Supporting Information

Enhanced Formation of Bioactive and Strong Silk-Bioglass Hybrid Materials Through Organic-Inorganic Mutual Molecular Nucleation Induction and Templating

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1. MATERIALS

B. mori silkworm cocoons were purchased from Jiaxing silk Co., China. Lithium bromide (LiBr), Tetraethyl orthosilicate (TEOS), calcium nitrate tetrahydrate (Ca $(NO_3)_2.4H_2O$), triethyl phosphate (TEP), sodium carbonate (Na_2CO_3), ethanol, hydrochloric acid (HCl), paraformaldehyde, Triton X-100, rhodomine phallodin, and 4, 6-diamino-2-phenyl indole (DAPI) were purchased from Sigma-Aldrich. Dialysis tube (molecular weight cut off: 3.5 KDa) was supplied by thermo scientific, Australia.

2. METHODS

Preparation of Regenerated SF. Aqueous SF solutions were prepared by degumming sliced silk cocoons in a boiling aqueous solution of 0.02 M Na_2CO_3 for 30 min twice. The degummed silk fibers were air-dried over night after being rinsed thoroughly with deionized water three times. Subsequently, the dried silk fibers were dissolved in a 9.3 M LiBr aqueous solution at 60 °C for 4 h. Then the SF solutions were filtered and dialyzed using a cellulose tube against deionized water for 48 h with 6 times of water change. The obtained solutions were centrifuged at 7000 × g at 4 °C for 30 min to remove any undissolved aggregates and kept at 4 °C for further use. All the concentrations of SF in this work were based on weight (wt%).

Preparation of BG sol. BG with a molar composition of 62.7% SiO_2, 33.2% CaO, and 4.1% P_2O_5 was prepared using an acid-mediated sol-gel method.[1] Briefly, sol was prepared by hydrolyzing 5.14 g of TEOS and 1 g of HCl acid (0.5 M) in 60 g of ethanol under stirring for

30 min at room temperature. Subsequently, triethyl phosphate (TEP) (0.6 g) was dissolved into the sol. After stirring for 30 min, 3.51 g of $CaNO_3.4H_2O$ was added to the mixture and stirred for 24 h to obtain a clear sol. The prepared sol was then aged at room temperature for another 24 hours to allow for complete hydrolysis.

Fabrication of hydrogel and aerogels. Hydrogels were fabricated by mixing SF aqueous solutions (2-6%) without or with BG sol of different volumes at 40 ^{o}C. The total volume of a gel is 2 mL. The samples in glass vials were kept in a water bath until complete formation of hydrogels. To determine the gelation time, the state of gelation was checked regularly by inverting the vials. A gel was considered to have formed if the content in the vial did not fall within 30 s. Aerogels were obtained with a critical point dryer (Leica CPD 300).

Morphological characterization of aerogels. An atomic force microscope (AFM, Bruker BioSciences Corporation) was used to image fibrous structure of hydrogels. 20 μL of diluted gels were dropped on freshly cleaved mica. A Scanasyst-air silicon nitride tip (Bruker) with a spring constant of 0.4 N/m was used in the tapping mode to scan the samples. The morphology and structure of SF/BG aerogels were observed with a scanning electron microscope (SEM, ZEISS SUPRA). The samples were gold sputtered and SEM images were taken at 5 KV. Energy dispersive X-Ray (EDS, Oxford Instrument Aztec) were employed to qualitatively investigate the presence of ion element in the aerogels.

Rheological characterization of hydrogels. In-situ rheological measurements of SF/BG gels were performed at 25 ◦C using a Discovery Hybrid Rheometer (TA Instrument) fitted with a cone-plate geometry (40 mm in diameter) and a 49 μm truncation gap at 25 ◦C. To avoid solvent evaporation during testing, the sample perimeter was covered with a low viscous oil. The oscillation frequency and the strain were set at 0.1 Hz and 0.05%, respectively. The storage modulus (G') and loss moduli (G") were demonstrated as a function of time during the gelation process for each gel. The oscillation frequency sweep was collected at 0.05% strain over a frequency range of 0.01 to 100 Hz.

Mechanical characterization of aerogels. The mechanical properties of prepared cylindrical aerogels were tested using an Instron 5967 universal mechanical testing equipment (Instron Corp, USA) with a 1000 N load cell at room temperature. Cylindrical scaffolds were compressed to 85% of their original heights at a cross speed head of 5 mm/min and all samples were measured in triplicates. Stress-strain curves were plotted in compression mode and the young modulus was calculated as the slope of the linear region in the stress- strain curves.

Atomic Force Microscopy (AFM). AFM images were obtained using a Multimode 8 AFM (Bruker BioSciences Corporation, USA) in tapping mode. Samples were diluted up to less than 0.1% and aliquot of the diluted solution was deposited on to a freshly cleaved mica and left to dry overnight. A Scanasyst-air silicon nitride tip (Bruker) with spring constant of 0.4N/m was used. AFM scans were taken at 512 × 512 pixels resolution and at a scan rate of 0.925 Hz.

