Outer-inner Dual Reinforced Micro/Nano Hierarchical Scaffolds for Promoting Osteogenesis

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Experimental Section

Preparation of scaffolds

The aqueous solution of SF was obtained from cocoons of B. mori, following the protocol previously described.[1] Briefly, de-sericin process was accomplished through boiling the cocoons in aqueous solution of Na_2CO_3 (0.5% w/w) (Aladdin, Shanghai, China) for 1 h. After the cocoons was fully washed and dried at 60 °C, 9.3 M of LiBr (Aladdin, Shanghai, China) solution was employed to dissolve the de-sericin cocoon for 4 h at 60°C. The obtained solution was dialyzed against deionized water that was changed twice a day for 4 days. Finally, the purified solution was concentrated through dialysis against polyethylene glycol powder (MW 20,000 g mol^{-1}; Biosharp, Shanghai, China) to obtain the final solution with 6% w/w concentration for further use.

Mesoporous nanoparticle of bioactive glass containing 80% SiO_2, 16% CaO, and 4% P_2O_5 was synthesized through a modified method as described previously.[2] The reaction was carried out at 60°C for 24 h, with Tris-HCl buffer solution (pH 8.0) (Aladdin, Shanghai, China) as catalyst and CTAB (cetyltrimethylammonium bromide, Sigma-Aldrich, USA) as template agent. The obtained nanoparticles were wash with ethanol and deionized water for 3 times, respectively, and later calcined at 500 °C to remove CTAB.

The microporous SF scaffolds were prepared through lyophilization. A modified crosslinking method was applied to for reduced formation of β-sheet in silk fibroin as reported previously.[3, 4] Briefly, the 6%(w/w) SF solution with or without MBG addition (mass ratio of SF and MBG: 20:1) was incorporated with glycerol (mass ratio of SF and

glycerol: 80:20) for crosslinking and further sonicated for 30 min before freeze-dryed at −80°C and subsequent lyophilization. The production was washed with deionized water for three times before further characterization.

The construction of hierarchical scaffold was carried out on the micro-scale scaffold prepared, following the aforementioned procedure.[5] The type I collagen from rat tail was dissolved in 0.1 M of acetic acid solution. The self-assembly of collagen was carried out in ice bath and began with diluting the collagen solution with phosphate buffered saline (PBS, 10×, Hyclone, USA) at a ratio of 6:1. Then, 0.5 M of NaOH (Aladdin, Shanghai, China) was applied to adjust the pH to 7.0 and the neutralized collagen solution was then dropped onto the microporous scaffolds to fully saturate the pore space, after which the scaffold were incubated at 37 °C for 30 min to allow the self-assembly process. The obtained scaffolds were washed with deionized water for three times before further characterization and experiment.

Characterization of the scaffolds

In order to verify the assembly of collagen as well as the synthesis of mesoporous bioglass, both assembled collagen and bioglass nanoparticles were subjected for TEM observation. In order to visualize the surface morphology of scaffolds and mesoporous bioglass nanoparticles, samples were mounted on the SEM sample stub with the help of conductive tapes and subjected for sputter gold coating process for 60s using gold sputter coating equipment (SC7620, Quorum Technologies, UK). Later, the observation was carried out using a scanning electron microscope (SEM, S-4800;

Hitachi, Japan) with a voltage of 5 kV.

In order to verify the incorporation of bioglass nanoparticles and the integration of collagen, the structure of scaffolds from different groups as well as pure collagen which was introduced as control were analyzed with FTIR spectroscopy (FTIR, Nicolet 6700; Thermo scientific, USA). Samples were grind with potassium bromide to produce tablets which were subjected for 128 scans with a resolution of 4 cm^{-1} ranging from 400 to 4000 cm^{-1}.

