Supplementary information

Silkworm spinning inspired 3D printing towards high strength scaffold for bone regeneration

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Content:

Preparation of SF:

15.0 g silkworm cocoon silk was soaked into 1.5 L concentration of 0.5 % Na_2CO_3 solution in 95 ℃ for 30 min to remove its sericin, repeating the above steps twice and drying it at 37 ℃ in an oven. The ternary system (anhydrous $CaCl_2$, ethanol and water) was prepared with the molar ratio of 1:2:8. The degummed silk was dissolved in the ternary system of 150 mL at 60 ℃ for 2 h. After completely dissolving, the silk fibroin solution was cooled down to room temperature naturally. A 4 days dialyzing was processed to remove the Ca^{2+}, then the pure spongy regenerated silk fibroin was finally approached after freeze-drying.

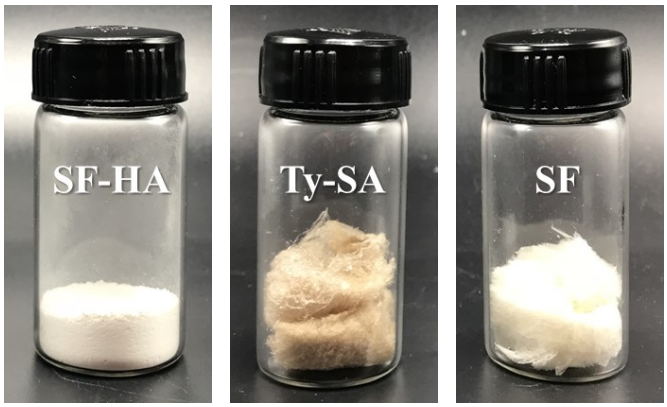

Fig. S1. Digital photos of three materials.

Synthesis of SF-HA Composites:

SF-HA composites were prepared based on the following coprecipitation synthesis procedure. 3.25 g degummed silk and 48.58 g ternary system (mentioned in the previous step) were placed into the three-neck flask. After the reaction at 75 ℃ for 2 h in a heat-collecting thermostatic magnetic stirre, the $(NH_4)_2HPO_4$ solution of 0.22 g m/L was added dropwise into the three-neck flask. The pH value was adjusted to 9-10 with ammonia water for a 24 h continual reaction. During the reaction, the solution was homogeneously mixed *via* magnetic stirring at a high speed (150 rpm). The SF-HAP suspension was pumped and filtered through a bouchard funnel after the completion of the reaction. The filtered SF-HAP materials were freezed at -20 ℃ for 24 h, then freeze-dried for later use.

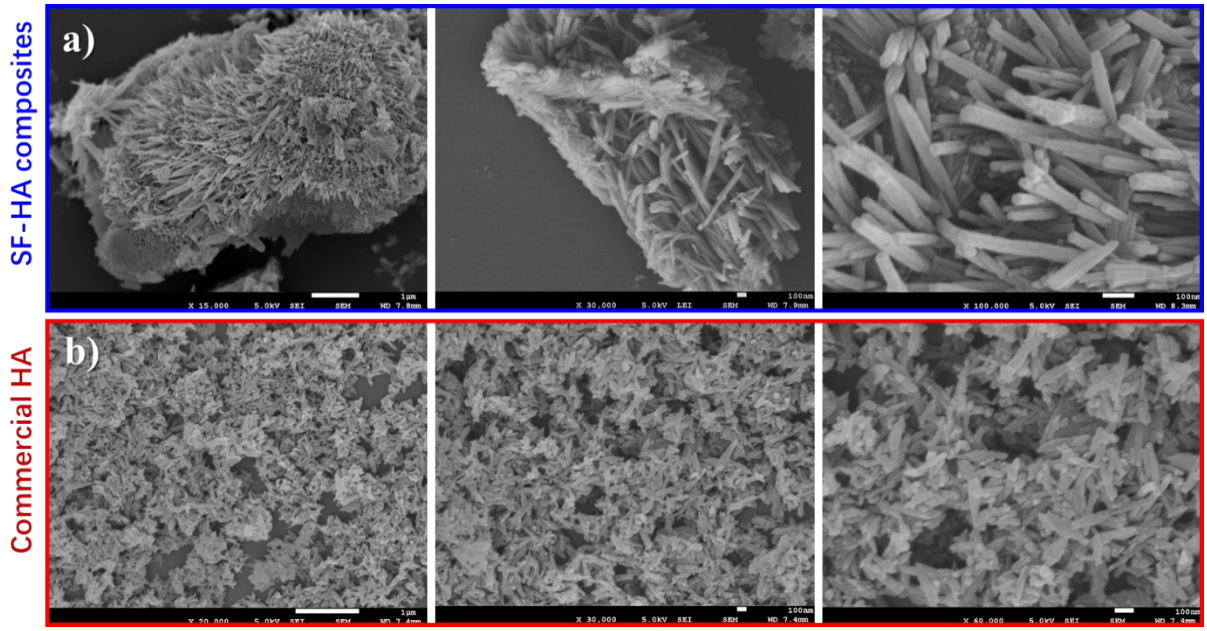

Fig. S2. SEM images of a) Synthesized SF-HA composites and b) Commercial HA.

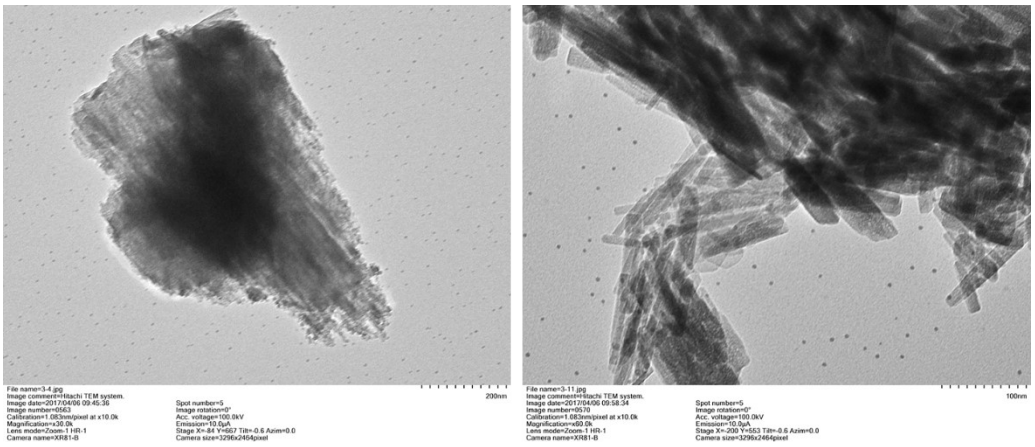

Fig. S3 TEM images of synthesized SF-HA composites.

