Supporting Information

A silk based high impact composite for core decompression of the femoral head

Jin Wang,[‡,a] Ruixin Mi,[‡,b,c] Guanglei Zhao,[‡,a] Jingsheng Shi,[a] Jie Chen,[a] Changquan Liu,[a] Zhengzhong Shao,[,b,c] and Jun Xia[*,a]*

a Department of Orthopedics, Huashan Hospital, Fudan University, Shanghai 200040, P. R. China.

b State Key Laboratory of Molecular Engineering of Polymers, Laboratory of Advanced Materials, Fudan University, Shanghai 200433, P. R. China.

c Department of Macromolecular Science, Fudan University, Shanghai 200433, P. R. China.

*Author to whom correspondence should be addressed Z. Z. Shao (E-mail: zzshao@fudan.edu.cn) and J. Xia (E-mail: 15715163094@163.com)

‡ These authors equally contributed to this work.

Experimental

1. Fabrication of Scaffolds

1.1 Preparation of regenerated silk fibroin (RSF) and HPMC aqueous solution

The Bombyx mori silk fibroin solution was obtained after degumming, dissolving and dialyzing process in accordance with a typical protocol described in available literatures and procedures, yielding a solution of protein concentration of 4 wt%, and stored at 4°C for further use. Although such regeneration process was applied, the term of silk fibroin (SF) in this work is still employed to refer the regenerated silk fibroin (RSF). The Hydroxypropyl methylcellulose (HPMC) is directly dissolved in deionized water at room temperature according to the required concentration (mass fraction).

1.2 Preparation of SF/HPMC gel

The regenerated SF solution was concentrated to the required concentration by polyethylene glycol (20 wt%) through reverse dialysis, followed by adding the HPMC solution (same mass fraction with SF) to prepare blending solutions with different mass ratios of SF:HPMC (9.5:0.5, 9:1, 8:2, 7:3, 6:4). Among these samples, we named the sample (10 wt% with SF : HPMC = 9:1) as “10-9-1”, named the sample (10wt% with SF : HPMC = 8:2) as “10-8-2”, and analogously named other samples. Finally, the hydrogel was formed by heating the above well-mixed solution at 70 ℃ for 2 hours.

1.3 Fabrication of solid core part

The matured composite hydrogel was obtained by immersing the hydrogel in a

solvent for 12 hours (such as absolute ethanol) to induce the conformational transformation of silk protein. After that, it is air-dried to a constant weight at room temperature. Then the solid silk bars with a length of 30 mm and a diameter of 3 mm were made by using a lathe and other tools.

1.4 Porous coating

The composite hydrogel formed by heating (70 ℃, 2h) was frozen at -20 ° C for 24 hours, utilizing the ice crystals to form the pores inside the gel. After taking out, it is immersed in a solvent for 12 h (such as absolute ethanol) that can induce the conformational transformation of silk protein. Then it was freeze dried to constant weight at room temperature for use.

2. Biomineralization

The biomineralization of SF/HPMC composite scaffolds was performed using Simulated body fluid (SBF) soaking method. The SBF was prepared according to the previously published protocol(45). The SF/HPMC composite scaffolds were immersed in the SBF at 37˚C for 1, 3, 5, 7 days, respectively. Then five groups of composite scaffolds (HAP-0d, HAP-1d, HAP-3d, HAP-5d, HAP-7d) were air-dried and stored.

3. Characterization

3.1 Mechanical testing

Mechanical Properties were measured by Instron. All samples were machined into cylinders with the diameter of 5mm and the height of 10mm, then tested under compression mode with the compressing rate of 10mm/min.

3.2 SEM scanning

Scanning microscopy (SEM) was performed with a TESCAN TS5136 MM at 20 kV of accelerating voltage (Au-coated prior to examination). The surface and cross-section of the samples were imaged, respectively.

3.3 TGA

Thermogravimetric Analysis (TGA) was performed on Perkin-Elemer Pyris 1 under air gas with flow rate of 40 cm^3 min^{-1} at a heating rate of 10°C per minute from 50°C to 800°C.

3.4 XRD

Powder X-ray diffraction (XRD) patterns were recorded on a Bruker D8 X-ray diffractometer (Germany) with Ni-ltered Cu Kα radiation (40 kV, 40 mA), applying a scanning rate of 0.02°s-1 in the 2θ range from 10° to 80°.