To study the bulk mechanical property, cylindrical-shaped scaffolds were immersed in PBS and later subjected for uniaxial compression test using universal mechanical test system (Hengyi, Shanghai, China). The tests were conducted with a strain going up to 60% and a speed of 5mm/min. Compressive modulus was obtained via calculating the slope of the stress-strain curve crossing zero point. As for the rheology study, self-assembled collagen and scaffolds were subject for the strain-dependent and frequency-dependent sweep at 37 °C on the HAAKE RheoStress 6000 (Thermos Scientic, USA). All mechanical tests were conducted with scaffolds fully saturated with phosphate buffer saline. When comparing the rheological performance of scaffolds, scaffolds without assembled collagen were saturated with water as control.

Evaluation of *in vitro* performance

Scaffold disks with diameter of 5 mm and thickness of 1 mm were placed in 96-well plates for in vitro evaluation. Bone marrow derived mesenchymal stem cells from SD rats were seeded on scaffolds with a density of 5×10^3 cells per well. The

morphology of cellular adhesion was observed using SEM on 1 day and 3 days post seeding and due to the inherent capacity of silk fibroin to absorb the coloring matter within cell counting kit-8, the proliferation rates were measured using a modified cell counting method, as previously reported,[6] at the points of 1, 3, 5 and 7 days post implantation. Briefly, the cell-seeded scaffolds were rinsed three times with PBS, followed by adding 50 μL of trypsin (0.25%, Gibco, USA) for 3 min incubation at 37 °C. After stopping digestion with culture media, scaffolds were fully washed with PBS for three times with the cell suspension collected and observed using a microscope. The amount of cells was counted for five times to obtain the average.

The capacity of scaffolds to induce osteogenesis were studied through detecting Alkaline phosphatase (ALP) activity and deposition of calcium nodules. Briefly, the scaffold disks were placed in the upper chambers of a 12-well Transwell plate with BMSCs cultured in lower chambers. ALP was stained with ALP staining kit (Beyotime, Shanghai, China) at 7 and 14 days after scaffolds immersion and the ALP activity was quantified at the point of 7 days using ALP quantification kit (Jiancheng, Nanjing, China). Calcium nodules were stained with Alizarin Red Staining kit (ARS, Cyagen, Guangzhou, China) 14 and 21 days after cell seeding.

In order to explore the underlying mechanism of osteogenesis effect resulted from structural cues, immunofluorescence staining was applied to study potential involved pathway. After co-cultured on scaffolds for different periods of time, BMSCs were fixed with 4% paraformaldehyde for 30 min, and later permeated with 0.3% Triton-X

(Aladdin, Shanghai, China) for 10 min. After blocked with bovine serum albumin (5% w/v) (BSA, Biosharp, Shanghai, China) overnight, the disks with cells were immunostained with primary antibody against β-integrin (Novus, USA), Vinculin (Novus, USA) and YAP (Cellsignal, USA), and further incubated with secondary antibody conjugated with fluorescent labels (Jackson, USA). After washing for three times with PBS, disks were subjected for observation using a confocal laser scanning microscopy (CLSM, Zeiss-LSM710, Carl Zeiss, Germany). It has to be mentioned that due to the inherent capacity of silk fibroin to absorb biomolecules, such as antibody, phalloidin and DAPI, silk scaffold was stained in multiple channels. However, the cells co-cultured with scaffolds remained distinguishable and comparable under CLSM.

BMSCs co-cultured with different scaffolds for certain periods of time were lysed with RIPA buffer (HXBC-BIO, Beijing, China), containing protease inhibitor (HXBC-BIO, Beijing, China) for 30 min, and latter centrifuged to obtain the supernatant. Protein concentration was determined by BCA protein assay kit (Beyotime, Shanghai, China) and equal amount of protein was electrophoreted on 5% sodium dodecyl sulfate polyacrylamide gels before transferred onto nitrocellulose membranes, which were further incubated in dilutes of the following antibodies: β-integrin (1:500, ABclonal, USA), vinculin (1:1000, ABclonal, USA), YAP (1:1000, ABclonal, USA), osteocalcin (OCN) (1:1000, Abcam, USA), Runt-related transcription factor 2 (RUNX2) (1:1000, Abcam, USA), followed by the incubation with corresponding horseradish peroxidase (HRP)-conjugated second antibodies (Jackson, USA) diluted by Tris buffered saline

with tween buffer (TBST) for 1 h at room temperature. Relative intensity was evaluated using BandScan software with GAPDH as control.