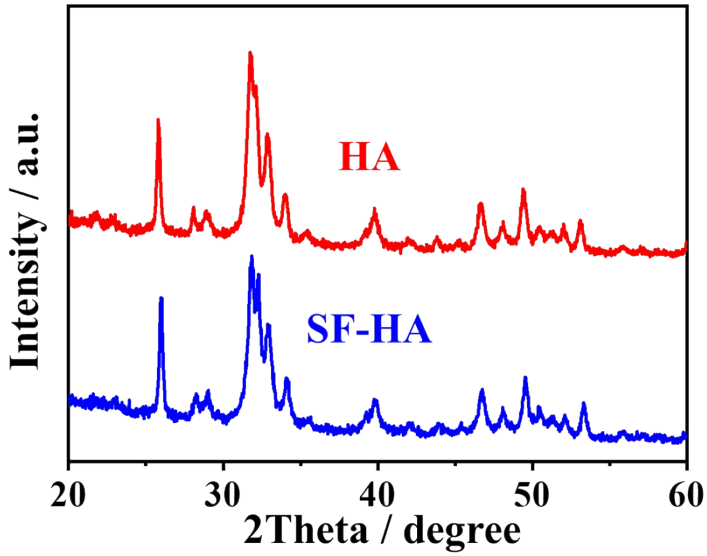

Fig. S4. XRD spectra of pure HA crystals and synthesized SF-HA composites.

Synthesis of Ty-SA:

1.0 g Sodium alginate (SA) was dissolved into 90 mL MES buffer. Then, 350 mg EDC, 132 mg NHS and 360 mg tyramine were added into the above SA solution, and stirred at 25 ℃ for 24 h to graft the tyramine (Ty) group onto the SA, under ordinary pressure. After that, the Ty-SA solution was dialyzed in a dialysis membrane (MW:10000) for 3 days. During dialysis, the dialysis was performed twice in 0.2 %NaCl solution, followed by ultra-pure water. The water was changed 3 times a day. Finally, the solution was freezed at -20℃ for 24h, and freeze-dried to obtain the spongy Ty-SA composites.

3D printing of scaffolds:

0.48 g Ty-SA was dissolved in 20 mL SF solution (0.04 g/mL). After complete dissolution, 0.8 mL Horseradish Peroxidase (HRP) solution (0.004 g/mL) and 8.0 g SF-HA were added subsequently. The mixture was kept stirring until semi-solid as the 3D printing ink. 10.0 g anhydrous $CaCl_2$, 1.5 mL H_2O_2, and 50 mL anhydrous ethanol were added into 150 mL deionized water and dissolved by stirring as the curing solution. The scaffolds with a specific shape were designed by 3ds MAX software and exported to STL format, sliced in Z direction and converted to G code printer instructions. A variable print speed and pressure of 20-50mm/s and 20-40 kPa, respectively, were used for 3D printing, at room tempreture. During extruded printing, the inner tube of the coaxial extrude-nozzle was loaded with printing ink, and the outer tube was loaded with cross-linked curing liquid, which was extruded on the base solution with an air pressure control device driving. After soaking in the 0.5 % $CaCl_2$ curing solution for 24 h, the printed scaffolds were washed with deionized water and dried in an oven at 40 ℃ for further use.

Compressive strength test:

A ZEISS (CMT5254) electronic universal testing machine was applied to obtain the compressive stress-strain curves of scaffolds. The test conditions were as follows: temperature 25 ℃, humidity 48 %, loading speed 0.5 mm/min. The scaffolds were printed into cylindrical scaffolds with 1.0 cm diameter and height.

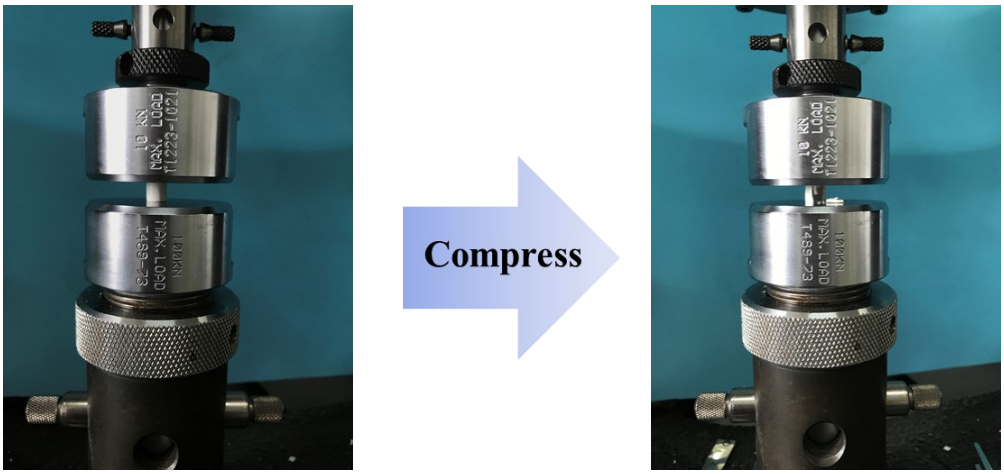

Fig. S5. The compressive stress-strain test on the ZEISS electronic universal testing machine.

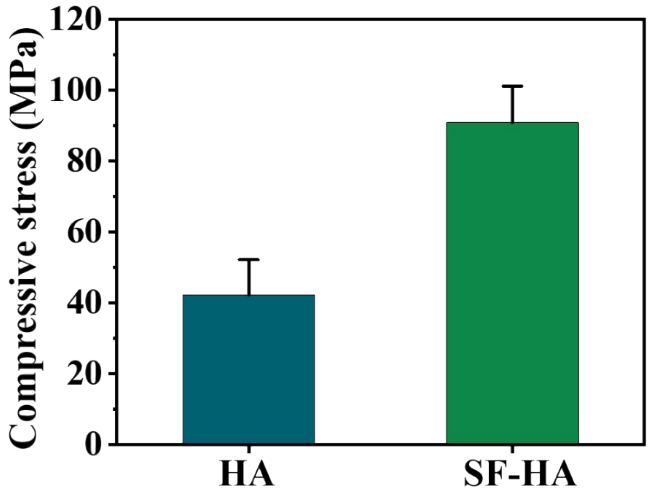

Fig. S6. Compressive strength of scaffolds printed with HA or SF-HA ink. The synthesized SF-HA composite shows 2-folds higher compressive strength than commercial HA.

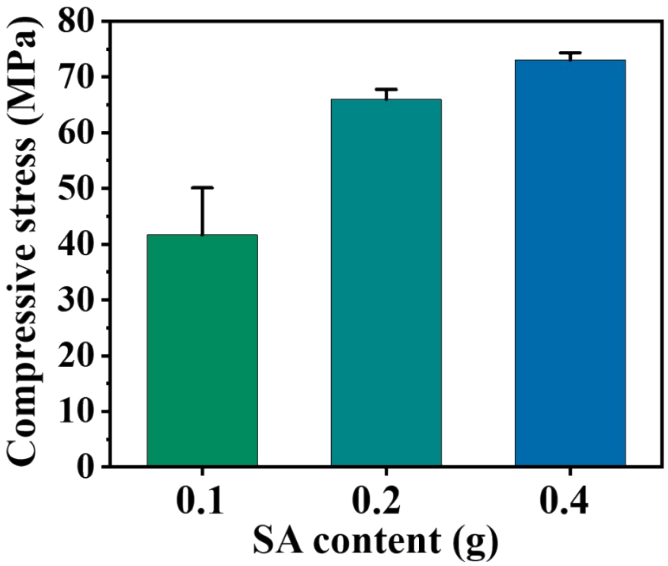

Fig. S7. The sodium alginate (SA) content influence to compressive strength of printed scaffolds.

