Dynamic and cell-infiltratable hydrogels as injectable carrier of therapeutic cells and drugs for treating challenging bone defects

Qian Feng\textsuperscript{a,b,†}, Jiankun Xu\textsuperscript{a,†}, Kunyu Zhang\textsuperscript{b}, Hao Yao\textsuperscript{e}, Nianye Zheng\textsuperscript{e}, Lizhen Zheng\textsuperscript{e}, Jiali Wang\textsuperscript{e}, Kongchang Wei\textsuperscript{b,d}, Xiufeng Xiao\textsuperscript{a}, Ling Qin\textsuperscript{c,*}, Liming Bian\textsuperscript{b,e,f,g,h,*}

\textsuperscript{a} Fujian Provincial Key Laboratory of Advanced Materials Oriented Chemical Engineering, College of Chemistry and Materials Science, Fujian Normal University, Fuzhou, China

\textsuperscript{b} Department of Biomedical Engineering, The Chinese University of Hong Kong, Hong Kong SAR, China

\textsuperscript{c} Department of Orthopaedic and Traumatology and Innovative Orthopaedic Biomaterial and Drug Translational Research Laboratory of Li Ka Shing Institute of Health, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong SAR, China

\textsuperscript{d} Empa-Swiss Federal Laboratories for Materials Science and Technology, Switzerland

\textsuperscript{e} Translational Research Centre of Regenerative Medicine and 3D Printing Technologies of Guangzhou Medical University, The Third Affiliated Hospital of Guangzhou Medical University, Guangzhou, Guangdong, China

\textsuperscript{f} Shenzhen Research Institute, The Chinese University of Hong Kong, China

\textsuperscript{g} China Orthopedic Regenerative Medicine Group (CORMed), Hangzhou, China

\textsuperscript{h} Centre for Novel Biomaterials, The Chinese University of Hong Kong, Hong Kong SAR, China

\textsuperscript{†}These authors contributed equally.
* Correspondence and requests for materials should be addressed to Prof. Liming Bian at: Department of Biomedical Engineering, Room 213, William M.W. Mong Building, The Chinese University of Hong Kong, Shatin, Hong Kong. Tel: +852 39438342; Fax: +852 26036002 Email: lbian@cuhk.edu.hk

Prof. Ling Qin at: Department of Orthopaedic and Traumatology, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong. Tel.: +852 35053071; Fax: +852 26377889; E-mail: lingqin@cuhk.edu.hk
**Figure S1.** $^1$H NMR of Ac-β-CD recorded in DMSO-$d_6$.

$^1$H NMR (400 MHz, DMSO) δ 6.26 (d, J = 49.2 Hz, 1H), 5.81 (d, J = 34.6 Hz, 4H), 4.85 (s, 1H), 4.45 (d, J = 11.7 Hz, 1H), 3.63 (s, 2H), 3.34 (s, 9H).

**Figure S2.** (a) Time sweep of dynamic rheology study on Ci-I gelatin hydrogels with different concentration of Ac-β-CD and 8% gelatin at 37 °C. (b) Time sweep of dynamic...
rheology study on Ci-I gelatin hydrogels with 10% Ac-β-CD and different concentration of gelatin at 37 °C. (close symbols for storage modulus $G'$ and open symbols for loss modulus $G''$).

As the Ac-β-CD content increases, both the storage and loss modulus of the Ci-I gelatin hydrogels increase (Figure. S2a). At the same, the storage and loss modulus of the Ci-I gelatin hydrogels also can be improved by increasing the concentration of gelatin. (Figure S2b). Interestingly, when the gelatin content increases from 8% to 10%, the storage and loss modulus of the Ci-I gelatin hydrogels do not show significant different. Therefore, based on these data, we choose Ci-I gelatin hydrogels with 8% gelatin and 10% Ac-β-CD for the following experiments.

**Figure S3.** (a) Cell viability staining of hMSC-laden Ci-I gelatin hydrogels before injection (hMSC-laden Ci-I gelatin hydrogels are cultured *in vitro* for 1 day). (b) Cell viability staining of hMSC-laden Ci-I gelatin hydrogels after injection (hMSC-laden Ci-I gelatin hydrogels are cultured *in vitro* for 1 day, injected with G21 needle, and then cultured *in vitro* for another 5 days). Scale bar: 100 µm (a, b).
Figure S4. Representative images of Ki67 staining. Scale bar: 200 µm.

