Supporting Information

3D-printable self-healing and mechanically reinforced hydrogels with host-guest non-covalent interactions integrated in covalently linked networks

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EXPERIMENTS SECTION

Materials: Gelatin (Type A, gel strength ~300 g Bloom, bioReagent), 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (I2959), methacrylic anhydride, acryloyl chloride, 1-bromoadamantane, Poly(ethylene glycol) diacrylate (PEGDA) and tin(II) 2-ethylhexanoate were obtained from Sigma-Aldrich. β-CD was purchased from Aladdin and recrystallized two times from DI water before use. Tetra-ethylene glycol (TEG), dimethylsulfoxide-d_6 (DMSO-d_6), triethylamine (Et_3N), N,N-dimethylformamide (DMF, anhydrous level), chloroform-d_1 ($CDCl_3$), and deuterium oxide (D_2O) were purchased from Aladdin (China). AOI was purchased from TCI. Carmine was purchased from Macklin (China). Dulbecco's phosphate-buffered saline (DPBS), calcein AM, streptomycin, penicillin, Dulbecco's-modified Eagle's medium (DMEM), ethidium homodimer and fetal bovine serum (FBS) were purchased from Gibco company (USA).

Synthesis of host molecule β-CD-AOI$_2$: β-CD-AOI_2 was synthesized according to the following method. β-CD (10 g, 7.6 mmol), which was recrystallized two times and dried under vacuum for one night, was fully dissolved in 50 mL of anhydrous DMF under a nitrogen atmosphere. Then, the mixture was added with AOI_2 (2.14 g, 15.2 mmol) dropwise. It was stirred vigorously before 50 μL of tin(II) 2-ethylhexanoate (a catalytic agent) was added into the solution. After it was stirred for 60 min at room temperature in the nitrogen atmosphere, the reaction temperature was increased to 40 °C and then the stirring was continued for 4 h until the reaction was completed. The resulting molecule (β-CD-AOI_2) was precipitated by adding 400 mL of cold acetone. The precipitate was repeatedly dissolved in 10 mL of DI water and poured into 200 mL of acetone several times to remove the DMF and unreacted AOI. The product was dried at 35 °C for 48 h in a vacuum oven. The substitution degree (DS) of β-CD was confirmed to be 2 using ^{1}H NMR and MALDI-TOF MS.

Synthesis of the guest-molecule A-TEG-Ad: A-TEG-Ad was prepared according to the reported literature.[1] In brief, 1-bromoadamantane (20 g, 0.093 mol) and Et_3N (0.0283 mol) were dissolved in 400 mL of TEG with stirring for 24 h at 110 °C. The resultant solution was cooled to room temperature and washed first with 1 M HCl (3 × 200 mL) and then with DI water (3 × 200 mL). Then, the product was extracted with 200 mL of dichloromethane (CH_2Cl_2). The combined organic phases were dried over anhydrous $NaSO_4$, and the solvent was evaporated to produce TEG-Ad as slightly yellow oil. Then the TEG-Ad (10 g, 30.5 mmol) and Et_3N (12.8 mL) were dissolved in 100 mL of CH_2Cl_2, and after cooling to 0 °C, acryloyl chloride (3 mL, 36.6 mmol) in CH_2Cl_2 (10 mL) was dropped into the mixture. The solution was warmed to 65 °C and refluxed for 2 h after the dropwise addition. The crude product was washed with DI water (4 × 50 mL) and 10 wt% NaCl (3 × 50 mL) several times. The organic layer was collected and dried overnight with anhydrous $MgSO_4$. Finally, the resultant product was filtered, evaporated, and purified using column chromatography to produce A-TEG-Ad as a light-yellow oil. The product was stored in a refrigerator at 4 °C (yield: 80%).

Preparation of the HGSM: β-CD-AOI_2 (5 g, 3.53 mmol) was added to 8.8 mL of DI water. After the dissolution was complete, yellow-oil A-TEG-Ad (1.35 g, 3.53 mmol) was placed and stirred for 24 h. The solution slowly changed from opaque to transparent, which represented the successful inclusion of the guest molecules into host molecules. The inclusion complexation between β-CD-AOI_2 and A-TEG-Ad was confirmed using 2D ROESY NMR and X-ray diffraction (XRD). The resulting mixture was lyophilized for further use.