Fourier transform infrared spectroscopy (FTIR). FTIR (Bruker Vortex 70, Bioscience Pty, Australia) spectra of SF aerogel without and with BG, were obtained in the transmittance mode by averaging 64 scans with a resolution of 4 cm^{-1} in the range of 4000-400 cm^{-1} to examine the chemical interactions between SF and BG. To determine secondary structure of SF, self-deconvolution of the infrared spectra in the amid I region (1595-1705 cm^{-1}) was carried out by OPUS 7.2 software. The original spectra was firstly smoothed with a five-point Savitsky-Golay smoothing filter. Then deconvolution was done using a Lorentzian line shape with a bandwidth of 25 cm^{-1} and noise reduction of 0.3. After baseline correction, the curve fitting procedure was applied, and the shape of each peak was changed to Gaussian. Finally, the software autofit program was used and the curve fitting was completed after the residual RMS error is less than 0.015. The relative area of each peak was expressed as a percentage (%) of total area.[2]

Zeta potential measurement. Zeta potential measurements were performed using a Zetasizer (Malvern Zetasizer Nano) to determine surface charges of SF without and with BG. To measure the zeta potential of a sample, 1 mL solution was loaded at room temperature. For SF/BG, the zeta potential was monitored in different time points and repeated three times.

X-ray powder diffraction. The composition of aerogels was characterized using an Xpert-Pro diffractometer with CuKα radiation, which was operated at 40 kV and 30 mA. The XRD data was acquired in the range of 10^{o}-70^{o} (2θ) with a scanning rate of 0.01^{o}/min.

Thermogravimetry (TGA) analysis. TGA (TA Q50) analysis of SF aerogels with and without BG was carried out by heating aerogels from room temperature to 700 °C with a heating rate of 10 °C/min under an inert nitrogen atmosphere.

In-vitro biomineralization. To examine the ability of the aerogels for promoting in-vitro biomineralization, aerogels were immersed in fresh simulated body fluid (SBF) prepared by following Kokubo's protocol[3] and incubated at 37 ^{o}C for 1 day, 3 days, 7 days and 14 days. The samples were retrieved at each time point, washed with deionized water, dehydrated using a series of ethanol, and then dried using a critical point dryer. The morphologies of the aerogels

were observed using SEM. XRD, FTIR and TGA were used to examine the structure and composition of aerogels after being soaked in SBF.

In-vitro cell study. Bone Marrow Stem (BMSC) cells were expanded in a standard culture medium. The scaffolds were sterilized and placed in a 24-well culture plate and cells at a density of 2×10^4 per scaffold were seeded and incubated at 37 ^oC in a 5% CO_2 atmosphere. After 1 day and 4 days' cell seeding, the scaffolds were washed with PBS three times and fixed with 4% paraformaldehyde for 30 minutes at room temperature. After rinsing the scaffolds with PBS again, the samples were dehydrated using a series of ethanol, air-dried, and imaged using SEM. Furthermore, to visualize the cytoskeletal organization of seeded cells on the scaffolds, the scaffolds with fixed cells were permeabilised using 0.1% Triton X-100 for 10 minutes at room temperature, followed by rinsing with PBS and incubated in 2% BSA for 20 minutes. After that, the scaffolds were rinsed with PBS and incubated in rhodomine phallodin for 30 min. After rinsing with PBS, cells were stained with DAPI for 10 min and the scaffolds were observed under the inverted fluorescence microscope (Olympus CX53). To assess the cell proliferation quantitatively, the cell counting Kit-8 (CCK-8) assay was used. At a specific culture time (1, 4 and 7 days), the scaffolds with cells were incubated with 110 μL of the medium containing 10 μL CCK-8 solution at 37 ◦C for 2 h. Subsequently, 100 μL from each well was removed into a new 96-well culture plate and the absorbance values at 450 nm were measured using a microplate reader.

3. SUPPORTING FIGURES

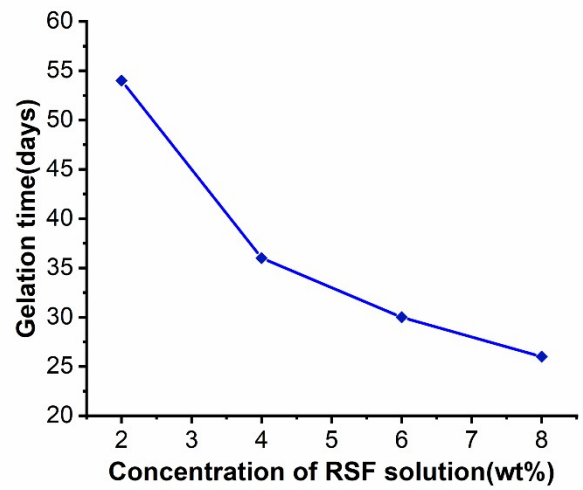


Figure S1. Gelation time of silk hydrogel as a function of SF concentration (room temperature).

