

Supporting Information

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Instructive conductive 3D silk foam-based bone tissue scaffolds enable electrical stimulation of stem cells for enhanced osteogenic differentiation

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Experimental Materials and Methods

Materials

Unless otherwise stated, all chemicals for synthesis and physicochemical analysis were of ACS grade, purchased from Sigma-Aldrich and used as received without further purification. Phosphate buffered saline (PBS) was at pH 7.4. Reagents for cell culture were purchased from Invitrogen (Carlsbad, CA) unless otherwise noted. Human mesenchymal stem cells (HMSCs) from a 24 year old drug- and disease-free male were purchased from Lonza (Gaithersburg, MD).

Preparation of silk foams

Aqueous solutions of silk fibroin were prepared in accordance with the literature.^[17] Briefly, silk cocoons of *B. mori* silkworms were degummed by boiling in an aqueous solution of Na₂CO₃ (0.02 M) for 20 min, followed by rinsing thoroughly with distilled water. The extracted silk fibroin was then dissolved in aqueous LiBr (9.3 M) at 60 °C for 4 h, and thereafter dialyzed against ultrapure water using a Slide-a-Lyzer dialysis cassette (MWCO 3,500, Life Technologies, Carlsbad, CA, USA) for 2 days. The solution was centrifuged at 9000 rpm (*ca.*

12,700 g) at 4 °C for 20 min, transferred to a fresh centrifuge tube, and centrifuged at 9000 rpm (ca. 12,700 g) at 4 °C for a further 20 min to remove any solids. The final concentration of silk fibroin aqueous solution was 8 w/v%, as determined by dry weight analysis. If necessary, solutions could be stored for a few weeks at 4 °C without the onset of premature precipitation. Glass petri dishes were almost filled with NaCl (particle size of 425-500 μm) and aqueous solutions of silk fibroin (8 wt%) were poured over the salt. The petri dish was covered and left at room temperature for 48 h, after which the petri dish was immersed in water (2L) and the NaCl extracted for 2 days changing the water at least three times a day (minimum 6 washes). Samples were cut to lengths appropriate for the various subsequent experiments using a disposable biopsy punch and a razor blade. The porous silk scaffolds were either stored in ultrapure water at 4 °C or lyophilized and stored at room temperature.

Preparation of conductive silk foams with interpenetrating networks of poly(pyrrole-co-(2-hydroxy-5-sulfonic aniline))

Interpenetrating networks of conductive polymers and silk fibroin were prepared by adaptation of the literature.^[10d, 18] Briefly, pyrrole was purified by passage over basic alumina. 2-hydroxy-5-sulfonic aniline (0.473 g, 2.5 mmol) was dissolved in HCl solution (1 M, 50 mL) in disposable 50 mL centrifuge tubes. Pyrrole (0.175 mL, 2.5 mmol) was added and the sample cooled to 0 °C. 60 silk foams (4 mm in height and diameter) were added and incubated at 0 °C for 1 hour. (NH₄)₂S₂O₈ (1.142 g, 5 mmol) and FeCl₃ (0.005 g, catalytic)^[19] were added, and the samples were shaken to assure dissolution of the ammonium persulfate and ferric chloride and then incubated for a further 24 h at 4 °C. The reaction mixture was decanted and the foams were washed with water, however, the interpenetrating network was inhomogeneous after this time. Therefore, the process was repeated, and a solution of 2-hydroxy-5-sulfonic aniline (0.473 g, 2.5 mmol) in HCl (1 M, 50 mL), pyrrole (0.175 mL, 2.5 mmol) was added to the foams, followed by (NH₄)₂S₂O₈ (1.142 g, 5 mmol) and FeCl₃ (0.005 g, catalytic).^[19] The samples were

shaken and then incubated for a further 24 h at 4 °C. Homogeneously black conductive foams were removed from the reaction mixture, placed in fresh distilled water, sonicated for 5 min, and then exhaustively washed (to remove monomers, oligomers and initiators) with deionized water until the water used to wash the materials was clear, colourless and the pH was neutral (*ca.* 3 days), after which they were washed with ethanol (1 day at 21 °C with two changes of ethanol). Conductive silk foams with an interpenetrating network of poly(pyrrole-co-(2-hydroxy-5-sulfonic aniline)) were dried under high vacuum at 21 °C (mass differences in the non-conductive and conductive foams were within experimental error negligible).

Porosity determination

The porosity of the samples ($n = 5$) was measured by liquid displacement.^[16c] Hexane was used as the liquid as it does not swell or shrink the sample. The sample was immersed in a known volume (V_1) of hexane in a graduated cylinder for 5 min. The total volume of hexane and the hexane-impregnated sample was recorded as V_2 . The hexane-impregnated sample was then removed from the cylinder and the residual hexane volume was recorded as V_3 . The total volume V of the sample was:

$$V = (V_2 - V_1) + (V_1 - V_3) = V_2 - V_3 \quad (1)$$

$$V_2 - V_1 \quad (2)$$

$$V_1 - V_3 \quad (3)$$

$V_2 - V_1$ is the volume of the polymer scaffold. $V_1 - V_3$ is the volume of hexane within the scaffold.