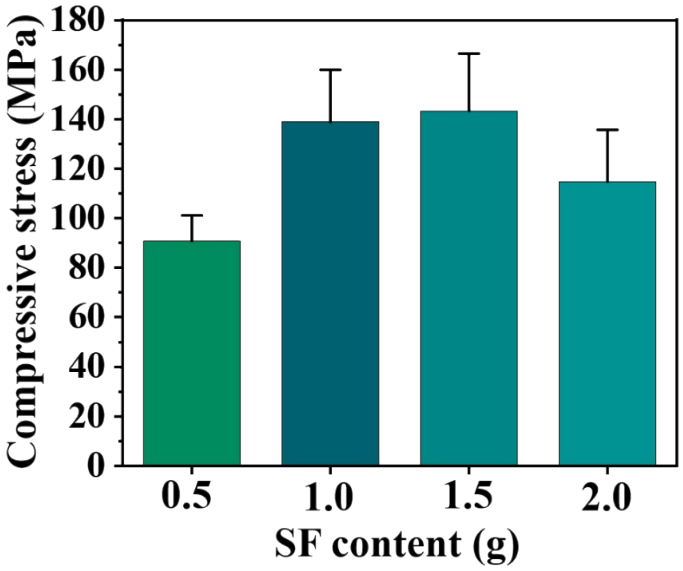

Fig. S8. The silk fibroin (SF) content influence to compressive strength of printed scaffolds.

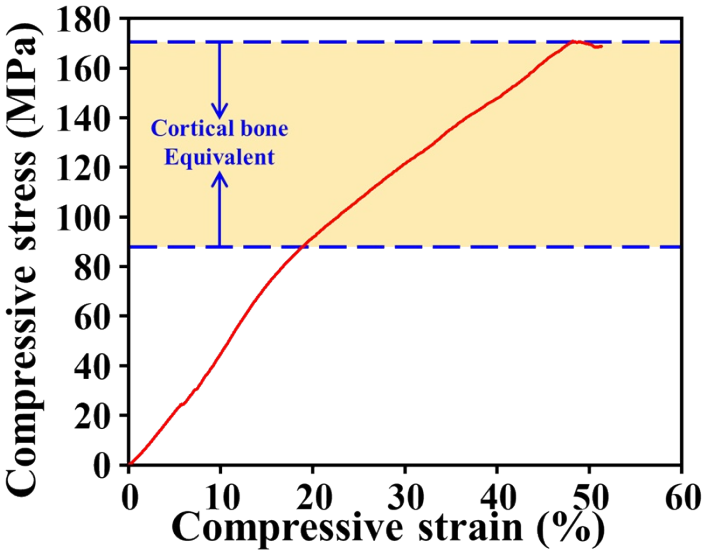

Fig. S9. Compressive stress-strain curves of SF-HA scaffold with the most optimized recipe (SF-HA/SA/SF=10/0.8/3), which can approach the cortical bone equivalent (90~170 MPa).

Bending strength test:

A ZEISS (CMT5254) electronic universal testing machine was adopted to test the bending strength of scaffolds on the condition of temperature 26 ℃, humidity 28 %, loading speed 2

mm/s and span 32 mm. The scaffolds were printed rodlike scaffolds with 6.0 cm length, 0.5 cm width and height.

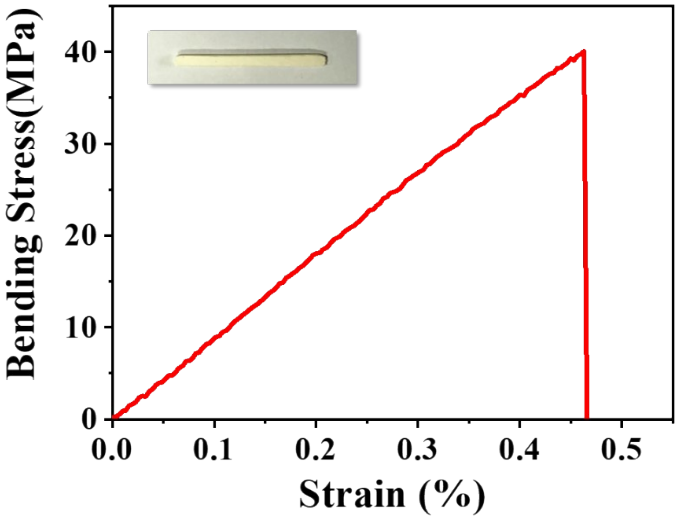

Fig. S10. Bending stress-strain curves of SF-HA scaffold with the most optimized recipe (SF-HA/SA/SF=10/0.8/1).

Scaffold stability investigation in the physiological environment:

The stability of the printed scaffolds in physiological environment was tested. The grid-like scaffold was printed to mimic the compact cortical bone and loose cancellous bone structure of human skeleton. The scaffolds were soaked in the simulated body fluid (SBF) or Dulbecco's modified eagle medium (DMEM) to simulate the physiological environment of human body respectively, and the macro morphological changes of the scaffold were observed. The scaffold could maintain its original morphology after soaking in PBS for three months, but it collapsed after soaking in DMEM medium for two days, which made it difficult to apply in the complex physiological environment of human body. In this regard, a method of cross-linking silk fibroin protein and Ty-SA with horseradish peroxidase (HRP), respectively, was performed to improve scaffold collapse in DMEM medium. Tyramine sodium alginate (Ty-SA) was adopted to perform the cross-linking with horseradish peroxidase (HRP). Meanwhile, the silk fibroin (SF) and HRP also underwent a widely and mildly enzymatic cross-

linking. The two cross-linking effects promoted the stability of SF-HA scaffolds in the physiological environment, where they still remained intact, after soaking in DMEM solution for 15 days.