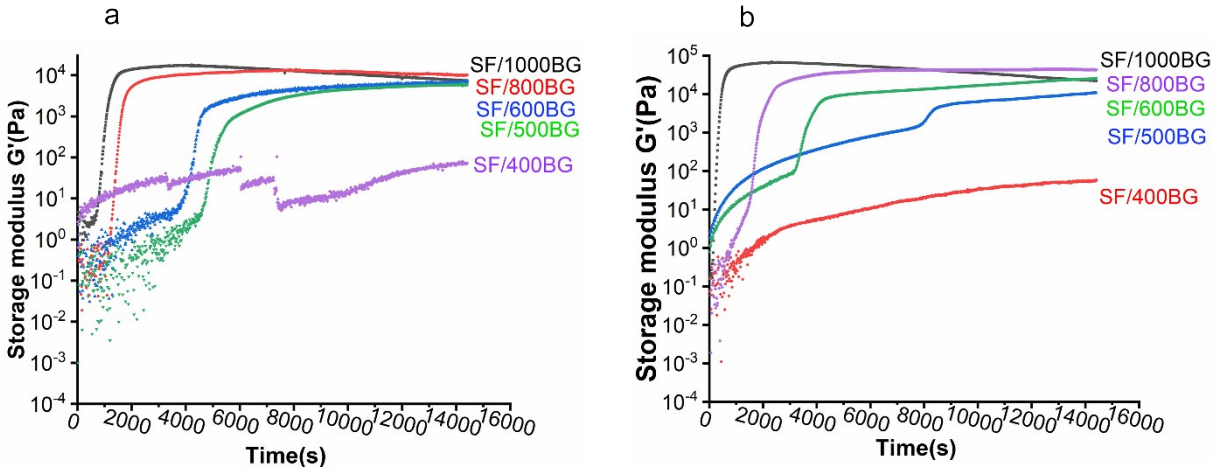


Figure S2. In-situ evolution of storage modulus (G') during silk hydrogel formation: a) 4% and b) 6% SF.

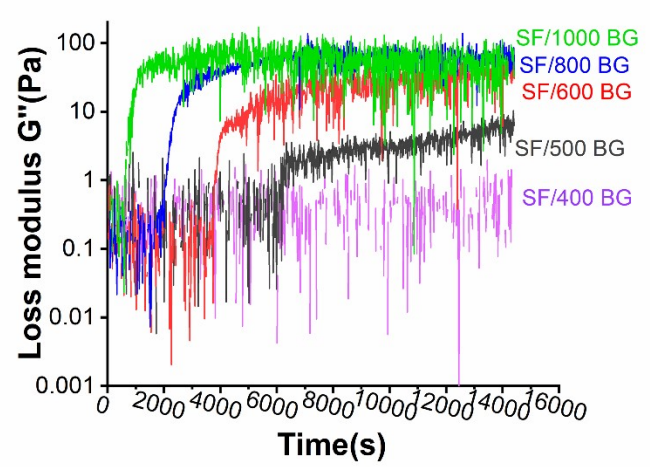


Figure S3. In-situ evolution of loss modulus of 2% SF hydrogel with different BG sol contents.