3.5 Pore size and porosity

The holes were randomly selected by the SEM image and more than 20 samples were repeated. the pore size and distribution of the stent are expressed by mean ± SD.

We use solvent replacement method to test the porosity of porous scaffolds. The weight of the silk protein-based porous scaffold material (diameter 10 mm, height 10 mm) were measured firstly, then immersed it in cyclohexane. Then the sample was weighed again after wiped the liquid on the surface. Calculate the porosity (%) of silk protein-based porous scaffolds according to the following formula:

$$\text{Porosity (\%)} = (M2 - M1) / \rho s \times 100\% \, Vm$$

M1 is the mass before material soaking, M2 is the mass after material soaking, ρs is

the density of the cyclohexane, and Vm is the apparent volume of the material.

4. Cell culture

The pre-osteoblast cell line MC3T3-E1 (subclone-14) was purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. The cells were cultured and passaged using α-modified minimal essential medium (a-MEM; HyClone) comprised of 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 U/ml streptomycin. The medium was changed every two days and the cells at passages 3~5 were used for subsequent experiments *in vitro*.

5. In vitro cellular response

5.1 Sample extracts preparation

The extracts of different scaffolds were prepared according to the GB/T 16175-1996. Briefly, samples were incubated in α-MEM cell culture medium at 37˚C and 5% CO_2 for 72h, with the sample weight to medium volume ratio was 0.2 g/ml. The extracts of different samples were subsequently used for cytotoxicity evaluation before filtered (0.22 μm) and disinfected with ultraviolet rays.

5.2 cytotoxicity evaluation

The MC3T3-E1 cells were seeded in the 96 well cell plate (2000 cells/well) and cultivated for 24h. Then the culture medium was replaced with sample extracts and the Cell Counting kit-8 (CCK-8, Dojindo, Japan) was used to quantify relative cell growth according to the manufacturer's instructions at 1, 3, 5 and 7 days. Briefly, 110μl CCK-8 test solution (100μl culture medium and 10μl CCK-8 regeant) were added to each well and incubated without light for 2h. Then the optical density (OD)

value was detected by a microplate reader (BioTek, Epoch2, USA) at 450 nm. A negative control was set in which the cells were cultivated in α-MEM culture medium all the time. The relative growth rate (RGR) were calculated with the following criteria:

Relative growth rate (RGR) = OD value in experiment group/ OD value in negative control group × 100 %.

5.3 Cell seeded

To prepare for cell seeding, the disc porous composite scaffolds were placed in the 24-well cell culture plate (one specimen a well) after autoclave sterilization (121˚C, 60min). Then a 50μl droplet of the suspended MC3T3-E1 cells (Passage 3) were seeded on each scaffold, containing 200,000 cells. Cells were allowed to penetrate and attach within the scaffolds for 2h before the addition of 950μl cell culture medium. Finally, the cell-scaffold composites were co-incubated at 37˚C and 5% CO_2, changing the medium every 2 days.

5.4 Cell viability and distribution

After the cells were cultured for 14 days, the samples were rinsed with PBS for 3 times and then stained with 4',6-diamidino-2-phenylindole (DAPI). Samples were put in the 24 well plates and 500μl DAPI solution was added into each well followed by cultivating in the dark for 30 minutes. Then the cell distribution was observed under fluorescence microscope (Olympus IX70, Germany) and the images were saved.

In addition, the three-dimensional (3D) distribution of MC3T3-E1 cells within scaffolds were visualized using a Confocal Laser Scanning Microscope (CLSM,

Zeiss, Oberkochen, Germany) with live/dead cell staining kit (Sigma-Aldrich, USA). In brief, samples were also put in the 24 well plates and 500μl working solution (5μl Calcein-AM and 20 μl Propidium iodide diluted in 10 ml PBS) was added into each well. The live cell (stained green) and the dead cell (stained red) were observed at the 490nm emission light.