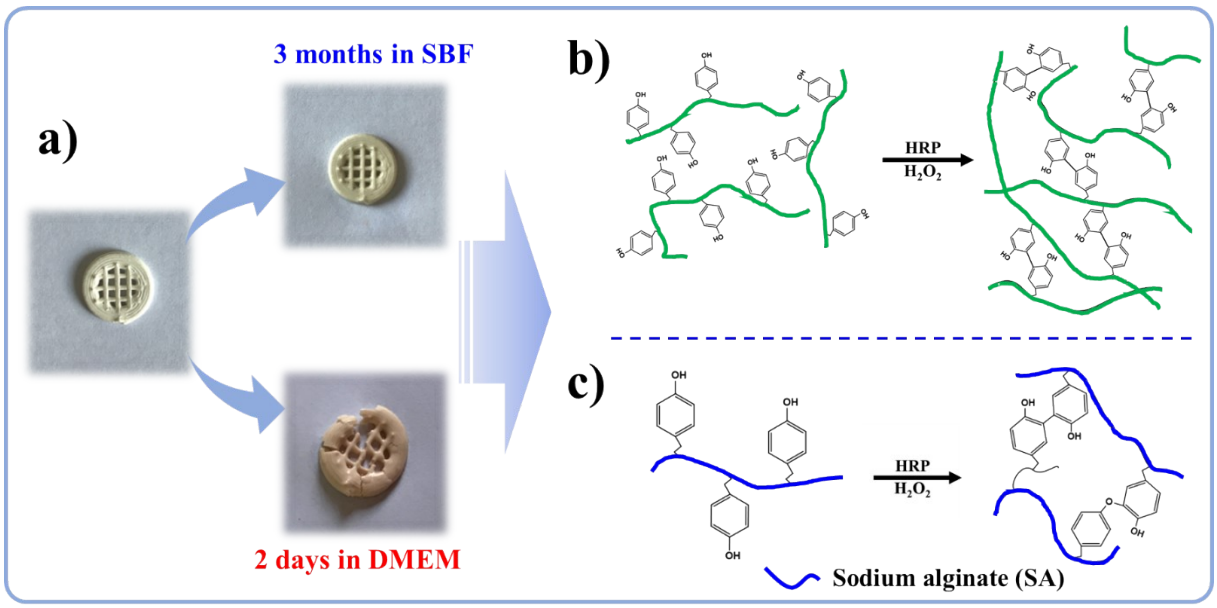

Fig. S11. a) Stability comparison of SA/HA/SF scaffolds in simulated body fluid (SBF) and Dulbecco's modified eagle medium (DMEM). In order to solve the collapse of the scaffold in DMEM. b) Cross-linking of silk fibroin (SF) and HRP. c) Cross-linking of tyramine sodium alginate (Ty-SA) and horseradish peroxidase (HRP).

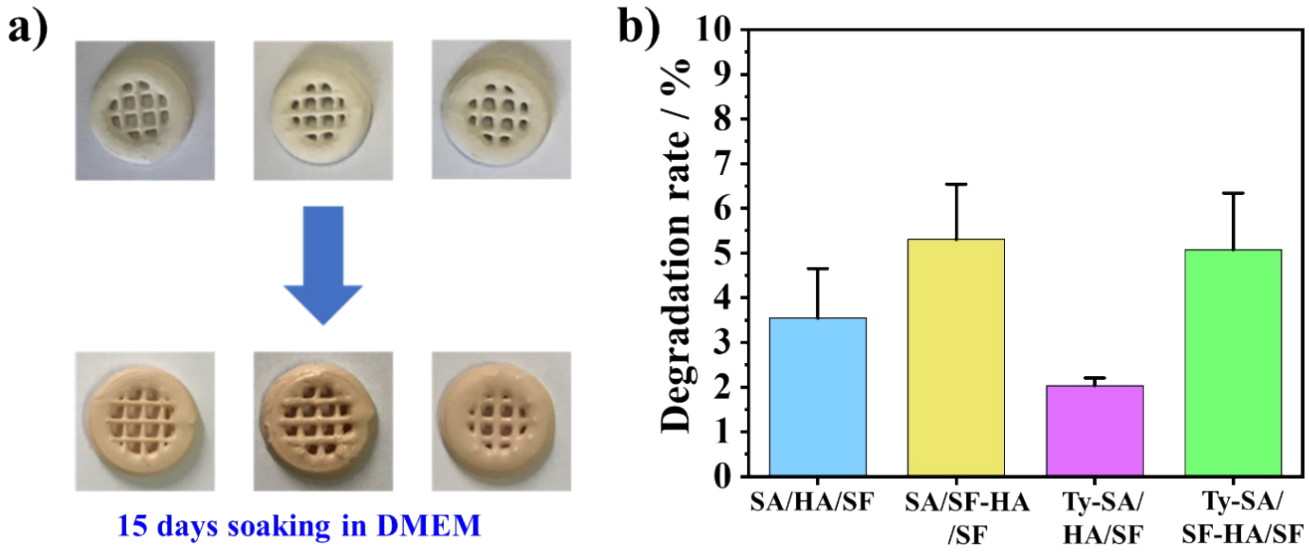

Fig. S12. a) Ty-SA printed scaffolds soaked in DMEM 15 days. b) Degradation of printed scaffolds in PBS after 4 weeks.

The molecular structure of SF investigation：

Circular dichroism (CD) spectrometer is a common method for analyzing conformational changes of proteins. We performed CD spectroscopy to investigate the secondary structural conformation changes of SF, during the preparing of printed scaffolds. Quartz sample pools with optical diameter of 1.0 mm were used in CD spectrometer (Chirascan plus), which was used to determine silk fibroin at 190-250 nm at room temperature. Each sample was scanned 2 times with 10mm the pathlength and 0.5 s time per point. The concentration of SF and Ty-SA were 0.01 g/L and 0.025 g/L, respectively. The circle of ethanol-water mixed solvent (scanned only once) with the same concentration (60 wt%) was determined under the same experimental conditions dichroism, and its effect has been subtracted from the final results.

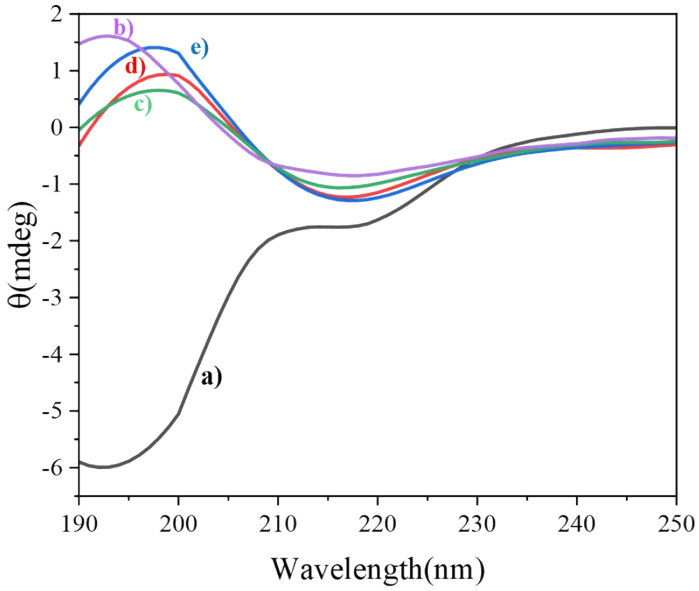

Fig. S13. The CD spectroscopic data of SF, SF in ethanol and SF/Ty-SA in ethanol. a) The SF. b) The SF in ethanol. c)The Ty-SA. d) The Ty-SA in ethanol. e) The SF and Ty-SA in ethanol.

Mechanical strength of printed SF-HA scaffolds:

The common cylindrical scaffolds with various cavity structures (grid, hollow, honeycomb) were printed and explored their mechanical supportability.