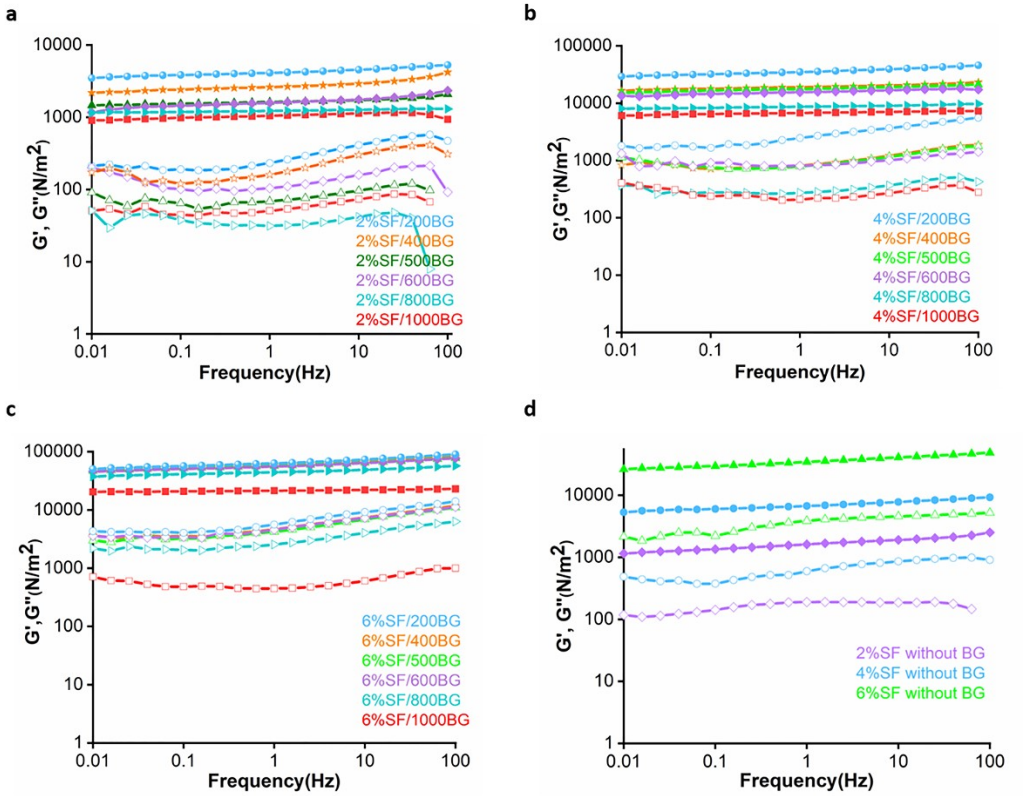


Figure S4. Frequency dependence of elastic modulus G' (filled symbols) and storage modulus G" (opened symbols) of silk hydrogels: (a) 2%, (b) 4%, (c) 6% SF hydrogels with BG and (d) SF hydrogels without BG.

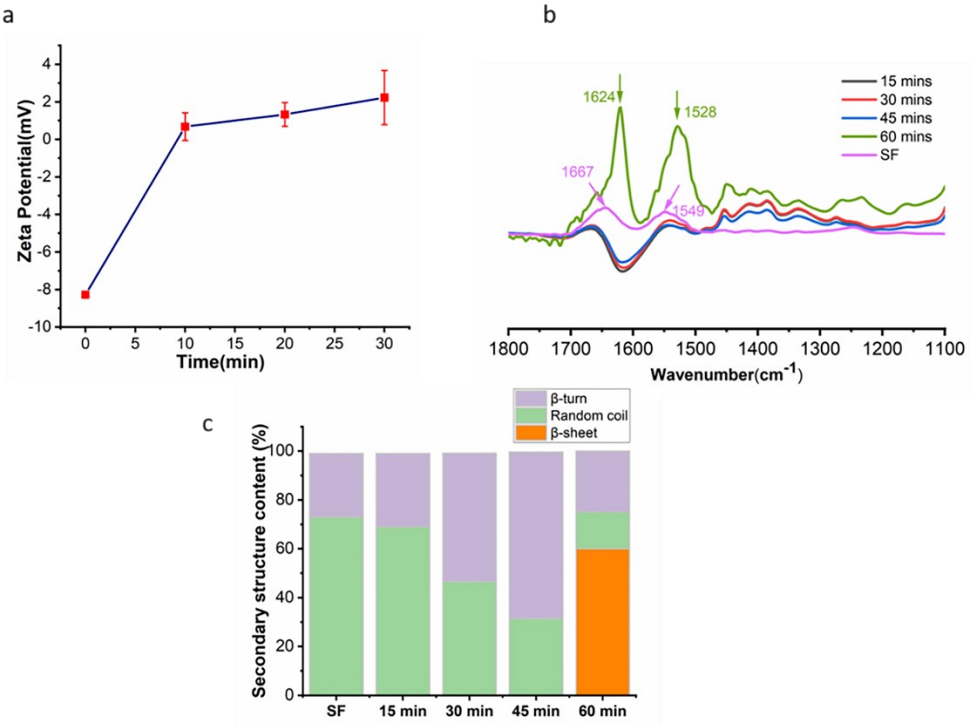


Figure S5. Evaluation of a) Z-potentials of silk solution without BG sol (t = 0) and after 10, 20 and 30 minutes of adding BG, b)Time-dependandant FTIR of SF solution before and after adding BG c) The secondary structure content (%) of SF before and after adding BG in different time obtained from Fourier transform self-deconvolution analysis of amide I regions. SF concentration was 2%, BG sol used was 500 µL/2 mL.

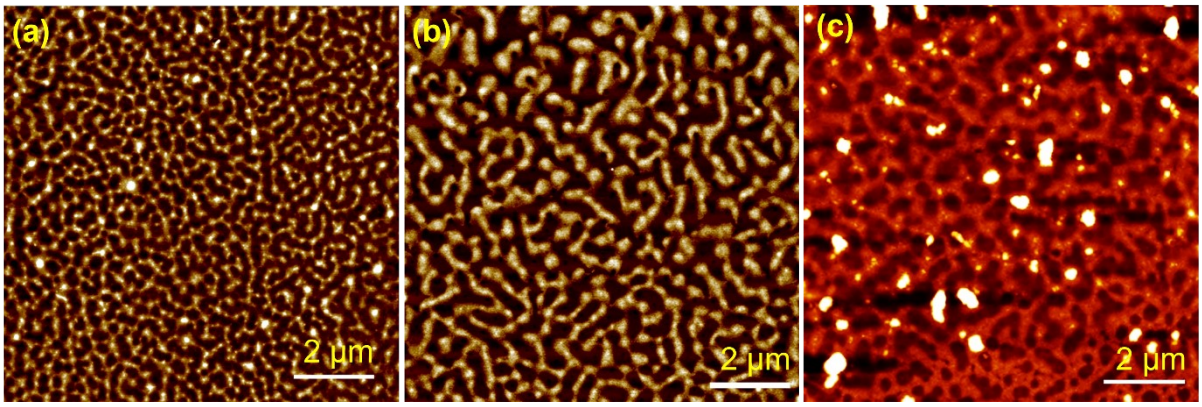


Figure S6. AFM images of SF/BG hydrogels at a lower magnification. BG sol content (a) 400 μL/2 mL, (b) 800 μL/2 mL and (c) 1000 μL/2 mL. SF concentration was 2%.