Figure S5. Negative control stains for the secondary antibodies used in the immunofluorescent staining at week 6 after SAON surgical treatment in SD rats. Scale bar: 200 µm.
**Figure S6.** The quantitative data for the Western Blot images shown in (a) Figure 5d and (b) Figure 5e. Blank repair was normalized as 1. The data were analyzed with One-way ANOVA with Tukey’s post hoc test. (n=3, *P<0.05, **P<0.01, ***P<0.001)

**Movie 1.** The 3D distribution of DAPI-stained human mesenchymal stem cell (hMSC) nuclei within the Ci-I hydrogels without icaritin encapsulation after 24 h of *in vitro* culture.

**Movie 2.** The confocal micrographs show the 3D distribution of DAPI-stained human mesenchymal stem cell (hMSC) nuclei within the Ci-I hydrogels with icaritin encapsulation after 24 h of *in vitro* culture.

**Materials**

β-Cyclodextrin (β-CD), acrylate chloride, and hydrogen peroxide (H₂O₂) were bought from Aladdin. Gelatin (type A, from porcine skin, isolectric point: 7~9, Cat. No.: G1890-500G, Sigma), dimethylsulfoxide-d6 (DMSO-d6), Icaritin, 2-hydroxy-4’-(2-hydroxyethoxy)-2-methylpropiophenone (I2959) methacrylic anhydride, 4’, 6-diamidino-2-phenylindole (DAPI), 3-(trimethoxysilyl) propyl methacrylate, silver nitrate, paraformaldehyde, Triton X-100, sodium thiosulfate, triethyl amine (TEA), ethidium bromide, calcein green, xylol orange, and hyaluronidase were purchased from Sigma. Dimethyl Formamide (DMF), dimethylsulfoxide, acetone, hydrochloric acid (HCl), and sodium hydroxide (NaOH) were purchased from Fisher Scientific. Poly (ethylene glycol) diacrylate (PEGDA) was purchased from Jenkem. Phosphate buffered saline (DPBS), α-minimum essential medium (DMEM), penicillin, streptomycin, L-glutamine, calcein AM, fetal bovine serum (FBS), and Trizol were obtained from Gibco.
BCA Protein Assay Kit, Calcium Colorimetric Assay Kit, and RevertAid First Strand cDNA Synthesis Kit were obtained from Thermo Fisher Scientific. DAB Peroxidase Substrate Kit and Vectastain ABC Kit were purchased from Vector Labs. All antibodies used in this study were purchased from Abcam. Human mesenchymal stem cells (hMSCs) were from Lonza(USA). Methylprednisolone (MPS) was purchased from Pharmacia & Upjohn (USA).

**Synthesis of acrylate β-cyclodextrin (Ac-β-CD)**

A total of 10g of β-CD were added to 150 mL of DMF and 7 mL of TEA were added to the solution. The mixture was stirred and cooled to 0 °C before 7mL of acrylic acid were added. After stirring for 12 h, the mixture was filtered to remove trimethylamine hydrochloride and the clear solution obtained was concentrated to about 20mL with vacuum rotary evaporation. Then the solution was dripped into 600 mL of acetone to precipitate the modified β-CD. The precipitate was washed several times with acetone and vacuum dried for 3 days. The substitution degree (DS) of CD was confirmed as 1.5 by $^1$H NMR (Bruker Advance 400 MHz spectrometer) recorded with DMSO-d$_6$ as the internal reference at 37 °C.

**Preparation of Ci-1 hydrogel**

Gelatin and Ac-β-CD were dissolved in Phosphate Buffered Saline (PBS) at 37 °C to produce solutions with fixed concentrations of gelatin (8% [w/v]) and Ac-β-CD (10% [w/v]). Then initiator I2959 was added at 0.05% (w/v). The mixture was pipetted into Polyvinyl Chloride (PVC) molds at 37 °C, cooled to 25 °C, and then exposed to 365 nm ultraviolet (UV) light (10 mW/cm$^2$, 10 min) at 25 °C to form supramolecular
hydrogels.

For different tests, PVC molds of different sizes were used.

**Rheological measurement**

Frequency sweep and shear thinning tests were performed on an Anton Paar MCR301 rheometer with 25-mm diameter plates (plate to plate) at a 0.2-mm gap size. The hydrogels were homogeneously distributed between the top and bottom plates of the rheometer. Frequency sweeps were recorded at a strain of 0.1% and a frequency that changed from 0.01 to 10 Hz. For the shear thinning test, the sample underwent sequential shear with a strain of 0.1% (for 120 s) and 1000% (for 120 s) for four cycles, and the recovery of storage (G’) and loss modulus (G’’) were monitored by time sweeps at a fixed frequency (10 Hz).