Synthesis of the GelMA: GelMA was prepared as previously reported.[2] GelMA was obtained as a white porous foam after lyophilization (-80 °C, 2 days) and was stored at -20 °C. The ^{1}H-NMR spectra of GelMA in D_2O were recorded. The actual degree of methacryloyl modification was calculated using a previously reported method as follows:[3]

Methacryloyl modification (%) = (Number of methacryloyl groups in GelMA/Number of amine groups in the unreacted polymers)×100%

Preparation of HGGelMA hydrogel: HGGelMA were prepared by initiating free radical copolymerization between GelMA and HGSM in DPBS at room temperature under ultraviolet (UV) light irradiation using Irgacure 2959 (I2959) as a photo-initiator. In detail, the concentration of GelMA was fixed at 15% (w/v) in PBS, and varying amounts of HGSM (3% (w/v), 6% (w/v), 9% (w/v), 12% (w/v), and 15% (w/v)) were dissolved in the solution. I2959 was added at 0.5% (w/v). The resulting mixture was injected into PDMS molds (9 mm diameter × 5 mm thickness) between two overlapped glasses slide at 37 °C. The mixtures were cooled to room temperature, and then placed in a UV crosslinker (UVP, CL-1000 L) with 365 nm UV light at a power desnity of 5 mW cm^{-2} for 10 min to produce HGGelMA hydrogels with various ratio of HGSM and GelMA, termed HG_XGelMA, where x indicated the concentration of HGSM in the hydrogel (w/v%). Unless noted, the concentration of GelMA in the HGGelMAs was fixed to be 15% (w/v).

Preparation of GelMA hydrogel: First, 15 wt % GelMA and 0.05 wt % I2959 as photoinitiator in water were added to PBS at 60 °C. The resultant solution was placed into PDMS molds and air-cooled to room temperature. Then UV irradiation (5 mW cm^{-2}) was applied to the solution for 10 min in a UVP. The resultant pure chemically crosslinked methacryloyl modified gelatin hydrogel was named GelMA. The Poly(ethylene glycol) diacrylate (PEGDA, 10% (w/v)) hydrogel was prepared using similar steps.

3D printing of the HGGelMA hydrogel: The 3D printed HGGelMA hydrogel was constructed from the hydrogel precursors as follow. First, we developed a direct-write "ink" that consisted of a constant concentration of GelMA (15% w/v), 9% HGSM, and 0.5% I2959. Then, the ink was loaded into an extrusion unit composed of a cartridge and a single channel needle (22G, 400m), and the temperature of the ink was decreased to 24 °C using a low-temperature and low-viscous dispensing head (LTV-Dispense Head). The LTV-Dispense Head was mounted on a X-Y-Z moving system on a

3D-Bioplotter™ (EnvisionTEC GmbH, Germany) to continuously deposit 3D computer-designed patterns of hydrogel fibers. When the ink phase was stable, a dispensing pressure of 8×10^4 Pa was exterted to the syringe by a nitrogen gas-pressurized cap, and the X-Y plotting speed was set to 18 mm s^{-1} to create full, straight and perfect strands. The distance between two adjacent deposited fibers was 800 μm. Finally, according to the designed rectangular block model (10 mm × 10 mm × 2 mm) using Bioplotter computer-aided manufacturing (CAM) software, 7 layers of HGGelMAs were produced in a layer-by-layer manner on a low-temperature platform. To maintain the shape of the deposited fibers and the structure of 3D printed hydrogel, the platform temperature was maintained at 5 °C to induce rapid solidification after dispensing. When the printing process was completed, the 3D printed HGGelMA scaffold was solidified secondarily by exposure to UV light (10 mW cm^{-2}) for 3 min. To evaluate the 3D printability of the HGGelMA, we designed two different architectures of hydrogel pore shapes by plotting fibers in a layer-by-layer fashion with a inter-layer angle of 45° or 90° (the corresponding scaffold was named HGGelMA-45 or HGGelMA-90).