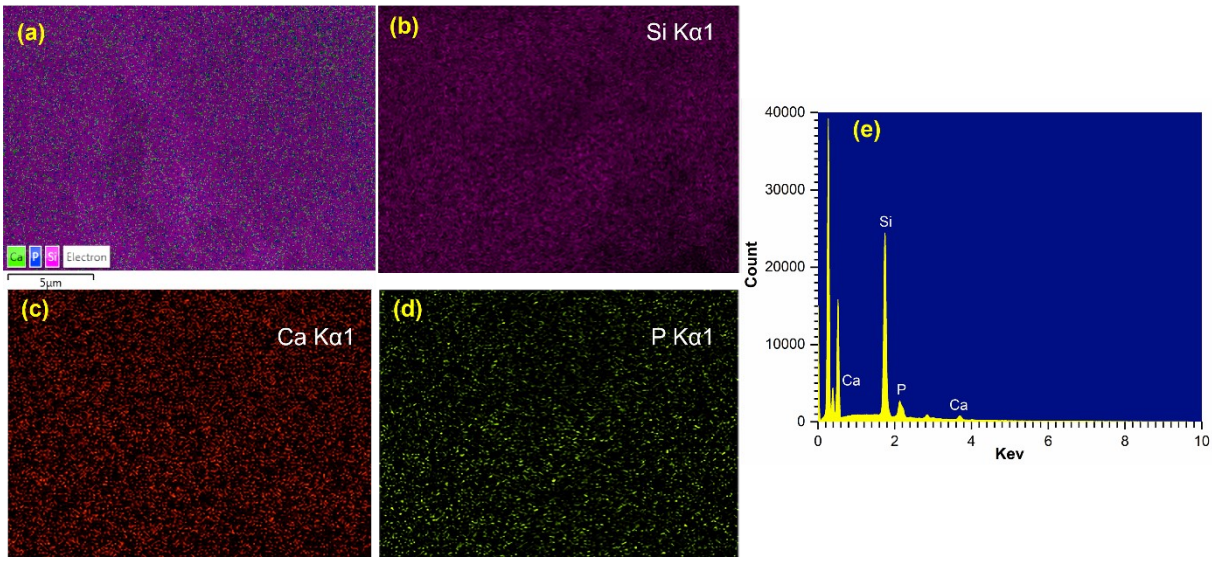


Figure S7. EDS mapping and spectra of SF/LBG aerogel (The concentration of SF is 2% and BG sol content:200 μL/2 mL)