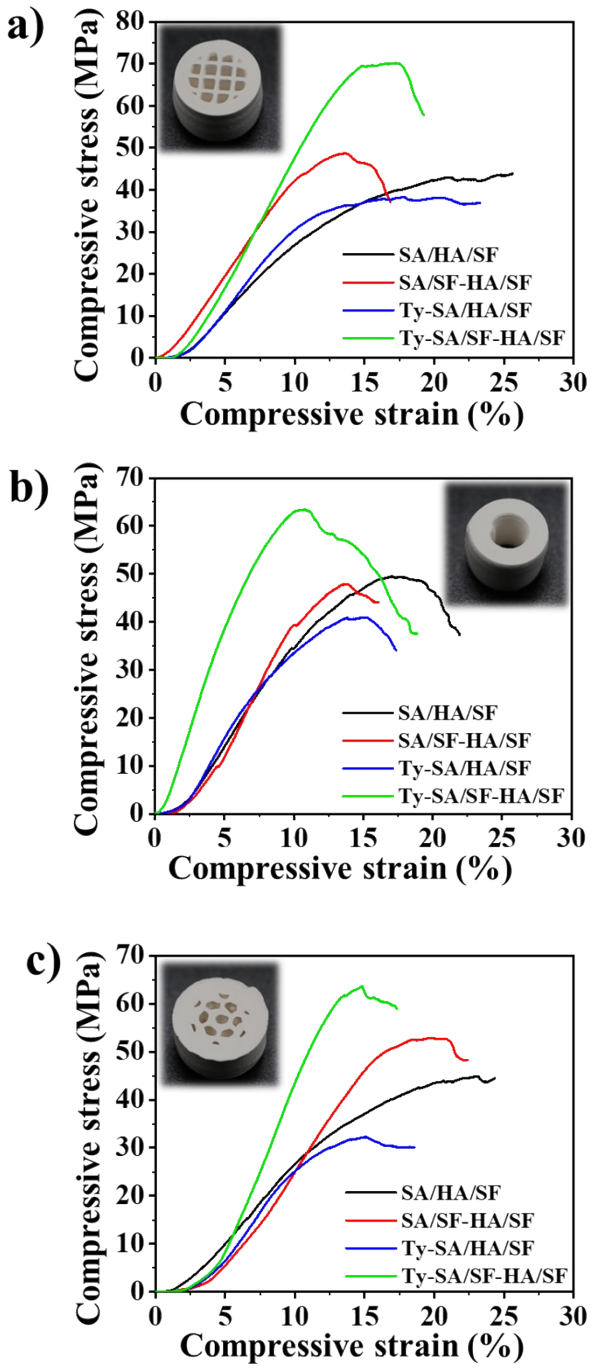

Fig. S14. Typical compressive stress-strain curves of four kinds of scaffolds with different shapes. compressive stress-strain curves of a) Grid, b) Hollow cylinder and c) Honeycomb scaffold, inset: digital photos of the tested scaffolds.

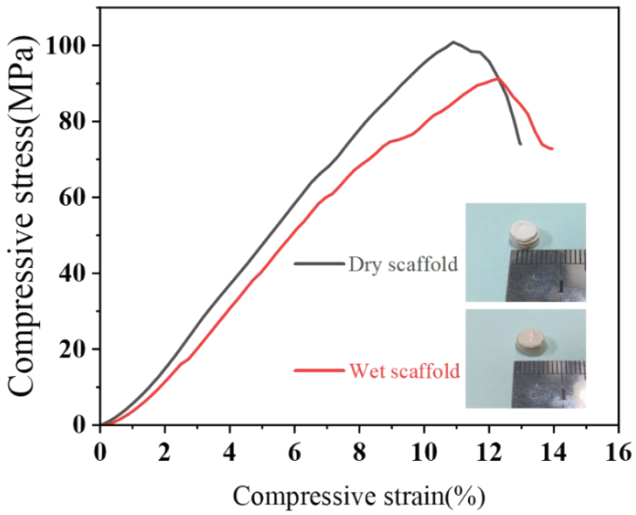

Fig. S15. Compressive strength comparison between wet and dry printed scaffolds.

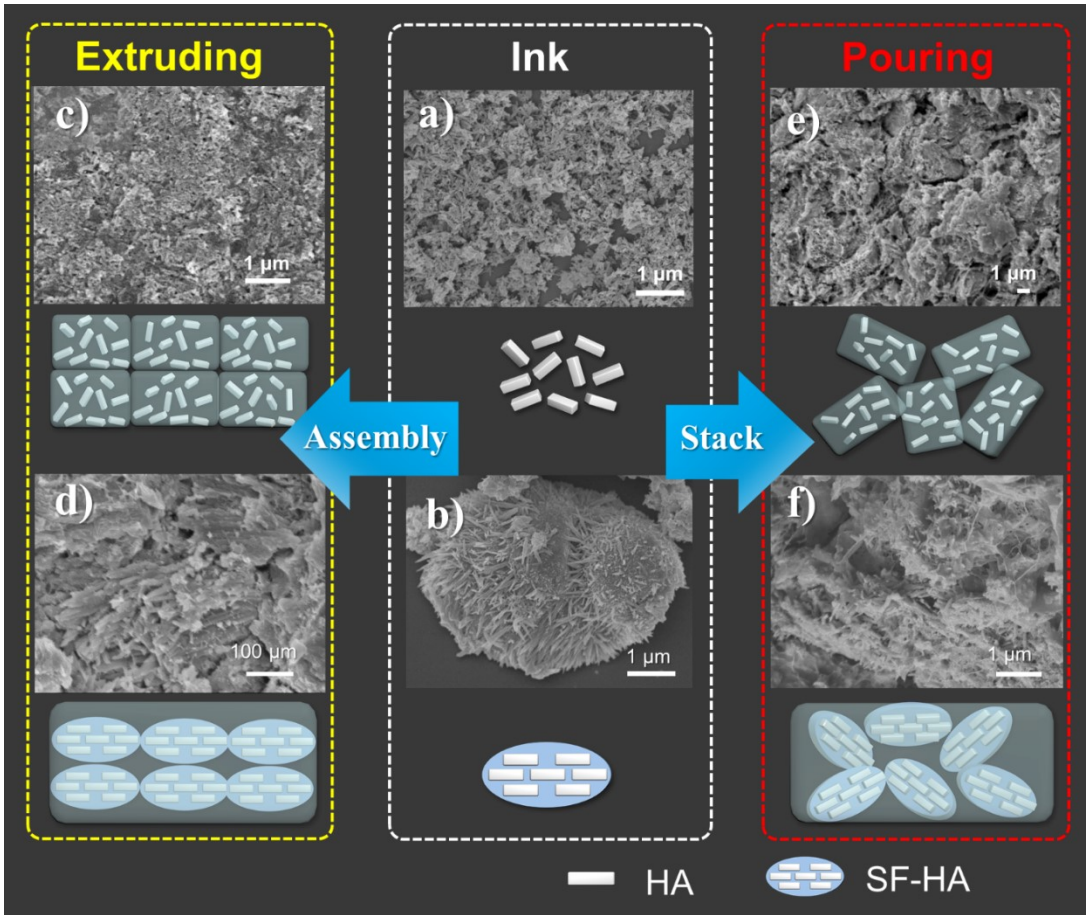

Fig. S16. Microstructures of scaffolds *via* different prototyping processes. SEM images of a) The random HA nanocrystals and b) Aligned crystalline SF-HA composites. The section view of c, d) SF-HA scaffold *via* extruding and e, f) HA scaffold *via* pouring. The extruding process directs the orientation of SF-HA micro-bricks to achieve the aligned assembly and compacted structure. And the pouring process results the random stacking.

