Supporting Information

Fabrication of Injectable BMSCs-laden Double Network Hydrogel Based on Silk Fibroin/PEG for Cartilage Repair

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1. Materials and methods

1.1 Materials

4-arm poly(ethylene glycol) amine (4-arm PEG-NH_2, 20 KDa) and 4-arm poly(ethylene glycol) carboxylic acid (4-arm PEG-CM, 20 KDa) were supplied by Xiamen Sinopeg Biotech CO., LTD. 6-amino-2-cyanobenzothiazole (CBT), Bocethylmercapto-L-cysteine (dicyclohexylammonium) salt, isobutyl chlorformate (IBCF), N-methyl morpholine (NMP), Dithiothreitol (DTT) were supplied by Sigma. Transforming growth factor-β1 (TGF-β1) and fibroblast growth factor (FGF) were obtained from Cloud-Clone Corp.. Fetal bovine serum (FBS), Dulbecco's modified eagle medium (low glucose) and TrypLE™ express enzyme were obtained from Gibco (Life Technologies, USA). BMSCs, PEG-CBT and PEG-d-Cys were prepared according to our previous methods.[1-3] All the other regents were obtained from Sinopharm Chemical Reagent Co. Ltd. and used as received.

^{1}H NMR spectra were recorded on a Varian NMR spectrometer at 400 MHz. WST assay was measured with BiotekCytation 3. The morphology of the hydrogel was observed under scan electron microscope (SEM, Quanta FEG 250). The cellular images were observed by confocal laser scanning microscopy (CLSM, Nikon A1). The storage modulus (G') and loss modulus (G″) of hydrogels were measured using a Haake rotational rheometer (RS6000).

1.2 Silk fibroin extraction

SF solution was extracted as previously described.[4] Briefly, cocoons of *Bombyx mori* were boiled in 0.02 M sodium carbonate solution for 40 min to remove sericin, following rinsing thoroughly with DI water. The resulting fibroin was air dried at ambient temperature, and then dissolved in 9.3 M LiBr solution to a final concentration of 20% w/v, at 60 °C for 4 h. Finally, the solution was dialyzed (MWCO = 3500) against DI water for 3 days to remove all salts and lyophilized.

1.3 Preparation of PEG-SF hydrogels

The compositions of different PEG-SF hydrogels are listed in Table S1. For each hydrogel group, the finial concentration of precursor was fixed at 10% (w/v), through changing the volume ratio of the PEG and SF solutions to yield blended precursor solutions of 100:0 (PEG), 70:30 ($PEG_{0.7}$-$SF_{0.3}$), 50:50 ($PEG_{0.5}$-$SF_{0.5}$) and 30:70 ($PEG_{0.3}$-$SF_{0.7}$), respectively. For the hydrogel preparation, 1 mL of pure SF (10% w/v) solution was treated under an ultrasonic cell crusher (at 50% amplitude for 1 min，5 s ultrasonication followed by a 5 s pause) to induce β-sheet formation. Thereafter, the PEG-CBT, PEG-d-Cys and DTT was quickly solubilized in SF solution with certain concentrations followed by incubating at 37 °C for 30 min before use.

To prepare the BMSCs-encapsulated PEG-SF hydrogels, Firstly, 4-arm PEG-CBT was dissolved in ultrasonicated SF solution and 4-arm PEG-d-Cys was dissolved in ultrasonicated SF solution containing DTT (final concentration: 2 mM). Then, BMSCs (1.5×10^6 cells/mL) were separately mixed with two precursor solutions

(finally total concentration: 10% w/v, (2.5% w/v for PEG-d-Cys, 2.5% w/v for PEG-CBT and 5% w/v for SF)). Next, these two precursor solutions mixed thoroughly. After the formation of PEG network of hydrogel, the hydrogels were placed in 37 °C to completely gel.