**Compression mechanical testing**

Compression tests were performed on samples of 3-cm diameter × 3-mm thickness at an extension speed of 1 mm/s using a MACH-1 Micromechanical System (Biomomentum Inc.).

**Analysis of the release of encapsulated icaritin from the hydrogels in vitro**

Icaritin laden HGM hydrogels (32 µg icaritin per 100 µL hydrogel n=4) were photocrosslinked in 6-mm diameter × 3-mm thickness PVC molds. The hydrogels were incubated in 350 µL of PBS. PBS solutions were collected and replenished at 1 h, 3 h, 6 h, 17 h, 1 day, 2 days, 4 days, 7 days, and 14 days. The absorbance of samples was measured at 242 nm for these solutions to calculate the percentage of icaritin released. To eliminate the effect of I2959, Ci-I hydrogels without icaritin were soaked in PBS as
a blank control.

**Analysis of hMSC migration in the hydrogels *in vitro***

The migration test was performed by using a 24-well transwell. Icaritin laden Ci-I hydrogels and Ci-I hydrogels without icaritin were each formed on the top of the transwell membrane. Then, the transwell setups were placed in a 24-well plate. The volume of each hydrogel was 30 µL. Next, 100 µL of media containing hMSCs (2 × 10^6/mL) were added to the top of the hydrogels, and 770 µL of growth media were added to the 24-well plates. After 24 h of incubation at 37 °C, the hydrogels were fixed with 4% paraformaldehyde and stained with DAPI. Confocal micrographs were obtained to visualize the distribution of the cells in the hydrogels.

**Culture of hMSC-laden hydrogels *in vitro***

hMSCs were expanded to passage 4 by using growth medium consisting of α-minimum essential medium with 16.7% FBS, 1% penicillin/streptomycin and 1% L-glutamine. For three-dimensional (3D) culture, 1 × 10^7/mL hMSCs were then photoencapsulated by irradiation at 365 nm UV (10 mW/cm², 5 min) in Ci-I or MeGel hydrogels. Media (α-minimum essential medium with 16.7% FBS, 1% penicillin/streptomycin, 1% L-glutamine, 10 mM β-glycerophosphate disodium, 50 µg/mL L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate, various amounts of dexamethasone (100 nM or 500 nM), and various amounts of icaritin (0 nM, 100 nM, and 500 nM)) were used for 3D culture *in vitro* with media refreshed every 3 days. Viability was tested on 3D cell culture samples by calcein AM (green, live cells) and ethidium bromide (red, dead cells). For the *in vitro* culture of a icaritin loaded Ci-I hydrogel, we loaded 16 µg icaritin
and hMSCs into the Ci-I hydrogels simultaneously.

**Analysis of the hMSC cell viability in the hydrogels after injection *in vitro***

The cylindrical hMSCs-laden Ci-I gelatin hydrogels was cultured in growth media for 1 day and then injected through a G21 needle to cylindrical molds to remold the hydrogels. After the injection, the remolded hMSCs-laden Ci-I gelatin hydrogels were culture in growth media for another 5 days. The cell viability was tested before and after the injection by using live/dead staining.

**Gene expression analysis *in vitro***

Samples were homogenized in Trizol reagent and then RNA was extracted according to the manufacturer’s instructions. The RNA concentration was determined using an ND-1000 spectrophotometer (Nanodrop Technologies). RNA from each sample (100 ng) was reverse transcribed into cDNA using RevertAid First Strand cDNA Synthesis Kit from Thermo following the manufacturer’s protocol. Polymerase chain reaction (PCR) was performed on an Applied Biosystems 7300 Real Time PRC system using Taqman primers and probes specific for *GAPDH* (housekeeping gene), osteogenic marker gene, and adipogenic marker gene. The relative gene expression was calculated using the ΔΔCT method, where fold difference was calculated using the expression $2^{-\Delta\Delta CT}$. Each sample was internally normalized to GAPDH, and every group was compared to the expression levels of Group 1, the quantitative value of which was determined to be 1.

**Surgical core decompression (CD) and hydrogel implantation in the SAON model of Sprague-Dawley (SD) rats**
Five-month-old male SD rats were used. The animal experimental protocol was reviewed and approved by the ethics committee of the Chinese University of Hong Kong. According to our established protocols1,2, every SD rat was injected with MPS (100 mg/kg per day) for 5 continuous days. At day 14, surgical CD of 1.2 mm diameter and 10 mm depth was performed in the mid-axis of the femoral neck of SD rats. Bulk cylindrical Ci-I hydrogels (15mm diameter, 3mm thickness) without icaritin, or loaded with icaritin (1.3 mM), or loaded with icaritin (1.3 mM) and MSCs (100 million cells per 100 µL hydrogel) were prepared. These bulk Ci-I hydrogels were washed in PBS for 3 minutes and then cultured in growth media for 1 hour. Then, the bulk Ci-I hydrogels was drawn into 3mL syringe iva suction and then immediately injected at 30 µL volume each time to a single bone tunnel created by the core decompression surgery. The total volume of hydrogel for each rat was ~ 30 µL. The injection duration was ~ 30 seconds.