The porosity of the scaffold (ε , %) was calculated from:

$$\varepsilon (\%) = ((V_1 - V_3) / (V_2 - V_1)) \times 100 \quad (4)$$

Swelling properties

Samples of dry weight (W_d) were immersed in PBS at room temperature for 24 h.^[16c] After excess water was removed, the wet weight of the scaffold (W_s) was determined ($n = 5$). The swelling ratio (SR) and equilibrium water content (EW_C) of the scaffold was calculated from:

$$SR = (W_s - W_d)/W_d \quad (5)$$

$$EW_C (\%) = \left(\frac{W_s - W_d}{W_s} \right) \times 100 \quad (6)$$

Mechanical properties

Compressive tests were performed using an Instron Materials Testing Machine 5543 Series Single Column System (Instron, Norwood, MA) with Bluehill 2 software. Samples (diameter 4 mm, height 4 mm) were stored in PBS (1 mL) for 24 hours prior to testing. Each sample was compressed to 90 % of its original height at a rate of 0.05 mm s⁻¹ using a 50 N load cell ($n = 16$). The compressive stress and strain were plotted and the average compressive strength as well as the compressive modulus and standard error of the mean determined. The elastic modulus was defined by the slope of the initial linear section of the stress–strain curve. The compressive strength was determined by drawing a line parallel starting at 1% strain. The point at which this line crossed the stress–strain curve was defined as the compressive strength of the scaffold.^[22c]

Scanning electron microscopy (SEM)

Samples were mounted on a Scanning Electron Microscopy (SEM) stub and sputter coated with Pt/Pd (15 nm) using a Cressington 208 Benchtop Sputter Coater. All samples were imaged using a Zeiss Supra 40 VP field emission scanning electron microscope.

X-ray photoelectron spectroscopy (XPS)

XPS was carried out on the samples to confirm that the surface chemistry of the scaffolds had changed after the growth of an interpenetrating network of the CP within the silk matrix. XPS was performed on a Kratos Axis X-ray photoelectron spectrometer (Kratos Analytical Ltd., Manchester, UK). The binding energy was calibrated using the C 1s photoelectron peak at 284.6 eV as a reference. The CasaXPS computer program was used for peak fitting of the C 1s and O 1s peaks in the XPS spectra. The reported spectra are representative of two measurements at different positions on a sample.

Fourier Transform infrared spectroscopy (FTIR)

A Thermo Scientific Nicolet 380 FTIR Spectrometer (Thermo Fisher Scientific Inc., USA) was used to record spectra in attenuated total reflectance (ATR) mode at 21 °C with a 1 cm⁻¹ resolution and 128 scans (corrected for background and atmosphere using the software provided with the spectrometer). Samples were secured in position on the ATR crystal using the built-in clamp.

In vitro enzymatic degradation assay

The degradation of the samples was evaluated using protease XIV (EC 3.4.24.31). Samples (4 mm diameter, 4 mm height) were immersed in PBS (1 mL) and incubated for 24h at 37 °C. Samples were washed with PBS and ultrapure water, dried on Kimwipes and their initial mass determined. Samples were incubated at 37 °C in PBS optionally containing the enzyme protease XIV (1U mL⁻¹). At specific times, samples were washed with PBS and ultrapure water, dried on Kimwipes and weighed. The enzyme solution was replaced with freshly prepared solution every 24 h (n = 5, error bars represent standard deviations).

Cell source & preparation

HMSCs were isolated from bone marrow aspirate (Lonza, Walkersville, MD) as described previously.^[28] Briefly, aspirate from a male donor under 25 years old was combined with HMSC proliferation medium (MEM alpha with 10% fetal bovine serum (FBS), 1% antibiotic/antimycotic, 1% nonessential amino acids (NEAA)) and cultured at 37 C with 5% CO₂ in a humidified environment. Flasks were rocked every day to allow HMSCs to adhere and medium was added every 3–4 days until HMSCs reached 80% confluence. Non-adherent cells were removed via PBS washes and the HMSCs were cultured in proliferation medium until either passaged or frozen.

Toxicity of the CPs as assessed via Cell Titer-Glo® luminescent cell viability assay kit

HMSC viability was assessed using a Cell Titer-Glo® luminescent cell viability assay kit (Promega, USA) in accordance with the with the manufacturer’s protocol, using a Synergy HT Multi-Mode Microplate Reader (Biotek, USA) to analyze the luminescence of the samples. As controls, cells seeded on tissue-culture treated Corning® Costar® tissue culture plates in pristine cell culture medium or cell culture medium containing 15% v/v ethanol were considered (n = 3, error bars represent standard deviations).