1.4 Characterization of hydrogels

First of all, FTIR spectroscopy was carried out to demonstrate the formation of β-sheet among SF in this hybrid hydrogel. Secondly, the structure of lyophilized hydrogel was observed by SEM (Quanta, FEG 250) after being sputter-coated with gold. Next, rheological tests of the hydrogels were investigated on a Haake rotational rheometer (RS6000) and the compression test were measured using an Instron 3365. Finally, swelling studies were carried out as following: the freeze-dried hydrogels were weighed as Wd. The wet hydrogels also were weighed (W_w) after about 2 days of immersion in PBS to reach equilibrium. The equation ((W_w-W_d)/W_d) was used to calculate the swelling ratio (SR) of hydrogels.

1.5 Proliferation of BMSCs within PEG-SF hydrogel

The as-prepared hydrogel was immersing in the culture medium and the media was replaced every 48 ~ 72 hours. Finally, Water Soluble Tetrazolium (WST) was used to test the proliferation of BMSCs at different time points. Meanwhile, the cell viability of encapsulated-BMSCs was also observed by LIVE/DEAD staining assay.

1.6 Chondrogenesis induction of BMSCs within PEG-SF hydrogels *in vitro*

Briefly, the BMSCs-encapsulated hydrogels was incubated in complete medium for 1 day, and then transferred to the inductive medium containing 50 μg/mL L-ascorbic acid, 100 nM dexamethasone, 40 μg/mL proline, 1 × ITS, 1 × sodium pyruvate, FGF (10 ng/mL) and TGF-β1 (10 ng/mL). The hydrogel was taken out at certain times, and rinsed by PBS three times for the following measurement.

1.7 Chondrogenic specific gene expressions

The mRNA expression levels of chondrogenic-related gene were then assessed at 0 day (cultured in complete medium for 1 day), 14^{th} day and 28^{th} day by qRT-PCR, including Collagen type II (Col II), Aggrecan (AGG), Sox 9. The relative expressions for the target genes were represented as a fold change to undifferentiated BMSCs (before encapsulation) and normalized to that of the reference gene GAPDH and the primers for qRT-PCR are listed in Table S2. At each time point, the hydrogel was taken out and rinsed by PBS three times. The total RNA was then extracted according to the protocol and the purity of the RNA was assessed using $A_{260/280}$ nm. Thereafter, 500 ng of RNA was reverse transcribed into cDNA using PrimeScript™ RT Reagent Kit (Takara). qRT-PCR was performed on Mastercycler® nexus (Eppendorf) using SYBR Green I PCR Kit.

1.8 Creation of articular cartilage defects

Sprague-Dawley rats (260~280g) were obtained from Nanjing Sikerui Biological Technology Co. Ltd. and acclimated under pathogen-free conditions for 1 week. All animal experiments were conducted in accordance with the relevant laws and institutional guidelines following the approval of the Ethics Committee of Chinese Academy of Sciences. Thereafter, the rats were divided into 3 groups: control group, hydrogel group and BMSCs-TGF-β1-laden hydrogel (B+T hydrogel) group. All the rats were anesthetized with 50 mg/kg pentobarbital sodium via intraperitoneal injection. The operation was carried out in the center of the condyle, and a full-thickness defect with the size of 3 mm × 3 mm was created. After irrigating with physiological saline, the holes were carefully dried. Then, the incision was then sutured for the control group, while for the experimental group, the hydrogel with/without BMSCs+TGF-β1 was quickly injected into the hole before solidification, and the incision was then sutured. After the operation, the rats were administered with antibiotics for 3 days, and had free access to food and water. Finally, the rats were sacrificed and the condyles were collected after feeding for 8 weeks.

1.9 Histological and immunohistochemical analysis *in vivo*

After sacrifice, the obtained condyles were fixed in 4% paraformaldehyde for 24 h. After rinsing with PBS, the condyles were subsequently decalcified in PBS containing 15% EGTA at 4 ^{o}C, and the solution was changed every 4

days. 2 months later, the tissue was washed with water followed by progressive replacement with a graded ethanol series. Thereafter, the samples were processed for embedding in paraffin and cut into 4-6 μm sections. To evaluate the morphology of the regenerated cartilage, hematoxylin and eosin (H&E), Safranin-O staining and Toluidine blue staining were used according to the standard procedure. The sections were blindly scored by three different investigators by using a common grading scale.[5-6]