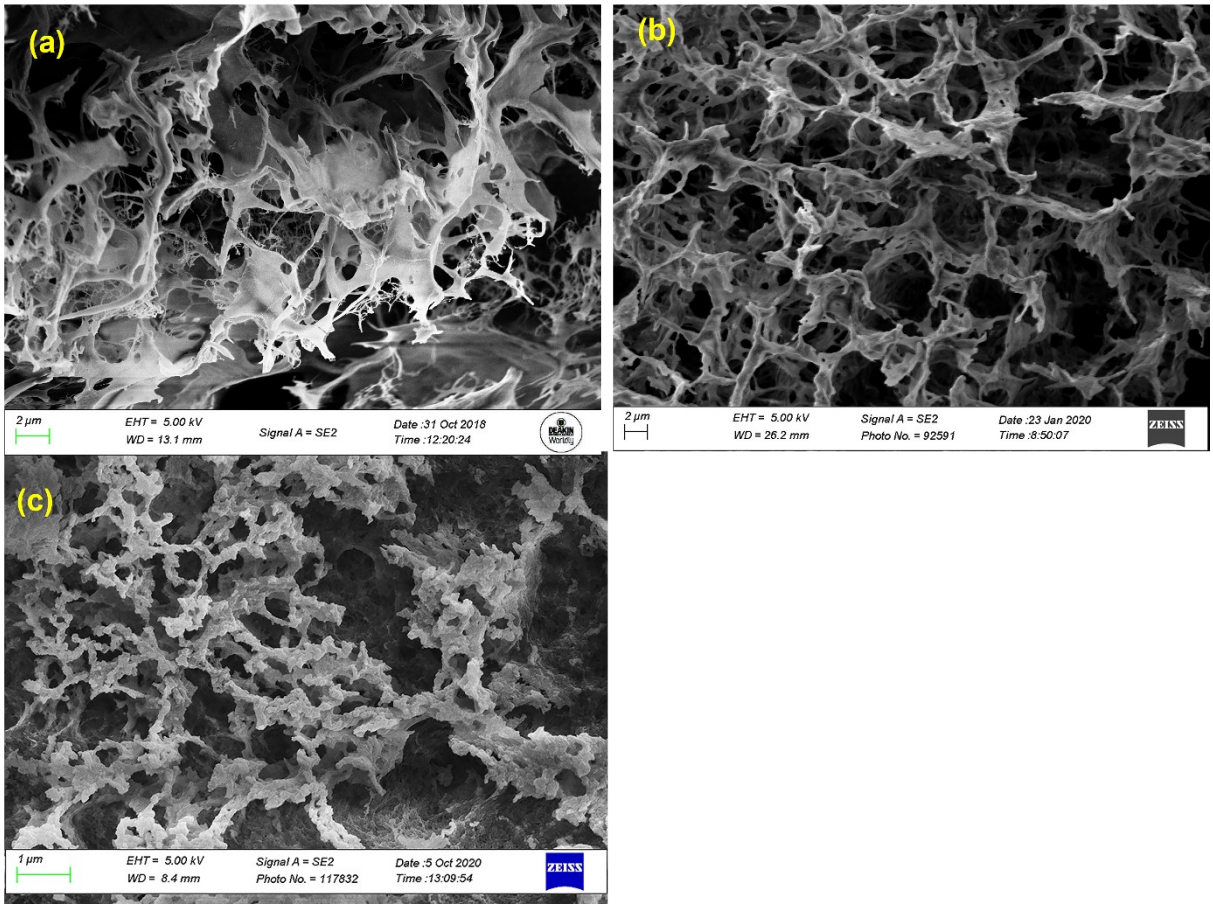


Figure S8. SEM images of xerogels obtained by direct freeze-drying of silk hydrogel (a), silk hydrogel with 200 BG (b) and silk hydrogel with 400 BG (c).

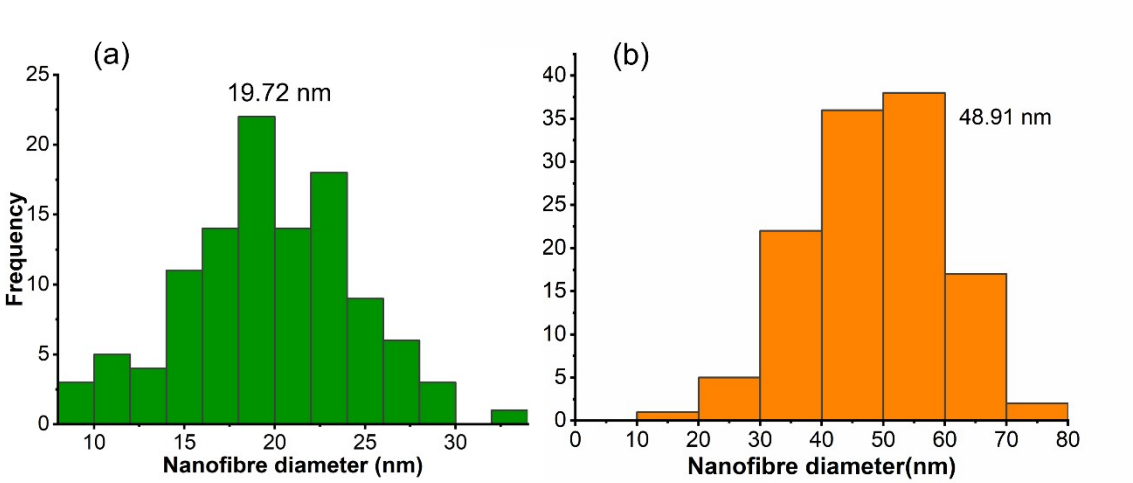


Figure S9. Distribution of nanofiber diameters of aerogels. (a) without BG, (b) with 200 BG.