MALDI-TOF-MS: MALDI-TOF-MS measurements were recorded using an Autoflex III SmartBeam (Bruker, Germany). β-CD-AOI_2 was dissolved in ultrapure water at a concentration of 1mg mL^{-1}, and α-cyano-4-hydroxycinnamic acid was employed as a matrix material.

2D ROESY NMR: To confirm the inclusion complexation effect within the HGSM, the prepared HGSM powder was dissolved in deuterium oxide (D_2O), and 2D ROESY NMR spectra were obtained using a 400 M NMR (AVANCE III HD 400, Bruker, Germany).

HGGelMA swelling experiments: To evaluate the effects of crosslinking on the swelling behavior of the HGSM, various concentrations of HGGelMAs were prepared in uniform round blocks (5 mm thick, Φ 9 mm; GelMA, HG_3GelMA, HG_6GelMA, HG_9GelMA, HG_{12}GelMA and HG_{15}GelMA represent 3% (w/v), 6% (w/v), 9% (w/v), 12% (w/v) and 15% (w/v) HGSM, respectively; the concentration of GelMA was fixed at 15% (w/v); n=5). The whole HGGelMA was freeze dried and weighed (W_0). Then, the HGGelMAs were immersed in DPBS and incubated at 37 °C. After different swelling periods (1, 12, 24 and 36 h), the weights of the HGGelMAs were measured (W_1, W_{12}, W_{24} and W_{36}). The swelling rate at the corresponding time point was calculated using the follow formula:

$$W_{swelling\ ratio} = (W_n - W_o)/W_o \times 100\%$$

where W_n indicated the weight of the HGGelMA after swelling for n hours in DPBS.

Rheological measurements: All rheological characterizations were conducted on a stress-controlled rheometer (Anton paar MCR302). For the HGGelMA hydrogels, The ultraviolet initiated polymerization process was investigated in a plate-plate geometry (plate diameter 25 mm, distance 0.3 mm) at 7.5 mW cm^{-2}. The mechanical spectra of different hydrogel concentrations (GelMA, HG_3GelMA, HG_9GelMA and HG_{15}GelMA) were recorded at a constant deformation of 0.5% strain and 10 rad s^{-1} at 25 °C. Other rheological measurements were conducted by oscillatory frequency sweeps (0.01-100 rad s^{-1}; 1% strain) and oscillatory strain sweeps (0.01-2000% strain; 10 rad s^{-1}). For shear self-healing experiments, a high shear strain-induced damage was assessed at a strain of 1000% and recovered at 0.5% strain, each at 10 rad s^{-1}.

To evaluate the rheological behavior of the HGGelMA bioinks, viscosity measurements of HGGelMA solutions as a function of the temperature (5-37 °C) were performed using rotational viscometry with a cone and plate geometry (plate diameter 50 mm, distance 0.108 mm at 5 rad s^{-1}. The viscosity under different shear rates was evaluated at 25 °C between 0.1-100 s^{-1}. Furthermore, the loss modulus (G") and storage modulus (G') of the HG_9GelMA inks were investigated as a function of the temperature by an oscillatory test at a constant frequency (6 rad s^{-1}) and strain (0.5%).

SEM: Cylindrical HGGelMAs (9 mm diameter × 5 mm thickness) were allowed to reach swelling equilibrium in PBS at 37 °C and then lyophilized under vacuum at -80 °C. Next, the dry HGGelMAs were fractured along the middle, and imaged by scaaning electron microscopy (SEM) after gold-coating.