5.5 Morphology of MC3T3-E1 cells on scaffolds

The cell-scaffold composite samples were rinsed with PBS for 3 times and fixed with 2.5% glutaraldehyde at 4 ˚C for one night. Then the samples were dehydrated using graded ethanol series (30%, 50%, 70%, 90%, 100%, 100%, 10 min at each concentration) followed by freeze-dried for overnight. Finally, the samples were sputter coated with gold (10mA, 120s) using the ion sputtering equipment (ION SOUTTER, E-1045, HITACHI, Japan) and then observed by the scanning electron microscopy system (SEM, S-4800, HITACHI, Japan).

5.6 Cell proliferation on scaffolds

MTT assay (MTT assay kit, Beyotime, China) were performed after the cell-scaffolds co-cultured for 1, 3, 5,7 days to semi-quantitatively evaluate the cell proliferation on all scaffolds. The samples were rinsed with PBS for three times and shifted to a new 24-wel cell culture plate. Then 500μl MTT solution (5mg/ml in PBS) was added into each well, and incubated for 4 hours at 37 ˚C. 1 ml dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. The OD value of each well was measured at the wavelength of 570 nm using a microplate reader (Bio-Rad, USA).

5.7 Cell Osteogenic differentiation on scaffolds

The osteogenic differentiation of MC3T3-E1 cells on the scaffolds were assessed by testing the Alkaline Phosphatase (ALP) activity and the relative expression level of osteogenic related genes. After cells were cultured for 14 days, the scaffolds were crushed into fragments the cell were lysed by the 1% Triton X-100 (Sigma, USA). The p-nitrophenylphosphate (pNPP) was added and incubated for 30 min (37˚C, 5% CO_2). The reaction was stopped by 1N NaOH. The absorbance of each well was read at 405 nm and the ALP activity was expressed as p-nitrophenol produced in nmol/min/mg protein.

The osteogenic related genes expression, including ALP, Run-related transcription factor (Runx-2), Collagen type 1 (COL-1) and Osteocalcin (OCN), were detected by Quantitative real-time polymerase chain reaction (qRT-PCR) after MC3T3-E1 cells were cultured on scaffolds for 14 days. In brief, total RNAs in each sample were extracted using the TRIzol Reagent (Invitrogen, USA) and the concentration was determined using NanoDrop (Thermo Scientific, USA). Then the cDNA was synthesized by reverse transcription reaction using a RT-reagent Kit (TaKaRa, Japan). The relative gene expression level was calculated by standard $2^{-\Delta\Delta Ct}$ method and normalized to the GAPDH. The primer sequences (obtained from Sangon, Shanghai) listed in Table S2.

6 In vivo bioactivity

6.1 Animals

All the experimental procedures were performed according to the National Institutes of Health guidelines for the Care and Use of Laboratory Animals and the protocol

was permitted by the Animal Care Committee of Fudan University.

A total of 25 healthy, mature male New Zealand White rabbits were purchased from the animal house of Fudan University (ranging in weight from 2.3 kg to 2.7 kg). All rabbits were housed and allowed free access to enough food and water in individual cages (25˚C, 40-60% humidity).

6.2 Pre-experiment

Because the morphological structure of the proximal femur of the rabbit is different from that of the human body, the X-ray scan was performed before the formal experiment. The parameters of the proximal femur were measured by imaging including the diameter of the femoral neck, the length of the femoral neck and the neck shaft angle (Fig S3a-b). Then a rabbit was selected randomly and euthanatized, the femur was dissected to further determined these anatomic parameters. And the bony marker of the drilling needle point and the size of the scaffolds were selected based on the pre-experiment results (Fig S3c-e).

6.3 Surgical procedure and Scaffolds implantation

The New Zealand rabbit were weighted and divided into four groups randomly: the control group, HAP-0d group, HAP-3d group and HAP-7d group (5 rabbits per group).

All rabbits were anaesthetized with 1.25% sodium pentobarbital (3ml/kg) through ear vein and then fixed on the operation table. The surgical regions were shaved and disinfected followed by routine draping. A 4 cm incision was made down along the femur to expose the greater trochanter. Then a bone tunnel (d=3.5mm, L=30 mm) was

drilled using the Kirschner wire from the point (1cm below the great trochanter) to the femoral head along the femoral neck. Then the composite scaffolds were inserted manually into the bone tunnel in experiment groups and the bone tunnels were left empty in control group. After the operation, the incisions were sutured layer by layer with 3-0 sterile suture and disinfected again with iodophor. To prevent infection, one dose of penicillin (30,000 U/kg) were injected intramuscularly for three consecutive days postoperatively. The rabbit general condition including diet, activity and wound recovery were observed closely until the termination time point of this study.