In vivo bone regeneration study

Sprague−Dawley (SD) male rats were purchased from the Experimental Animal Center of Soochow University (Suzhou, China). Surgical procedures and pre-/ and post-surgical handling were carried out in accordance with protocols approved by the Ethics Committee at the First Affiliated Hospital of Soochow University.

The SD rats were anaesthesized through intraperitoneal injection of pentobarbital sodium (2%). After fully shaving and iodophor disinfection of the operation area, a longitudinal incision was made to expose the calvarium and a bone defect with a diameter of 5 mm was created using a dental trephine. Scaffolds were place into the defect as demonstrated in **Figure 7**A. Penicillin was applied once a day postoperatively through intramuscular injection for three days.

In the 8 and 12 weeks after surgical procedure, SD rats implanted with scaffolds were euthanatized to harvest the calvarium specimens, which were fixed in 10% formalin before further characterization. Regenerative condition within the defect space was evaluated with micro-CT (SkyScan 1176, SkyScan, Belgium) under the following settings: 65 kV, 385 mA, and 1 mm Al filter and three-dimensional structures were reconstructed with Mimic software. The region of interest (ROI) defined as a cylinder space with a diameter of 5 mm was applied to measure bone volume/tissue volume (BV/TV), bone mineral density (BMD) and trabecular number using CT Analyzer

(Skyscan).

In the 4 and 12 weeks after surgery, the fixed specimens were decalcified in 10% formic acid for 1 week at room temperature after which samples were dehydrated through gradient alcohol and embedded in paraffin. 5 μm-thick histological sections were cut at the center of the embedded specimens, followed by staining with hematoxylin and eosin (H&E) and Masson trichrome to evaluate the bone formation detail, and new bone fraction was analyzed with ImageJ (USA). Deposition of collage I was evaluated via immunohistochemical staining. Samples were stained against COL-I antibody (1:200 ditlution, abcam, USA) at 4 °C overnight and later incubated with secondary antibody (Jackson, USA) for 1 h at 37 °C followed by staining with DAB solution (Dako, Germany) for 10 min. Images were obtained under a bright field microscope (Zeiss Axiovert 200, Carl Zeiss Inc., USA).

Statistical Analysis

The data was presented with the form of means ± standard deviations. Statistical analysis (GraphPad Software, USA) was evaluated using one-way or two-ways analysis of variance followed by Tukey's multiple comparison test to evaluate the differences between the groups. A probability value (p) less than 0.05 was considered to be statistically significant.

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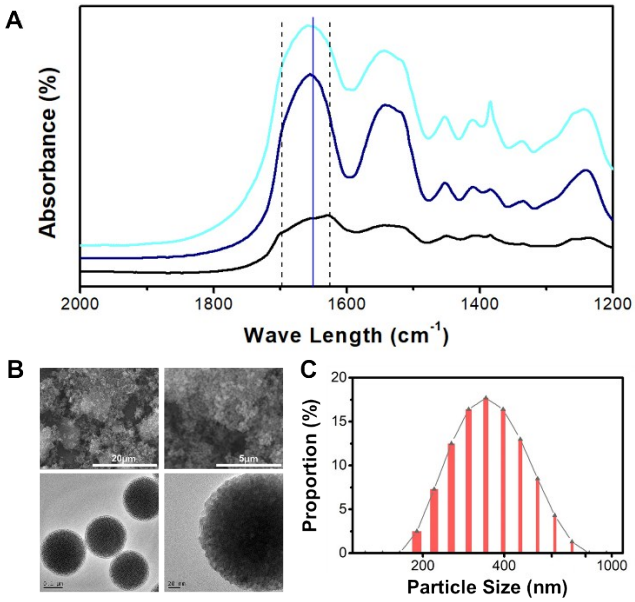

Fig. S1. (A) The formation of β-sheet within silk fibroin scaffold with increasing amount of glycerol (0%, 10%, 20%). (B) SEM and TEM morphology of mesoporous particles. (C) Particle size measured by DLS.