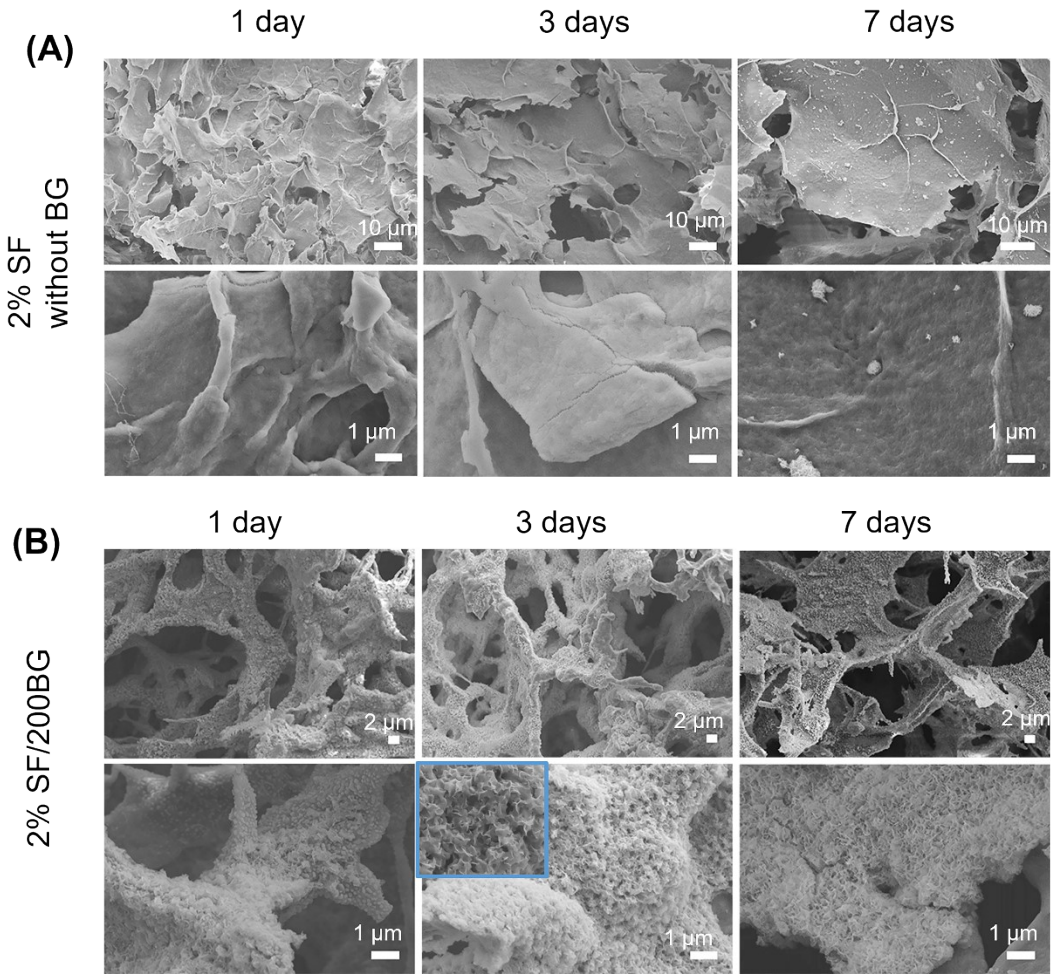
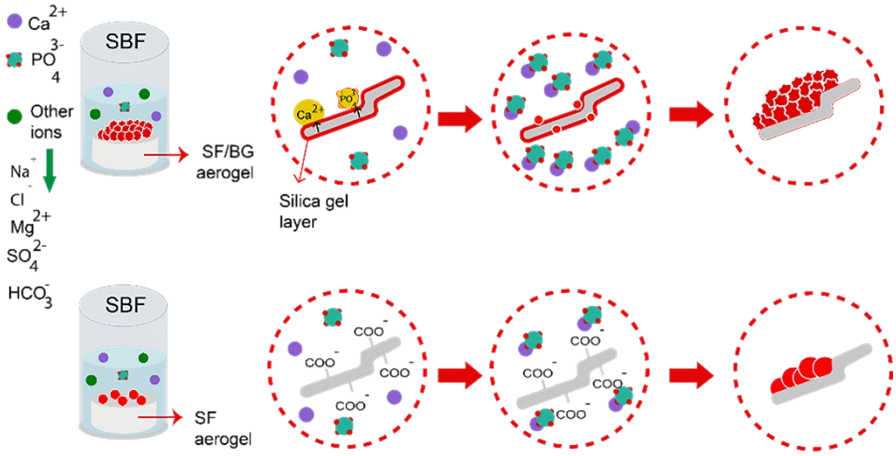


Figure S10. Biomeralization with xerogels. 2% SF xerogel (a) without BG and (b) with 200 BG. The inset in the middle image of the lower row in (B) is an magnified image showing peddle-like apatite crystals.



Scheme S1. Schematic description of apatite formation on SF/BG and SF nanofibrous aerogels. In the presence of BG, a layer of silica is formed, Ca^{2+} and PO_4^{3-} ions are released from BG (the ions in yellow circles) to promote nucleation of apatite, leading to formation of a large quantity of apatite.

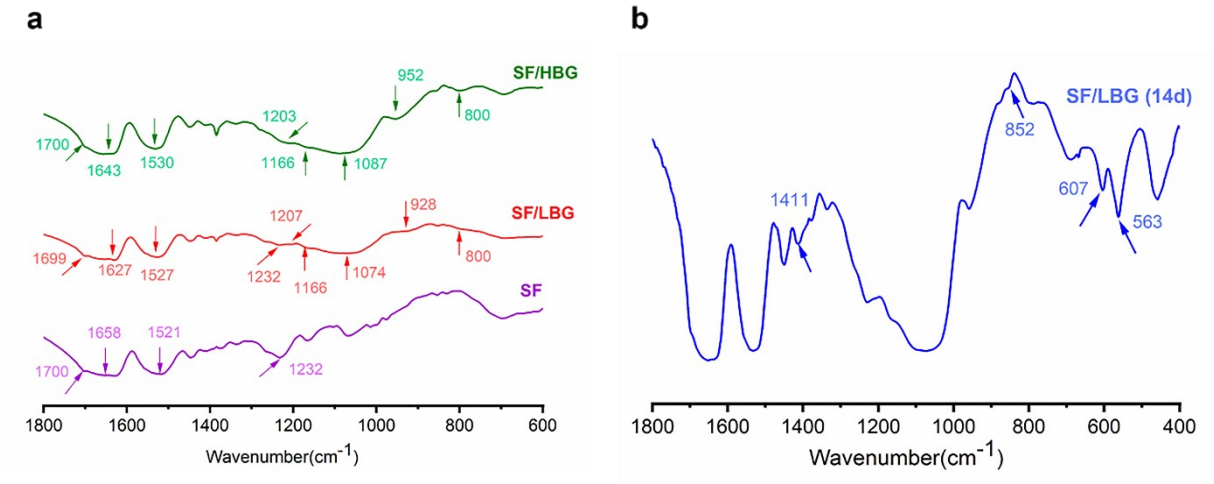


Figure S11. More detailed FTIR spectra of aerogels before (a) and after mineralization (b).