***In vitro* bioactivity of silk extruding biomimetic 3D printed scaffolds:**

All the experiments were in accordance with the Guangdong Medical Experimental Animal Center (License Number: SCXK(Guangdong)2018-0002) and approved by the Animal Protection and Utilization Committee of Jinan University (License Number SYXK(Guangdong)2020-0230) Bone marrow stem cells (BMSCs) were isolated and cultured according to the method reported previously. In simple terms, the eight-week-old C57BL/6 male mice (body weight 20-25 g) were sacrificed. The femur was removed after disinfection and the bone marrow cavity was washed with Dulbecco's modified Eagle's medium (DMEM, HyClone, China). After centrifugation and suspension, the cell suspension was cultured in DMEM, supplemented with 10 % fetal bovine serum (Invitgen) and 1 %penicillin-streptomycin solution (Invitgen), at 37 ℃ and 5 % CO_2 incubator. The extracts of scaffolds were prepared with DMEM medium, and the preparation of material extracts was in accordance with biological evaluation criteria for medical devices (GB/T 16886.12-2005). The extraction medium was medium containing serum (0.1 g/mL) at 37℃ for 24 h. The extracts were filtered with a needle filter then exposed to UV lamp for 30 min for sterilization. The proliferation activity of BMSCs was detected by CCK-8 method. CCK-8 solution was prepared with the volume ratio of CCK-8 reagent to DMEM medium of 1:10, firstly. BMSCs were inoculated into the extract of scaffolds in 48-well culture plate and cultured for 1, 3 and 5 days, respectively. The well plates were taken out, and 500 μL CCK-8 solution was added to each well, culturing in a 37 ℃, 5 % CO_2 cell incubator for 2 h. The absorbance at 450 nm was measured with a microplate reader (TECAN, Tecan/Spark 10M). Each experiment was performed at least three times. All the results are expressed by the optical density minus the absorbance of the blank trap.

Hemolysis test:

Mouse red blood cell (ShuoHeng Biotechnology, GuangZhou) was centrifuged with 3000 rpm for 5 min and washed with PBS solution for 5 times, firstly. Pure red blood cell was prepared into 5 % red blood cell suspension with PBS. Red blood cell suspension only added with PBS was used as negative control group, and the suspension with ultra-pure water was acted as positive control group. After ultrasonic treatment, four different kinds of scaffolds powder suspension (100 μg/mL, experimental groups,) mixed with red blood cell suspension (0.25 mL : 0.25 mL) was cultured at 37 ℃ for 8 h. The samples in each group were centrifuged at 1500 RPM for 5 min and photographed then the supernatant was added 250 μL into each well of the 96-well plate. Finally, the absorbance was measured with a microplate reader at 570 nm. The hemolysis rate *Z* of the sample is calculated according to the following equation:

$$Z\,(\%) = \frac{D_s - D_n}{D_p - D_n} \times 100\%$$

Z is the hemolysis rate of the sample to be tested, D_s is the absorbance of the test group, D_n is the absorbance of the negative control group, D_p is the absorbance of the positive control group.

Flow cytometry apoptosis experiment:

Annexin V/PI double staining method was used to detect apoptosis, where cells at different stages of apoptosis could be distinguished. The experimental procedures were as follows: The extract of printed scaffold was placed in 6-well plate, then 3 mL DMEM medium and 150,000 BMSCs were added into each well. After culturing at 37 °C in a 5 % CO_2 incubator for 24 h, apoptosis was induced in the specified way. The adherent cells were digested with trypsin without EDTA and centrifuged. After re-suspension, the staining was carried out using Annexin V/PI staining kit according to manufacturer's recommendations, and the apoptosis rate of the cells was analyzed by flow cytometry.

Osteogenic activity:

20000 BMSCs cells and the printed scaffolds added in a 24-well plate culturing in the osteogenic induction medium (supplemented with 10 mM β-glycerophosphate, 10 mM dexamethasone, and 50 ug/mL ascorbic acid) for 7 and 14 days were fixed with 4% paraformaldehyde (Jiancheng Technology, NanJing) for 20 min and washed 2-3 times with 1 x PBS. Then the cells were prepared to ALP staining under the requirements of the BCIP/NBT kit (Beyotime Biotechnology) instructions, observed to be gray-brown deposition under an optical microscope. Meanwhile, the cells were lysed with 1 % Tritonx-100 (Jiancheng Technology, NanJing) for 40 min, 30 μL and 20 μL of the cell suspensions were respectively dropped into a 96-well plate measuring the OD values at 520 and 562 nm to determine the ALP activity by the ALP (Jiancheng Technology, NanJing) and BCA protein kits (Keygen Biotechnology, NanJing). In calcium deposition test, BMSCs cells were cultivated on the printed scaffolds in osteogenic induction medium for 14 days. Then, after stained with 1% alizarin red, the stained cells were desorbed with 10% CPC in PBS to measure the quantity of calcium deposition with enzyme-linked immunosorbent assay plate reader at 540 nm. Finally, all results of staining were observed under a stereo microscope (Stemi 2000-C, Carl Zeiss, Germany).