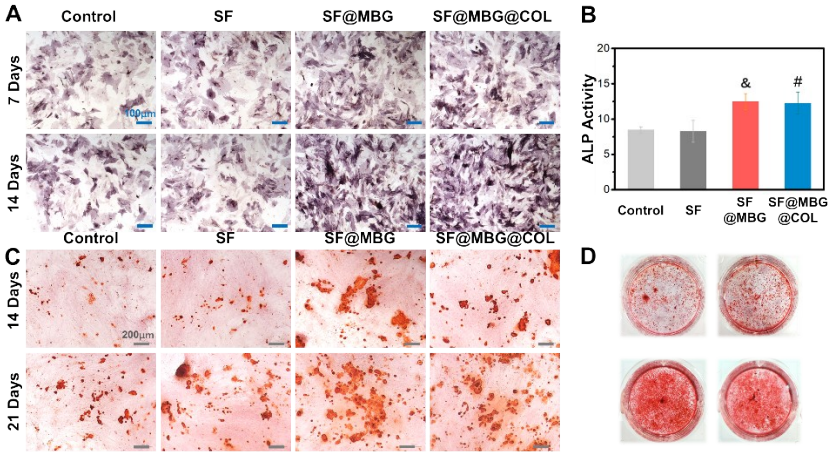

Fig. S2. (A) representative optical images of ALP staining at both 7 and 14 days followed by (B) quantification after 7 days' co-culture. (C) Representative optical images of Alizarin red staining at 14 and 21 days. (D) Representative macroscopic view of Alizarin red staining at 21 days. (Statistical differences are indicated with *$p<0.05$ when comparing SF@MBG@Col and SF@MBG, #$p<0.05$ when comparing SF@MBG@Col and SF, and &$p<0.05$ when comparing SF@MBG and SF.)

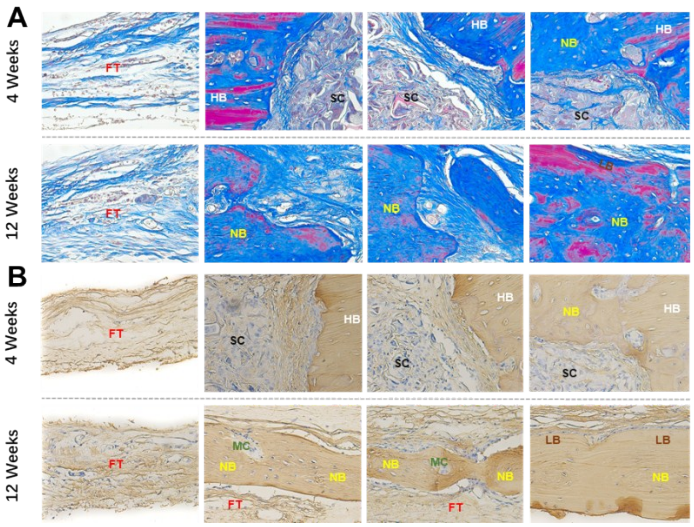

Fig. S3. (A) Masson's trichrome of the defect area at 4 and 12 weeks after surgery. (B) immunohistochemical staining of collagen I in defect area at 4 and 12 weeks after surgery. Details are demonstrated with HB indicated as host bone, NB as newborn bone, LB as lamellar bone, SC as scaffold and FT as fibrous tissue.