6.4 Micro-CT evaluation and histological analysis

All rabbits were euthanatized 2 months postoperatively and all femurs specimen were harvested followed by fixed in 4% (v/v) paraformaldehyde for 48h. The microcomputed tomography systems (micro-CT, resolution 10um, Skyscan Bruker, Germany) were used to observe the new bone regeneration around the composite scaffolds in the bone tunnel. After the micro-CT scan, all the samples were fixed for 72h in Million's fixative solution, and dehydrated by graded ethanol (40%, 70%, 95%, 100%, 100%) following with rinsing in water overnight, and then transparent by xylene. After that, the samples were embedded in acrylic resin, and the target areas were sectioned into 10 μm thickness, and stained using Goldner's Trichrome method (Solarbio, China) and visualized by microscope (Zessi, Germany).

Statistical analysis

Data were presented as mean ± standard deviation (S.D.). All the statistical analysis was performed by IBM SPSS Statistics 22.0 software (SPSS Inc., Chicago, IL, USA). The One-Way ANOVA with the Tukey's post-hoc test was employed to discern the statistical differences between groups and $P < 0.05$ was considered to be a statistical difference.

Supplementary Figures

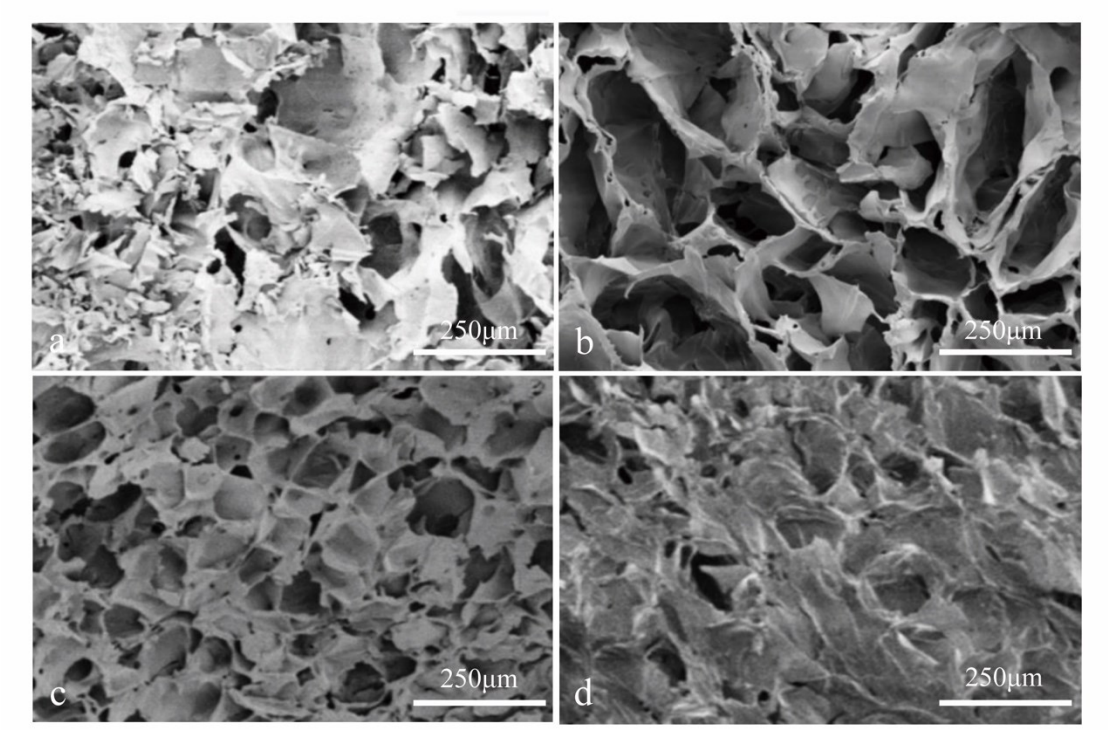

Figure S1. SEM images of scaffolds fabricated with different solid content. (a) 2.5 wt%; (b) 5 wt%; (c) 10 wt%; (d) 15 wt%.