1.10 Statistical analysis

All the experiment results were reported as mean ± standard deviation for *in vitro* and *in vivo* studies. The statistical data analysis was conducted using Origin Pro 8.5 program and P values < 0.05 were considered statistically significant. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

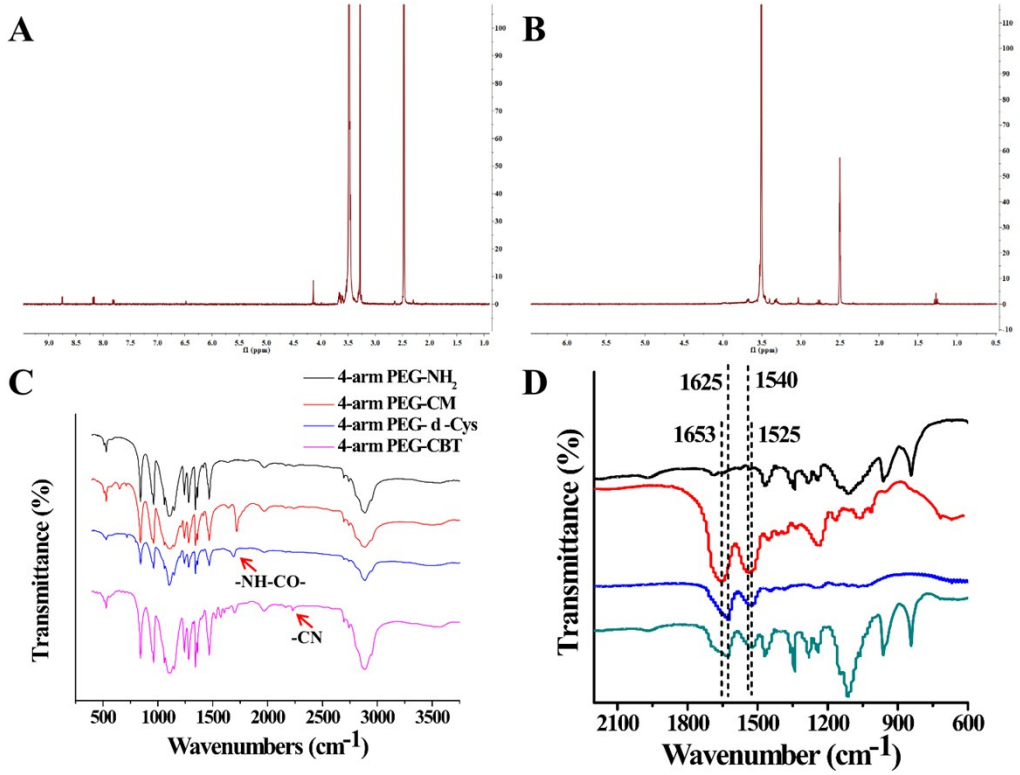

Fig. S1 ^{1}H NMR spectra of PEG-CBT (A) and PEG-d-Cys (B); FT-IR spectra of 4 arm PEG-NH_2, 4 arm PEG-COOH, 4 arm PEG-d-Cys and 4 arm PEG-CBT (C); FT-IR spectra of PEG hydrogel, SF (without β-sheet), SF hydrogel (with β-sheet) and PEG-SF hydrogel (D).

