaffolds were lyophilized and then immersed in a graduated cylinder of known volume of hexane (V_1). A series of quick evacuation-depressurization cycles were performed to completely evacuate entrapped air and to impregnate the scaffold with hexane; thereafter, the volume in the cylinder was recorded (V_2). The hexane-impregnated scaffold was removed and the volume was recorded again (V_3). Any change of volume due to evaporation during the evacuation cycles was checked using another cylinder without the scaffold. The porosity of the scaffold is expressed as

$$[(V_1 - V_3)/(V_2 - V_3)] \times 100\%. \quad \textbf{[S1]}$$

Mechanical Properties. Unconfined compressive mechanical testing of hydrated silk-fiber reinforced-HFIP scaffolds was performed on an Instron 3366 testing frame equipped with a 0.1 kN load cell. Tests for all scaffold types both unseeded and cell-seeded were carried out in 0.1 (M) PBS bath (BioPuls; Instron Corp.) at 37 °C under hydrated conditions. Separate silk scaffold discs were punched out for compressive tests, with dimensions of 4 mm diameter and 3 mm height. For cell-seeded silk scaffolds, each type was individually seeded with 10^6 human bone marrow-derived mesenchymal stem cells (hMSCs) at day 1 and cultured for 28 d in osteogenic medium. All tests were accessed with a conventional open-sided (nonconfined) configuration and were performed using a displacement control mode at a rate of 5 mm/min following ASTM standard D1621-04a (standard test method for compressive properties of rigid cellular plastics). After the compression tests, the compressive stress and strain were graphed based on the measured cross-sectional area and sample height

(nominal ~4–5 mm, measured automatically at 0.02 N tare load), respectively. The elastic modulus was calculated based on a linear regression fitting of the small strain section that preceded an identifiable plateau region.

Isolation of hMSCs. hMSC isolation and expansion was carried out following our previously published protocols (3). A 25-mL bone marrow aspirate (Lonza) was obtained from a 27-y-old male donor and was diluted in 75 mL of PBS. Cells were separated by density gradient centrifugation and 20 mL aliquots of the bone marrow suspension were overlaid onto a poly sucrose gradient (1,077 g/cm^3 Histopaque; Sigma) and centrifuged at 800 g for 30 min at room temperature. The cell pellet was resuspended in Eagle's minimum essential medium (α-MEM; Gibco BRL) supplemented with 10% FBS (Gibco BRL), 100 U/mL penicillin G (Gibco BRL), and 100 μg/mL streptomycin (Gibco BRL). Cell number and viability were determined using a trypan blue exclusion test. The resuspended cells were plated at a density of 1.5×10^5 cells/cm^2 and placed in a 5% CO_2 incubator at 37 °C. The culture medium was changed every other day and cells were passaged three times (P3) before use in experiments.

Cell Proliferation and Osteogenic Differentiation on Silk Scaffolds. Osteogenic potential of silk-fiber reinforced-HFIP scaffolds was evaluated by differentiation of hMSCs in osteogenic media. Approximately 10^6 cells were seeded onto each reinforced silk-fiber scaffold of dimension 3 × 2 mm per group of four ($n = 4$) followed by addition of growth medium (DMEM + 10% FBS+ antibiotics) after initial cell attachment. Seeded hMSCs were cultured for 3 d at 37 °C and 5% CO_2 before transferring into osteogenic media consisting of DMEM supplemented with 10% FBS, 0.1 mM nonessential amino acids, 50 μg/mL ascorbic acid-2-phosphate, 100 nM dexamethasone, 10 mM β-glycerolphosphate in the presence of 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL Fungizone. Cultures were maintained at 37 °C in a humidified incubator supplemented with 5% CO_2. Half of the medium was changed every 2 d. Scaffold discs were removed for analysis after 4 wk. hMSC proliferation on three-dimensional silk scaffold constructs were monitored by Alamar blue dye reduction assay (Invitrogen) after 1, 7, 14, 21, and 28 d following the manufacturer's protocol.