Mechanical testing: Compression and tension tests were performed using samples with various concentrations of HGGelMAs and conducted in a thermostated room at 23 ± 2 °C. All hydrogels were swollen to equilibrium in PBS at 37 °C before experimentation. Young's modulus was calculated from the initial linear segment of the stress–strain curves between 3-15%. More than five samples were used to get the average Young's modulus. Cylindrical HGGelMA samples (9 mm diameter × 5 mm thickness) prepared for the compression tests were subject to a compression speed of 1 mm/min on a universal material testing machine with 500 N load cells. The 3D printed HGGelMAs (rectangular block, 10 m × 10 mm × 2 mm) used identical parameters. For the HGGelMA tensile tests, a dynamic mechanical analyzer (DMA) was used to examine the tensile properties of the hydrogels. All HGGelMAs were prepared as dumbbell shapes with dimensions of 15 mm (L) × 2 mm (W) × 1.5 mm (T), which was precisely recorded by a digital caliper micrometer. The two arms of the gel samples were fixed by clamps to perform the tensile tests under an extension rate of 1 mm min^{-1}. About the evaluation of resist compression ability, two different weights (500 g and 1 kg) were pressed on the GelMA and HGGelMA hydrogels respectively and held the pressure for minutes to observe the state of the hydrogels. In addition, cyclic elongation tests were performed on the dumbbell-shaped hydrogels using DMA. The fracture energy[4] of hydrogels (n=5) was calculated as

$$\Gamma = \mathrm{d}\int_1^{\lambda_c} s\,d\lambda$$

The hydrogel films were pulled at a constant speed of 1 mm/min with a tensile strain of 0-35% for 10 cycles.

Self-healing testing: The self-healing abilities of the HGGelMAs were evaluated. First, the HGGelMAs (cylindrical) at swelling equilibrium were equally cut into two parts with a scalpel. Half of the samples were dyed red with Ponceau 4R (food colorant, Shanghai LionHead), and the other half did not experience any treatment. Then, the two halves were joined along the incision part. Approximately 60 min later, the self-healing hydrogels were pulled with tweezers to observe the self-healing state. The self-healing process of hydrogel incisions was observed by 3D rotational microscopy. Self-healing efficiency of HGGelMA was evaluated by a mechanical tensile test based on fracture energy.

Evaluation of the ease of processability and reproducibility: To investigate the reproducibility of the HGGelMAs, prepolymer mixtures of HGGelMAs were injected into molds with various shapes (pentagram, triangle, cordiform, square) that were clamped between two overlapping glass slides at 37 °C. HGGelMAs with characteristic shapes were obtained after polymerization under UV light (5 mw cm^{-2}). The hydrogels were fully swollen in PBS to achieve equilibrium and then dried naturally at room temperature. Finally, the dried hydrogels were soaked in PBS to reach swelling equilibrium again. The morphologies of the dried and reswollen gels were subsequently observed.

In vitro culturing of mBMSCs on the 3D printed scaffolds: mBMSCs (ATCC CRL12424) at passage 3-4 were cultured in a conventional medium, which consisted of DMEM supplemented with 10% fetal bovine serum (FBS), at 37 °C and collected using a 0.25% trypsin-EDTA solution (Gibco, USA) in 75 mL tissue culture flasks. The hydrogel was developed into a porous rectangular block scaffold (10 m × 10 m × 2 mm) using 3D printing. After lyophilization, the 3D printed hydrogel scaffolds were sterilized by exposing to gamma radiation (15 kGy). Subsequently, the scaffolds were re-swollen in sterile PBS to achieve equilibrium for further cytological evaluation.

Live/dead viability: For the encapsulation of cells, the mBMSCs and uncrosslinked hydrogel solutions (HGGelMA, GelMA or 10% PEGDA) were mixed to get the cell-loaded pre-polymer mixture. Then, the mixture was injected into a 48-well plates and exposed to UV to form cell-loaded hydrogels for 5 minutes. Then, 400 μL culture medium was placed to each well and incubated. After cultured for 1, 4 and 7 days, the BMSC-laden hydrogels were rinsed twice with PBS. Subsequently, the cell-loaded hydrogels were incubated in 500 μL of PBS containing calcein AM and ethidium homodimer at 25 °C for 30 min. The cell-loaded hydrogels were then washed twice with PBS and observed using a confocal laser scanning microscope (LSCM, Leica TCS SP8, Germany). The live and dead mBMSCs on the 3D hydrogel scaffolds were stained green and red with calcein AM and ethidium homodimer, respectively. The Live/dead fluorescence staining process of the encapsulated mBMSC cells on GelMA and HGGelMA printed fibers was similar.