In vitro culture of human Mesenchymal stem cells without electrical stimulation

Foams were sterilized by electron beam sterilization (Nutek Corporation, Hayward, CA, USA). After sterilization, the samples were incubated for 30 minutes in 24 well plates containing HMSC growth medium that was composed of: high glucose Dulbecco’s Modified Eagle Medium (DMEM, 440 mL); fetal bovine serum (50 mL); antibiotic-antimycotic (5 mL); non-essential amino acids (5 mL), and 2 ng mL⁻¹ basic fibroblast growth factor. Medium was aspirated and replaced prior to HMSC seeding. Cell viability before starting the experiment was determined by the Trypan Blue exclusion method, and the measured viability exceeded 95 % in all cases. HMSCs were seeded at *ca.* 0.5 x 10⁶ cells per foam, and incubated at 37 °C, 95 %

humidity, and a CO₂ content of 5 %. After 3 days the medium was aspirated, the materials were washed gently with PBS and transferred to a fresh 24 well plate containing osteogenic medium that was composed of: high glucose Dulbecco's Modified Eagle Medium (DMEM, 425 mL); fetal bovine serum (50 mL); antibiotic-antimycotic (5 mL); non-essential amino acids (5 mL), dexamethasone (100 nM), β -glycerol phosphate (10 mM) and ascorbic acid (50 μ M). Thereafter the osteogenic medium was aspirated and replaced every 2 days until the samples were analysed.

In vitro culture of human mesenchymal stem cells with electrical stimulation

Electrical stimulation of human mesenchymal stem cells was achieved employing a custom built setup. A conductive composite composed of a dispersion of multiwalled carbon nanotubes (0.1 g) and Pellethane® 2363-80A TPU (Lubrizol, Wickliffe, OH, USA) (1 g) in chloroform (10 mL) was painted on the tip of copper wires and carefully embedded in the top and bottom of the conductive foams. The tip of the wire was bent to act as a hook inside the foam and secured in position by the application of more nanotube-loaded Pellethane®. The chloroform was allowed to evaporate and the samples were dried under high vacuum, yielding waterproof and conductive electrical contacts enabling the passage of an electrical current through the samples.

Foams were sterilized by electron beam sterilization (Nutek Corporation, Hayward, CA, USA). After sterilization, the samples were incubated for 30 minutes in 24 well plates containing HMSC growth medium that was composed of: high glucose Dulbecco's Modified Eagle Medium (DMEM, 440 mL); fetal bovine serum (50 mL); antibiotic-antimycotic (5 mL); non-essential amino acids (5 mL), and 2 ng mL⁻¹ basic fibroblast growth factor. Medium was aspirated and replaced prior to HMSC seeding. Cell viability before starting the experiment was determined by the Trypan Blue exclusion method, and the measured viability exceeded 95 % in all cases. HMSCs were seeded at *ca.* 0.5 x 10⁶ cells per foam, and incubated at 37 °C, 95 %

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The tips of the copper wires attached to the foams were wound around alligator clip-terminated wires attached to the multipotentiostat (CH Instruments, Austin, TX, USA). The counter and reference electrodes were connected together and clipped to the copper wire protruding from one end of the sample, and the working electrode was clipped to copper wire protruding from the other side of the sample. Wires and alligator clips were secured in position with adhesive copper tape (Ted Pella, Inc., Reading, CA, USA) and wrapped in Parafilm® to render them electrically insulating and waterproof (i.e. suitable for use inside an incubator). A potential step of 100 mV/mm was placed across the samples for the duration of 4 h per day for 6 days, after which the wires were disconnected and the substrates cultured as normal. Throughout the electrical stimulation experiments the osteogenic medium was aspirated and replaced every 2 days. Thereafter the osteogenic medium was aspirated and replaced every 2 days until the samples were analysed.

Biochemical assays

Cell viability on the scaffolds at various time points was assessed with an AlamarBlue® assay kit and a Synergy HT Multi-Mode Microplate Reader (Bio-tek US, Winooski, VT) in accordance with the manufacturer's protocol, and numbers of cells adhered to the samples studied herein are reported relative to their initial seeding density, which was assigned an arbitrary value of 100%. The DNA content and Alkaline Phosphatase (ALP) activity of samples that were broken up in a buffer of 0.2% Triton X-100 were quantified concurrently, using the PicoGreen® assay (Life Technologies, Thermo Fisher Scientific Inc., USA) for DNA

quantitation in accordance with the manufacturer's protocol, a SensoLyte® pNPP Alkaline Phosphatase Assay Kit (AnaSpec, Inc., Fremont, CA, USA) for ALP quantitation in accordance with the manufacturer's protocol, and a Synergy HT Multi-Mode Microplate Reader (Bio-tek US, Winooski, VT). The calcium content of samples was quantified using a Calcium Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA) in accordance with the manufacturer's protocol, and the collagen content of samples was quantified using a Sircol™ Assay Kit (Biocolor Ltd., Carrickfergus, UK) in accordance with the manufacturer's protocol, and a Synergy HT Multi-Mode Microplate Reader (Bio-tek US, Winooski, VT). Data were normalized to DNA quantity, (n = 13, error bars represent standard errors of the mean).