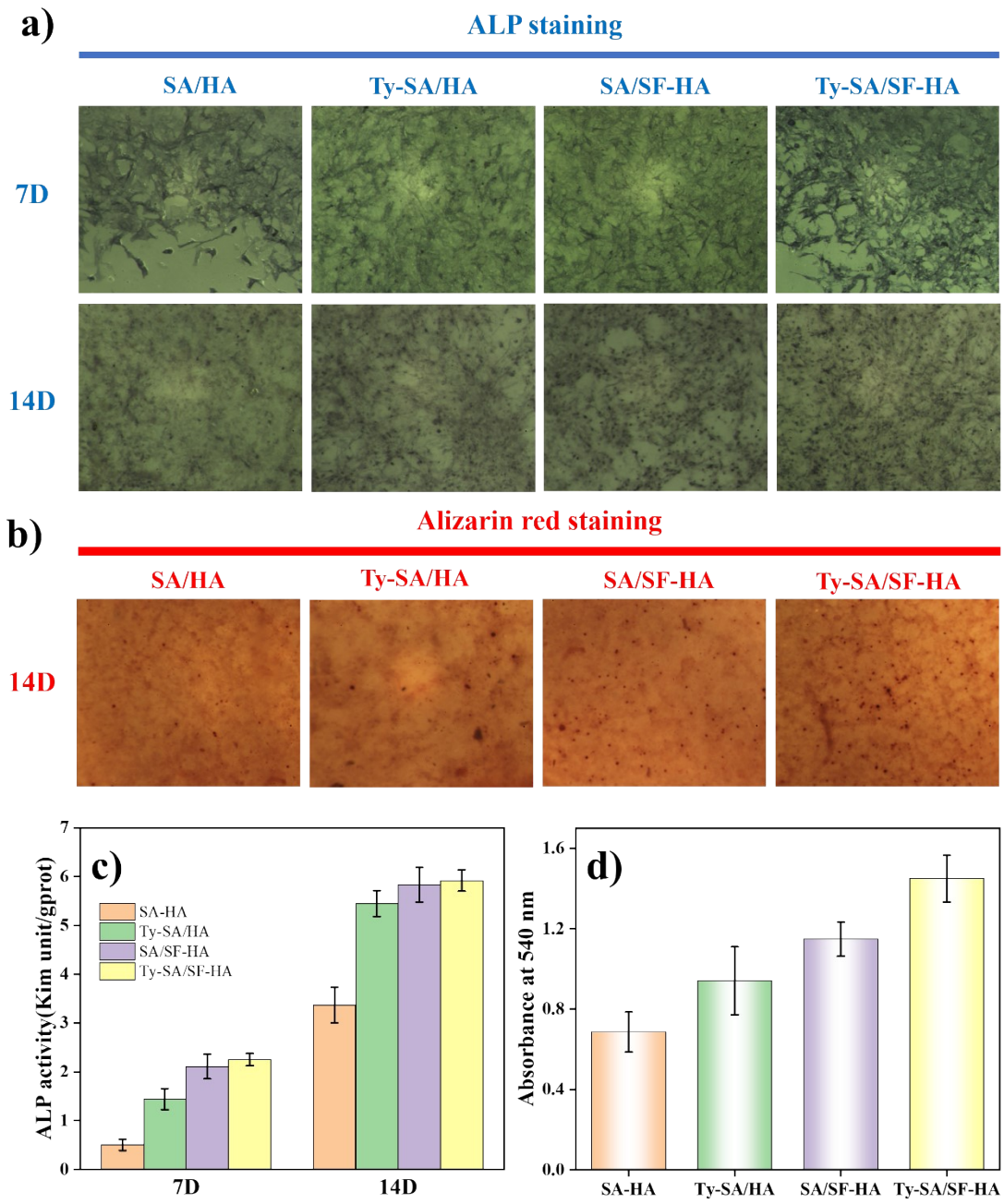

Fig. S17. The osteogenic capability of printed scaffolds was evaluated by ALP staining, ALP activity and cultured in osteogenic media for an additional 14 days to determine mineralization assay. a) Optical photograph views of ALP staining (magnification 100x). b) Optical photograph views of alizarin red staining (magnification 40x). c) Quantitative analysis of ALP activity. d) Quantitative analysis of alizarin red.

The photographs of 3D printed scaffolds' size before implantation:

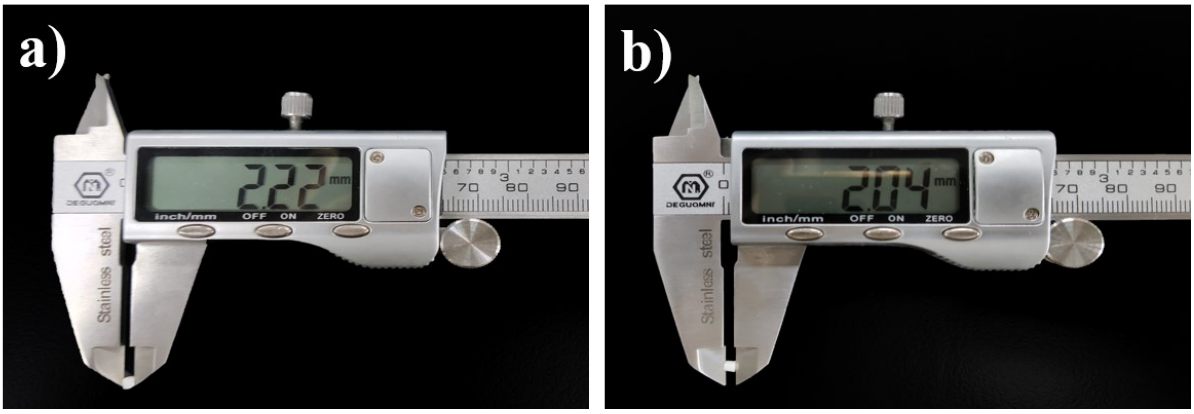

Fig. S18. The size of printed scaffolds implanted *in vivo*. a) The diameter; b) The height

***In vivo* osteogenic activity analysis of silk extruding bionic 3D printed scaffolds:**

All the experiments were in accordance with the Guangdong Medical Experimental Animal Center (License Number: SCXK(Guangdong)2018-0002) and approved by the Animal Protection and Utilization Committee of Jinan University (License Number SYXK(Guangdong)2020-0230). Eight specific pathogen-free male Sprague-Dawley rats (6-8 weeks old) were selected. The rats in the model control group were anesthetized and the right leg was selected as the experimental leg. After the operation, the femur defect of the external bone was not treated (drilling a bone hole with about 2 mm in diameter and height). After suture and disinfection, the rats were fed routinely. The rats in the experimental group were anesthetized and the femur defects were made by the same method, but they were filled with Ty-SA/SF-HA scaffold. After 8 weeks, the rats were sacrificed. For the standard histologic, after limb harvesting and removal of adjacent soft tissue, the specimens were immersed in the 4 % paraformaldehyde (P0099,100 mL) fix solution for 24 h. Then, scanner software of Micro-CT (Skyscan1174) was used to scan each sample. The experiment steps were as follows: The experimental samples were fixed in the fixator along the long axis, the 70KV voltage, the 7 W power, superposition of four frames, the 0.72 °, the 100 μs exposure time, and the PRJ image was obtained. After reconstruction, the cylindrical rigid interest area near the window was

selected for analysis. Followed by decalcification in 10 % EDTA (Servicebio) for 2 weeks in the constant temperature shaker, where the temperature was controlled between 25 ℃ and 30 ℃ and the rate of the shaker was about 110-120 rpm. The decalcification solution was replaced once 2-3D. The softening bone defect tissues then were paraffined and sectioned at a 4 μm thickness for subsequent histological analysis. Slides were hydrated *via* an ethanol gradient. Finally, these paraffin sections underwent histological staining, including H&E, saffron O, Alcian blue and Van Gieson staining, which suggested the scaffolds we designed significantly promoted new bone formation in the bone defect site, after microscopic examination, image collection and analysis.