Cell proliferation: The cell viability on the HGGelMAs was determined by Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, USA). The mBMSCs were seeded on the 3D printed hydrogels with a density of 10^5 cells mL^{-1} (n = 5) and incubated for 1, 3, 5 and 7 days. PBS was added to rinse the gels twice, and then the cells were incubated for 60 min with 1 mL of CCK-8 working liquid in each well. Subsequently, 100 μL of the supernatant medium was moved to a 96-well plate, and the optical density value was quantified at 450 nm absorbance with a multifunctional microplate reader (Thermo Scientific 3001, USA).

Cell adhesion: To observe the cell adhesion on the 3D hydrogel scaffolds and PEGDA hydrogel (10% (w/v)), mBMSCs were incubated in a CO_2 incubator for 24 h for vinculin staining and 3 days for focal adhesion kinase (FAK) and integrin β1 staining with an initial density of 10^6 cells per mL. After the cell media were gotten rid of, PBS was used to wash the cells twice. Then, mBMSCs were fixed with 4% neutral formaldehyde (AR, Aladdin) for 40 min and then washed with PBS. The mBMSCs were then incubated with 0.1% Triton X-100 for 15 min to increase their permeability except for FAK staining before washing with PBS twice. Afterwards, the cells were blocked with PBS containing 3% bovine serum albumin (Biochemical, Aladdin) and 0.1% tween (Biochemical, macklin) for 1 h and washed three times with PBS. Subsequently, the samples were incubated with primary antibodies including anti-vinculin (rabbit monoclonal to vinculin, 1:50; Abcam, China), anti-FAK (rabbit monoclonal to FAK, 1:200; Abcam, China) and anti-integrin β1(rabbit monoclonal to integrin β1, 1:200; Abcam, China) overnight at 4 °C, respectively. The nonbound primary antibodies were removed by washing twice with PBS, and Alexa 488 goat anti-rabbit IgG secondary antibody (red fluorescence, 1:500, Abcam, USA) was placed and incubated for 1 h in the dark. Similarly, the cytoskeletons were stained with the Cell Navigator™ F-Actin Labeling Kit (AAT Bioquest, USA) for 1 h in vinculin and integrin β1 staining and rinsed with PBS. Next, the cell nuclei were labeled blue after staining for 5 min using DAPI. Finally, the morphologies of the cells on the 3D printed HGGelMA scaffolds were captured using an LSCM (Leica TCS SP8, Germany).

Animal experiment evaluation via subcutaneous implantation of HGGelMA scaffolds: The 3D-printed GelMA and HGGelMA scaffolds were subcutaneously implanted into the backs of nude mice (two different pockets for every nude mouse). 20 days and 40 days post-implantation, the implanted scaffolds were histologically analyzed. All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the South China University of Technology and performed under the guidelines of National Institutes of Health.

Histological analysis: The harvested scaffolds were fixed in paraformaldehyde for one day. The tissue scaffolds were paraffin-embedded and processed into histological sections (4 μm thick). Hematoxylin and eosin (H&E) staining, immunohistology staining and real-time PCR tests and was implemented on the sections following the manufacturers' instructions.

Statistical analysis: SPSS V17.0 statistical software was utilized to analyze the results. All data were expressed as the averages ± standard deviations with significant P values (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