Histology

Slices of the samples (thickness of 10 µm) were prepared on a Leica CM-1950 UV cryostat (Leica Microsystems Inc., Buffalo Grove, IL, USA). Hematoxylin & Eosin (H&E) staining: samples were dehydrated in xylene and ethanol, rehydrated in water, dipped in Harris Hematoxylin solution to stain nuclei, rinsed in water and placed in Scott's solution until they turned blue, rinsed again in water, dipped in Eosin solution to stain the ECM, then dehydrated prior to being covered with a coverslip in xylene-based mounting media. Alizarin red staining: samples were dipped in 1% Alizarin red stain with a pH of 4.1-4.3 for 2 minutes, followed by 20 dips in acetone, followed by 20 dips in 50/50 Acetone/Xylene, then Histomount was used to cover each slide and the samples were left to dry overnight before imaging (images are representative of 3 samples).

Table S1. Physicochemical properties of the foams. Errors quoted are standard deviations.

Sample	Non-conductive silk foam	Conductive silk foam
Porosity, ϵ (%)	89.6 ± 0.6	70.8 ± 4.2
Swell ratio	14.0 ± 1.4	12.5 ± 1.4
Equilibrium water content (%)	93.3 ± 0.7	92.5 ± 0.8
Compressive modulus (kPa)	99.3 ± 30.4	74.7 ± 31.9
Compressive strength (kPa)	9.2 ± 4.2	7.4 ± 3.9

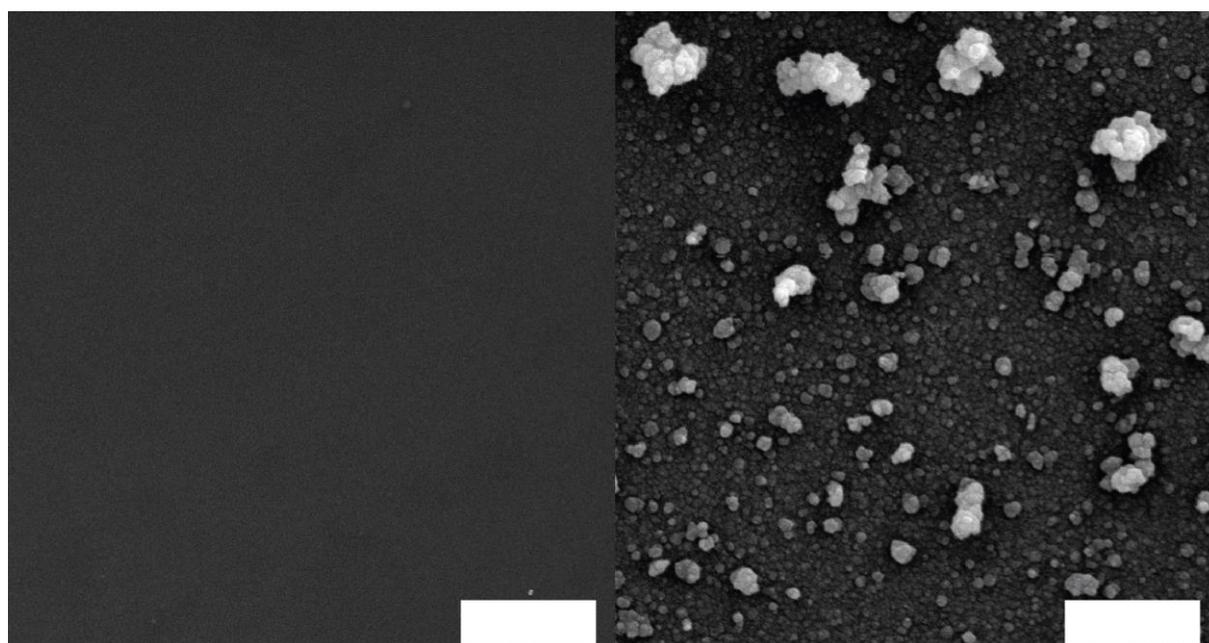


Figure S1. High magnification SEM image of the smooth underside of the foams in contact with the petri dish during foam preparation (foams are subsequently removed from the petri dish template during salt leaching). Left) non-conductive silk foam. Right) conductive silk foam clearly shows the presence of CP-based nanoparticles on the surface of the silk. Scale bar represents 1 μm .