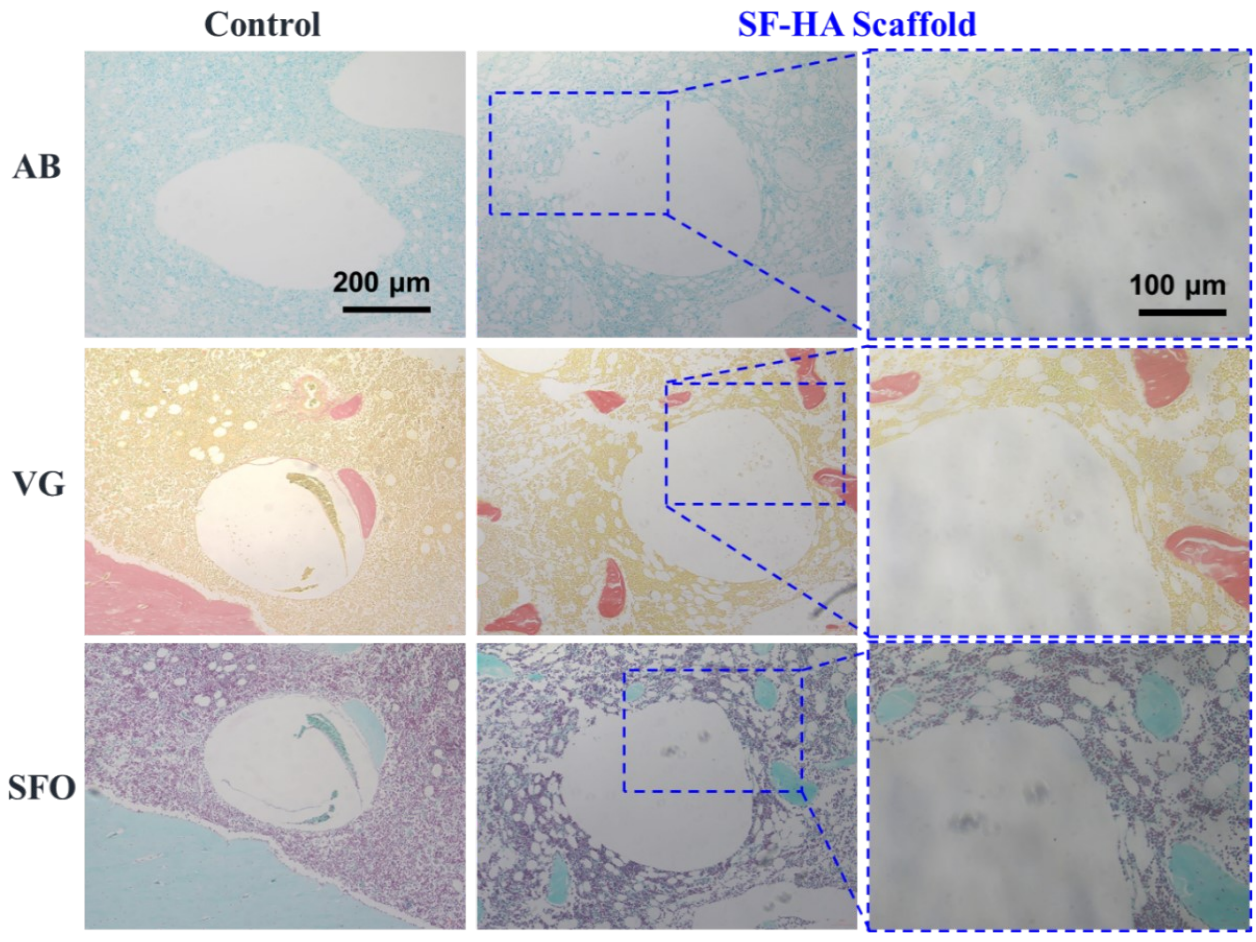

Fig. S19. *In vitro* histological evaluation for Sprague-Dawley rat femoral tissue engineering. The histological sections with Aniline blue (AB), Van Gieson (VG) and Safranin-O (SFO) pathological staining.

Small angle X-ray scattering of printed scaffolds:

The SAXS studies were carried out in Bruker D8 Advance. The spot size andwavelength of the monochromatic X-ray beam were 25 µm × 25 µm and 1.54 Å, respectively. Experiments were performed at ambient temperature, a sample-detector distance of 7573 mm, q range of 0.1933 $Å^{-1}$ to 6.030 $Å^{-1}$, and beam stop of 4 mm.

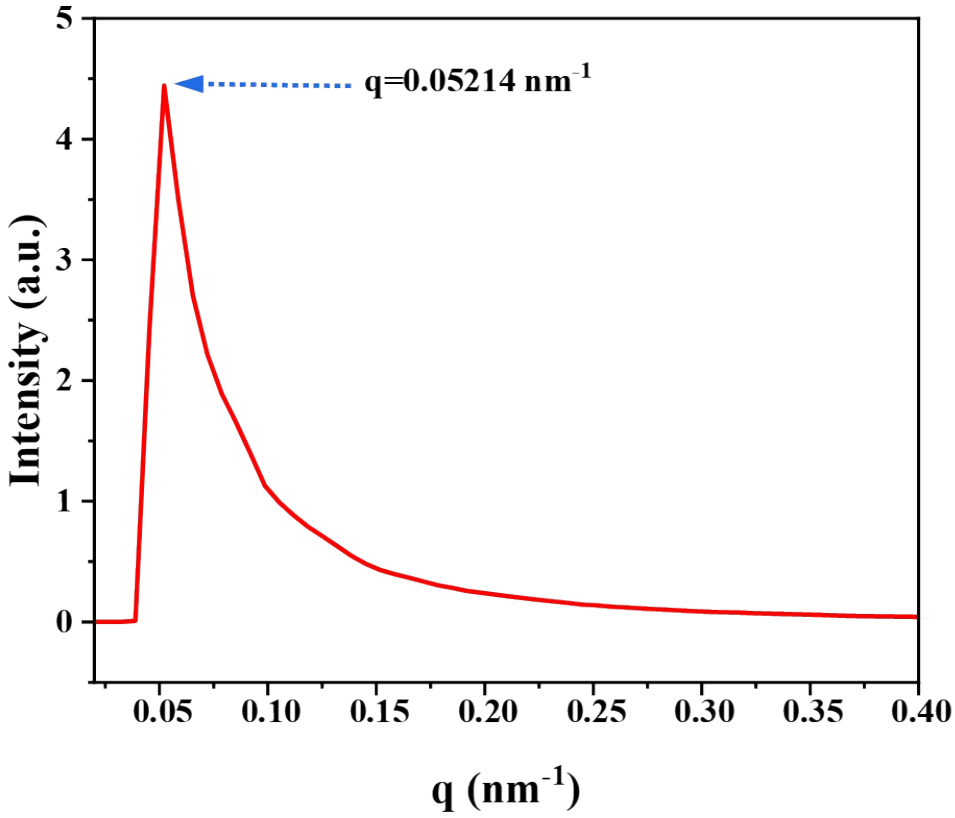

Fig. S20. The 1D SAXS diffraction pattern of printed scaffolds.