Real-Time PCR. Reinforced silk-fiber scaffolds with cells ($n = 4$ per group) were transferred into 2-mL plastic tubes, then 1.0 mL of Trizol was added. Scaffolds were chopped into pieces with microscissors on ice. The tubes were centrifuged at 12,000 × g for 10 min, after which the supernatant was transferred to a new tube. Chloroform (200 μL) was added to the solution and incubated for 5 min at room temperature. Tubes were then centrifuged at 12,000 × g for 15 min, and the upper aqueous phase was transferred to a new tube. One volume of 70% ethanol (vol/vol) was added and applied to an RNeasy mini spin column (Qiagen). The RNA was washed and eluted according to the manufacturer's protocol. The RNA samples were reverse transcribed into cDNA using oligo (dT)-selection according to the manufacturer's protocol (High Capacity cDNA Archive Kit; Applied Biosystems). Collagen type Ia1 (Col Ia1), alkaline phosphatase (ALP), bone sialoprotein (BSP), and osteopontin (OP) were quantified using the M 3000 Real-Time PCR system (Stratagene) for osteogenesis. PCR reaction conditions were 2 min at 50 °C, 10 min at 95 °C, and then 50 cycles at 95 °C for 15 s/60 °C for 1 min. All data were normalized to the expression of the housekeeping gene, GAPDH. The GAPDH probe was labeled at the 50 end with fluorescent dye VIC and with the quencher dye TAMRA at the 30 end. Probes for human GAPDH (Hs99999905_m1), collagen type Iα (Col Iα1, Hs00164004_m1), ALP (Hs00758162_m1), BSP (Hs00173720_m1), and OP (Hs00167093_m1) were purchased as Assay-on-Demand Products (Applied Biosciences).

Biochemical Analysis. For each study group, DNA content and ALP activity were analyzed using scaffolds chopped with microscissors on ice. Crushed samples ($n = 4$) were extracted twice with 0.2% (vol/vol) Triton X-100/5 mM $MgCl_2$ solution. DNA content was measured using the PicoGreen assay (Molecular Probes), according to the manufacturer's protocol. Samples were measured fluorometrically at an excitation wavelength of 480 nm and an emission wavelength of 528 nm. ALP activity was checked on the same samples using a biochemical assay from Stanbio Laboratory based on conversion of p-nitrophenyl phosphate to p-nitrophenol, measured spectrophotometrically at 405 nm. ALP activity was normalized by DNA content of the sample.

In Vivo Subcutaneous Implantation in Mice. All procedures were conducted under animal care protocols approved by Tufts Institutional Animal Care and Use Committee. All animals used in this study were 5–7-wk-old balb/c female mice (Charles River breeding labs). The mice were distributed by three experimental groups each with two time points: 7 d and 4 wk. The mice were randomly assigned to the experimental groups and silk-fiber reinforced-HFIP scaffold samples were subcutaneously implanted in lateral subcutaneous pockets of each mouse under general anesthesia using a mixture of oxygen (0.6l/ min) and 1.5–3 vol % of Isofluran. The healing process at the incision region was coarse monitored during all study periods and no deaths were registered during the experiment. To check for inflammatory responses, mice were euthanized by CO exposure after 7 d and 4 wk post-implantation and samples collected along with the overlying tissue for histological examination.

Histology. Histologic sections of individual scaffold types were examined to assess the extent of degradation and for local inflammatory responses at the implant-host interface, such as for neovascularization, fibrosis, and the presence of inflammatory cells. After collection, samples were immediately immersed in 10% neutral buffered formalin for 24 h before histological analysis. Samples were processed through a series of graded ethanol, embedded in paraffin, and sectioned at 5–7 μm thickness. For histological evaluation, sections were deparaffinized, rehydrated, and stained with H&E.

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3. Park SH, Gil ES, Kim HJ, Lee K, Kaplan DL (2010) Relationship between degradability of silk scaffolds and osteogenesis. *Biomaterials* 31:6162–6172.