We can see from the Fig.S1 that the pore size of the obtained material decreased with the increase of the blending solution concentration / gel solid content. However, when the solid content is too low, more fracture marks appear and no complete pores could be formed due to the poor mechanical properties (Fig. S1a); when the concentration is too high, although the mechanical properties of the material itself could be guaranteed, but the obtained porous scaffold is too dense for the gel internal network structure in the previous step is too strong, leading to the restricted ice crystal growth (Fig. S1d); when the concentration is 5 wt% (Fig. S1b)) or 10 wt% (Fig. S1c), the

pores were uniformly distributed and the structure is intact. Generally, the pore diameter of the scaffold suitable for cell growth is about 100 to 400μm. Therefore, we choose a sample with a concentration of 5-9-1 for the preparation of composite materials.

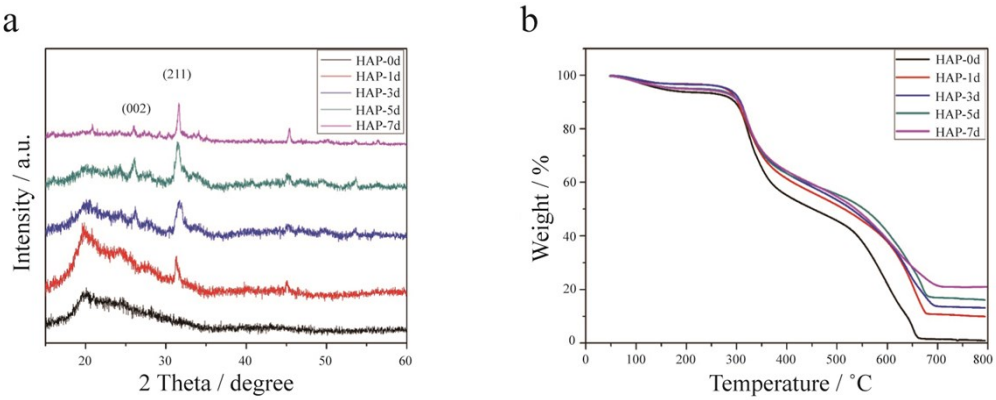

Figure S2. The XRD curves (a) and the TGA curves (b) of SF/HPMC scaffolds with different content of HA. The different samples were as follows: HAP-0d, without treatment with simulated body fluid (SBF); HAP-1d, immersed in SBF for 1 day; HAP-3d, immersed in SBF for 3 days; HAP-5d, immersed in SBF for 5 days; HAP-7d, immersed in SBF for 7 days

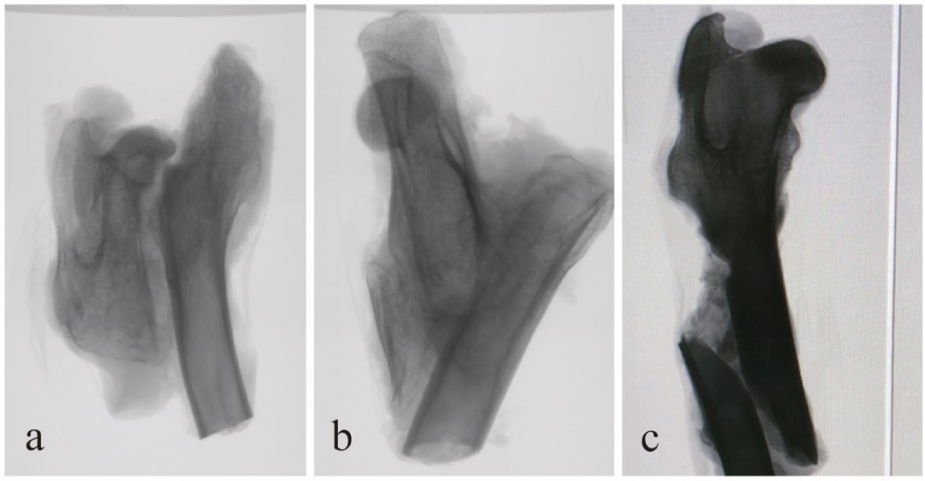

Figure S3. The X-ray images of the bone fracture in three rabbits. All these three rabbits were received core decompression of the femoral head without scaffolds inserted.