Micro-CT imaging

The femora were collected and fixed in 70% ethyl alcohol prior to bone scanning fusing a high-resolution peripheral computed tomography instrument (HR-pQCT, Scanco Medical, Brüttisellen, Switzerland) with a source voltage of 70 kV and current of 114 µA, according to our established protocols3. 3D images of the peri-tunnel and tunnel at week 3 and week 6 were reconstructed. Bone tissue volume fractions (bone volume/total volume, BV/TV) were measured using the built-in software of the HR-pQCT system.

Histological analysis
In vitro samples were fixed in 4% paraformaldehyde for 24 h, embedded in paraffin, and processed using standard histological procedures. The histological sections (5 µm thick) were stained for targets of interest. Hematoxylin and eosin (HE) staining and Mason’s Trichrome staining were prepared following the manufacturers’ instructions, respectively. For the Runx2 and osteocalcin (OCN) immunochemical staining, the sections were stained using the Vectastain ABC Kit and the DAB Substrate Kit for peroxidase. Briefly, sections were predigested in 0.5 mg/mL hyaluronidase for 30 min at 37 °C and incubated in 0.5 N acetic acid for 4 h at 4 °C to swell the samples prior to overnight incubation with primary antibodies, mouse monoclonal anti-collagen type I (Takara), at dilutions of 1:200. Non-immune controls underwent the sample procedure without primary antibody incubation.

The femoral head samples were bisected along the coronal plane including the trochanteric region. Half of each bisected region was decalcified and embedded in paraffin while the other half was embedded in methyl methacrylate (MMA) without decalcification. The decalcified sections with a thickness of 5 µm were cut and subjected to HE to evaluate the newly formed bone under a light microscope. Immunofluorescence staining was used to determine the expression of Osterix and Runx2. Sequential fluorescence labeling was used for the evaluation of the new bone formation rate as previously reported. MMA sections of 10 µm thickness were used for Goldner’s Trichrome staining, according to our established protocol.

**Bone formation rate measurement**

According to our previous protocol, on the first day of week 3 or week 6 post surgery,
calcein green (CG, 10 mg/kg) was subcutaneously injected into each rat. Then, after 7 days, xylenol orange (XO, 90 mg/kg) was injected into the rat through the same way. After another 3 days, rats were sacrificed, and their femoral heads were collected and embedded in MMA without decalcification and sectioned into thick sections (200 μm). The tissue sections were further polished to 100 μm for the evaluation of the new-bone formation rate under a fluorescence microscope. The spacing between the CG area and the XO area was measured by using ImageJ (supplied by National Institutes of Health, USA), and this spacing equaled to the distance of new bone formation after CG injection but before XO injection (7 days). Then, the bone formation rate at week 3 or week 6 post surgery was the calculated spacing divided by 7 days.

**Superparamagnetic iron oxide (SPIO) labeling**

SPIO labeling was performed on rat MSCs according to our established protocol. SPIO nanoparticles were coated with silica and functionalized with amines, suspended in serum-free α-MEM, and then sonicated for 30 min at 100 V to disperse aggregated particles. MSCs were washed thoroughly with PBS for 16 h to remove the serum prior incubation with SPIO. The labeled MSCs (100 μL suspension of 1 million pre-labeled MSCs) were injected into the bone marrow cavity of the proximal ipsilateral tibia during SAON surgery for histological analysis.

**Western blot**

According to our established protocols, the concentration of protein was measured by using a BCA protein assay kit. 50 μg of protein was separated by 10% SDS-PAGE and transferred to a PVDF membrane by a buffer-tank-blotting apparatus. After blocking
with 5% fat-free milk for 1 h, the membrane was incubated with primary antibody β-catenin, β-actin, c-Src, PCNA CEBPα, PPARγ, Osteocalcin (OCN), and colony stimulating factor-1 receptor (CSF-1R) overnight at 4 °C, respectively. Then it was incubated with secondary antibody conjugated to horseradish peroxidase for 1 h. After washing with tris-buffered saline and Tris-buffered saline Tween-20, the protein was detected and visualized using Amersham ECL Plus™ Western Blotting Detection.

No unexpected or unusually high safety hazards were encountered.

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