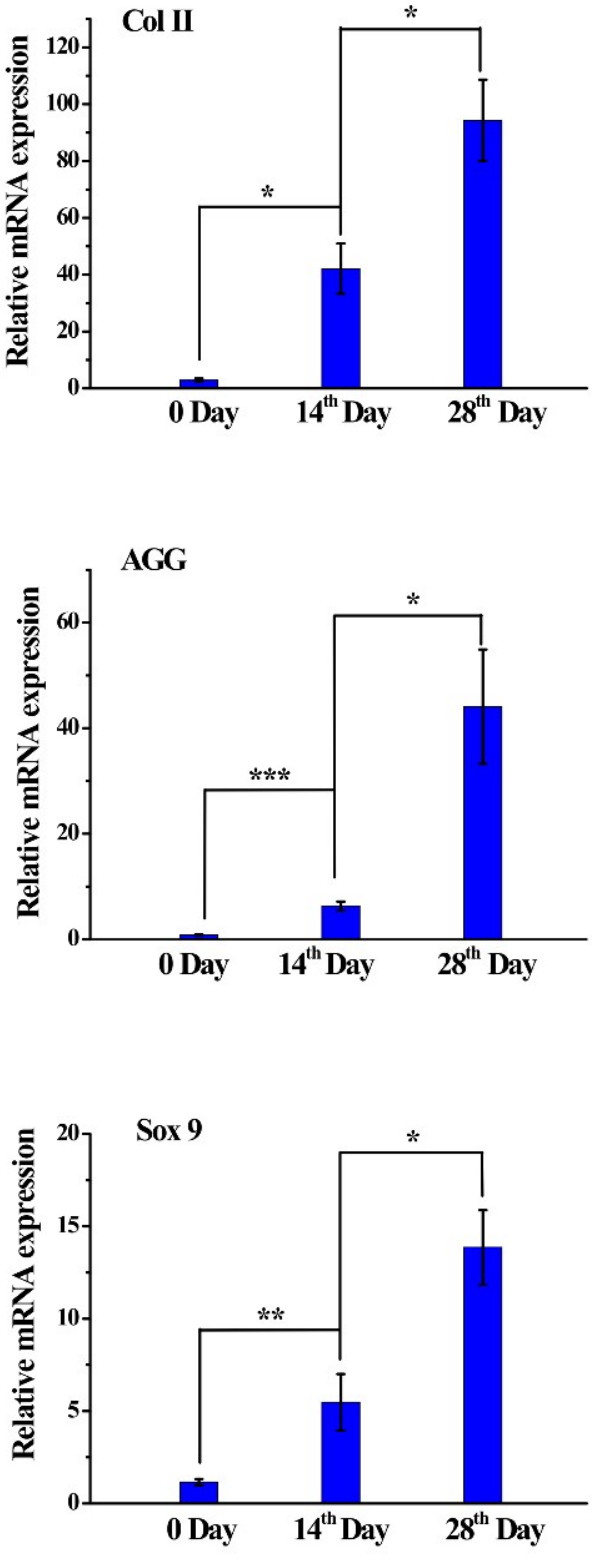

Fig. S2 qRT-PCR analysis of chondrogenic gene expression level: Col II (A), AGG (B) and Sox 9 (C). The relative mRNA expression of undifferentiated BMSCs was set as 1.

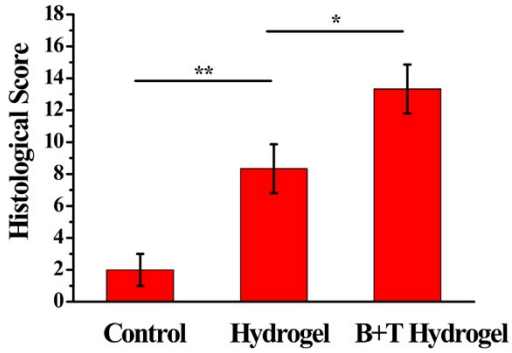

Fig. S3 Histological scores for repaired cartilages at 8 weeks after surgery.

Table S1 The compositions of different PEG-SF hydrogels.

Hydrogels	Concentrations (w/v)			
	PEG-CBT	PEG-d-Cys	DTT	SF
PEG	5%	5%	7.5 mM	0
$PEG_{0.7}$-$SF_{0.3}$	3.5%	3.5%	5.25 mM	3%
$PEG_{0.5}$-$SF_{0.5}$	2.5%	2.5%	3.75 mM	5%
$PEG_{0.3}$-$SF_{0.7}$	1.5%	1.5%	2.25 mM	7%

Table S2 Primers for qRT-PCR.

Gene	Prime sequence
Col II	F-CACCGCTAACGTCCAGATGAC R-GGAAGGCGTGAGGTCTTCTGT

AGG	F-GGAATCCCTAGCTGCTTAGCAG R-GAGTCATTGGAGCGAAGGTTC
Sox 9	F-AGGAAGCTGGCAGACCAGTA R-ACGAAGGGTCTCTTCTCGCT
GAPDH	F-TGGAGTCTACTGGCGTCTT R-TGTCATATTTCTCGTCCTTCA

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