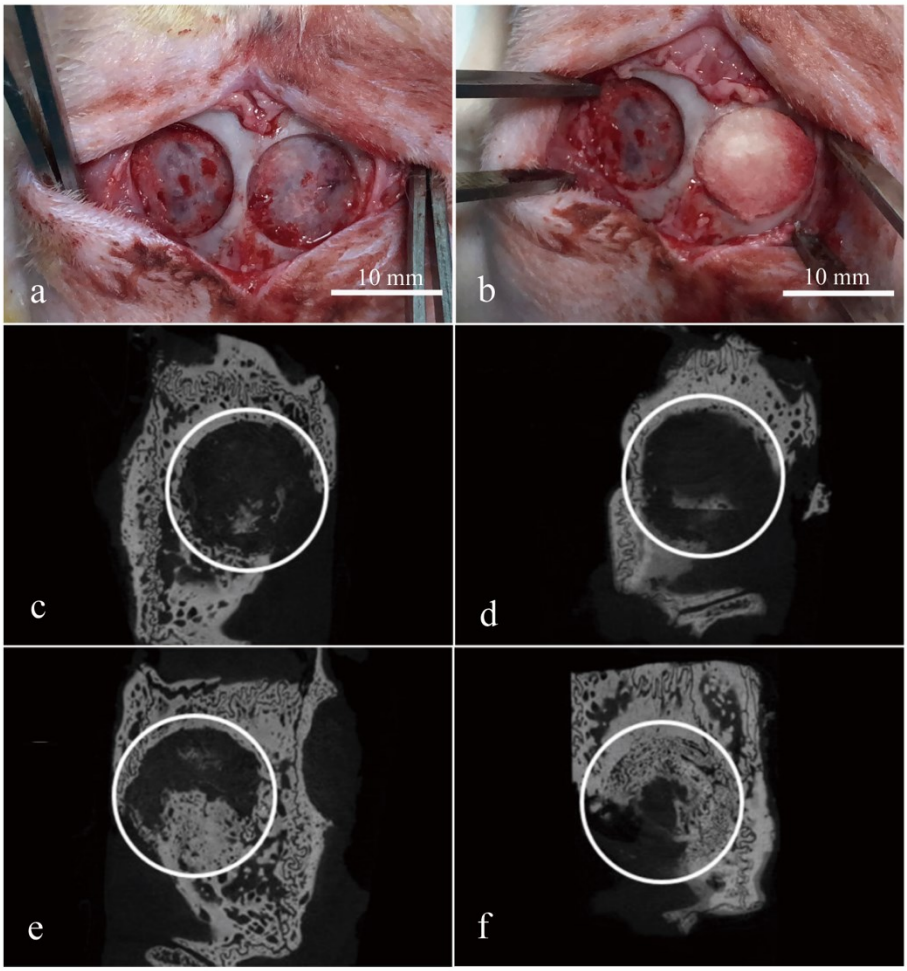

Figure S5. Implantation of the porous SF/HPMC based scaffolds in critical rabbit skull defects (10 mm diameter) and representative micro-CT reconstruction images of the rabbit skull at the time of sacrifice. (a) the critical skull defects; (b) scaffolds implantation; (c) micro-CT reconstruction image of control group without scaffold; (d) micro-CT reconstruction image of HAP-0d scaffold; (e) micro-CT reconstruction image of HAP-3d scaffold; (f) micro-CT reconstruction image of HAP-7d scaffold

From micro-CT reconstruction images, there was only a small amount of new bone tissue appeared in the samples of the control group (Fig.5c) and the HAP-0d group

(Fig.5d), while the original damage recovery / regeneration was good in HAP-3 group (Fig.5e) and HAP-7 group (Fig.5f). It indicated that the structure of this porous scaffold plus mineralized minerals has strong osteogenesis induction.

Supplementary Tables

Table S1. Mechanical property comparison table of silk-based materials with different RSF/HPMC ratios and solid contents of its precursor (mean±S.D.; n≥3).