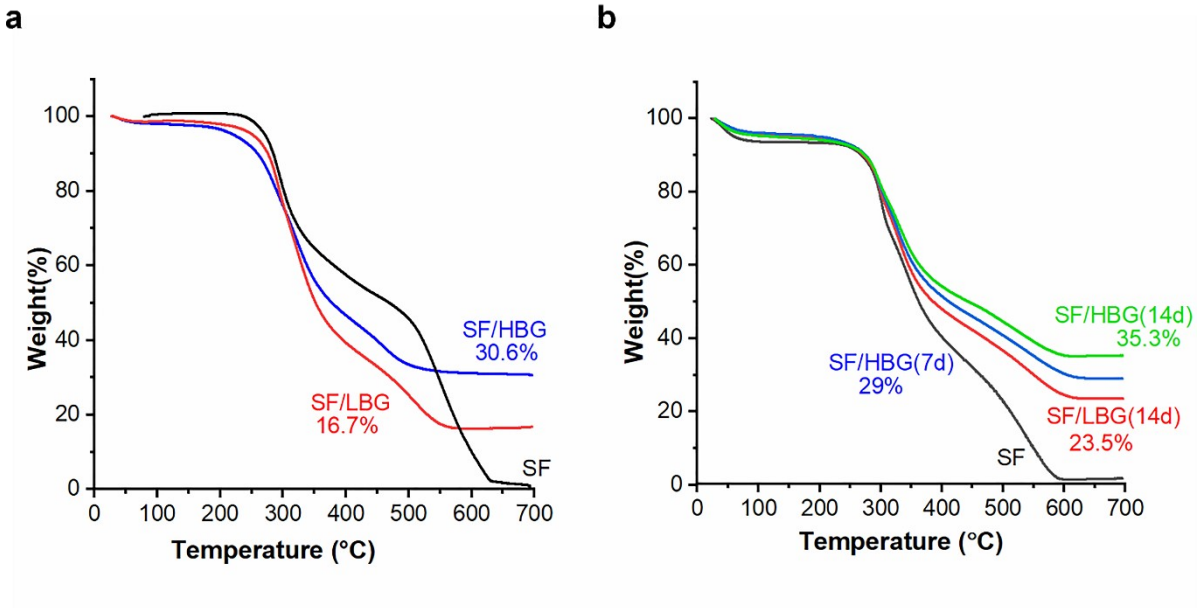


Figure S12. TGA analysis of SF aerogel and SF/BG aerogels (a) before and (b) after being soaked in a SBF for different durations.

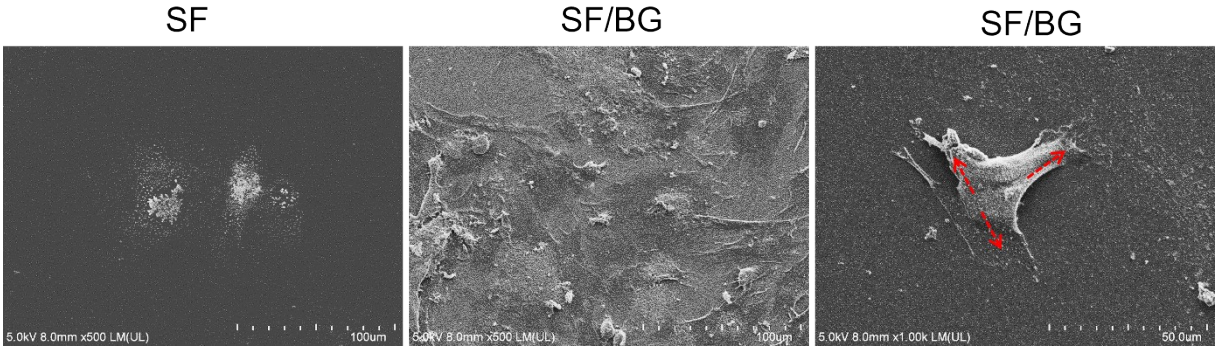


Figure S13. SEM images of BMSCs attachment and morphology on SF and SF/BG aerogels after 4-day incubation.

4. References

[1] H. Luo, Y. Zhang, Z. Wang, Z. Yang, J. Tu, Z. Liu, F. Yao, G. Xiong, Y. Wan, *Chem. Eng. J.* **2017**, *326*, 210.
[2] X. Hu, D. Kaplan and P Cebe, Macromolecules, 2006, 39. 6161-6170.
[3] T. Kokubo, H. Takadama, Biomaterials, 2006, *27*, 2907.