Sample (wt%-SF-HPMC)	Compression Modulus (MPa)	Yield Strength (MPa)
10-9.5-0.5	1192 ± 14	27.9 ± 1.3
10-9-1	864 ± 12	25.9 ± 1.7
10-8-2	720 ± 21	19.2 ± 1.3
10-7-3	513 ± 17	12.6 ± 1.1
10-6-4	352 ± 23	4.7 ± 0.5
15-9-1	1624 ± 15	46.2 ± 2.4
12.5-9-1	1403 ± 11	41.1 ± 1.2
7.5-9-1	569 ± 21	17.5 ± 5.2
5-9-1	500 ± 32	13.4 ± 5.5
2.5-9-1	236 ± 43	8.8 ± 6.0

The samples are named follow the sequence of gel mass fraction (wt%)-silk fibroin mass-HPMC mass (for example the 10-9-1 group means that the gel mass fraction is 10wt%, the mass ratio of SF to HPMC is 9:1)

Table S2. Weight residue of the five group of scaffolds which are mineralized with different time after being heated to 800°C.

Sample	HAP-0d	HAP-1d	HAP-3d	HAP-5d	HAP-7d
Weight residue (%)	0.94	9.93	13.16	16.12	21.99

HAP-0d, scaffolds without mineralization; HAP-1d, scaffolds mineralized for 1 day; HAP-3d, scaffolds mineralized for 3 days; HAP-5d, scaffolds mineralized for 5 days; HAP-7d, scaffolds mineralized for 7 days.

Table S3. Evaluation Standard of Cytotoxicity Score

(a) GB/T 16175-1996:

Score (Cytotoxicity Grade)	Relative Growth Rate (%)
0	≥100 %
1	75-99 %
2	50-74 %
3	25-49 %
4	1-24 %
5	0 %

Grade 0-1 indicates that the material is biomedical qualified and has no cytotoxicity; Grade 2 indicates that the material has a certain cytotoxicity; Grade 3-5 indicates that the material has obvious cytotoxicity and unqualified.

(b) ISO 10993-5:2009:

A decrease in number of living cells results in a decrease in the metabolic activity in the sample. This decrease directly correlates to the amount of blue-violet formazan formed, as monitored by the optical density at 570 nm. To calculate the reduction of viability compared to the blank Equation is used:

$$\text{Viab. \%} = \frac{100 * OD570e}{OD570b}$$

OD_{570e} is the mean value of the measured optical density of the 100 % extracts of the test sample; OD_{570b} is the mean value of the measured optical density of the blanks. The lower the Viab.% value, the higher the cytotoxic potential of the test item is.

If viability is reduced to < 70 % of the blank, it has a cytotoxic potential. The 50 % extract of the test sample should have at least the same or a higher viability than the 100 % extract; otherwise the test should be repeated.

Table S4. The primer sequences of the osteogenic related genes for qRT-PCR. ALP, alkaline phosphatase; Runx2, runt-related transcription factor 2; OCN, osteocalcin; COL-1, collagen-1.

Target gene	Sense and antisense primer sequence
GAPDH	5’- GGTGGACCTCATGGCCTACAT-3’
	3’-GCCTCTCTCTTGCTCTCAGTATCCT-5’
ALP	5’- TGAATGACGGGCCTGATGAC-3’
	3’-GTTCCGGGCCCTATTCATGG-5’
Runx2	5’- CCCTGAACTCTGCACCAAGT-3’
	3’-TAGGGGTAGGTAGGTGAGGT-5’
OCN	5’- TGACCTCACAGATGCCAAGC-3’
	3’-CATCACTTGTCTGAGGCCGC-5’
COL-I	5’- CCAGCCGCAAAGAGTCTACA -3’
	3’-GGGTTACCACTCTGCACCTT-5’

Table S5. The Bone histomorphometrical analysis of different composite scaffolds implanted in the bone tunnel after core decompression of the femoral head.

Bone histomophormetry	Control	HAP-0d	HAP-3d	HAP-7d
vBMD /g.mm-3	0.067±0.004	0.063±0.002	0.097±0.004	0.090±0.007
BV/TV /%	16.48±2.51	16.28±1.77	19.44±4.30	20.37±4.56
Tb.Th /mm	0.18±0.06	0.19±0.08	0.19±0.09	0.15±0.02
Tb.Sp /mm	0.10±0.03	0.15±0.07	0.14±0.08	0.12±0.06