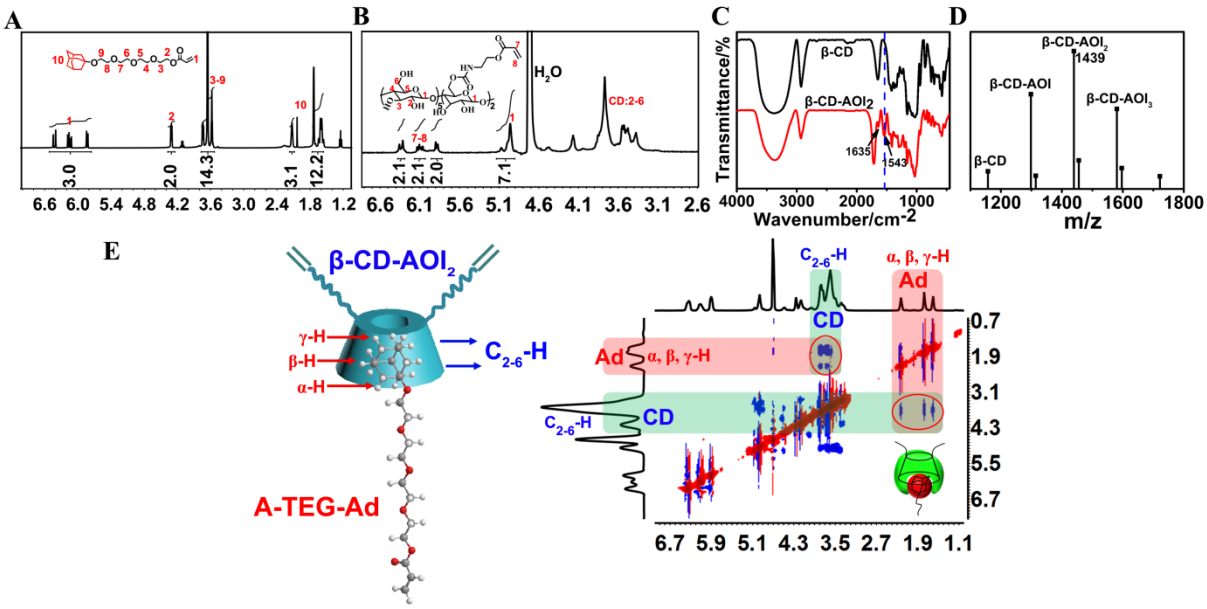

Figure S1. Chemical characterization of the HGSM. (A) The ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of A-TEG-Ad. (B) The ^{1}H NMR spectrum (400 MHz, DMSO-d6) of β-CD-AOI_2. (C) FT-IR spectra of different samples (β-CD, β-CD-AOI_2). (D) The MALDI-TOF MS spectrum of β-CD-AOI_2. (E) 2D ROESY NMR spectrum of HGSM.

As shown in the ^{1}H NMR spectroscopy measurements (Figure S2A), the ratio of the signal integral area between C1-H of the β-CD (d, 7H, 4.9 ppm) and the double bond (m, 6H, 5.8~6.4 ppm) was determined to be 7 : 6, which indicated the successful synthesis of β-CD-AOI_2 with about 2 double bonds per molecule. As seen in the IR spectra (Figure S2B), the characteristic absorption IR bands were found at 1635 cm^{-1} and 1534 cm^{-1}, corresponding to the stretching vibrations of the secondary amides. This result indicated the successful grafting of AOI. The MALDI-TOF MS (Figure S2C) spectrum also supported this point. As shown in the results of MALDI-TOF MS, the products were mainly β-CD-AOI_2 but also contained a small proportion of β-CD-AOI and β-CD-AOI_3. The data showed that the β-CD modification process was efficient and resulted in a high yield (≥85%).

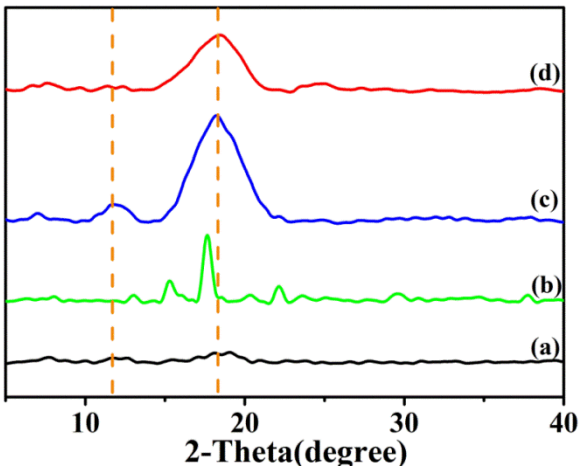

Figure S2. X-ray diffraction (XRD) patterns of A-TEG-Ad (a), β-CD-AOI_2/A-TEG-Ad solid system obtained by physical mixture (b), Host-guest interaction supramolecules of β-CD-AOI_2 (c), and A-TEG-Ad (d).

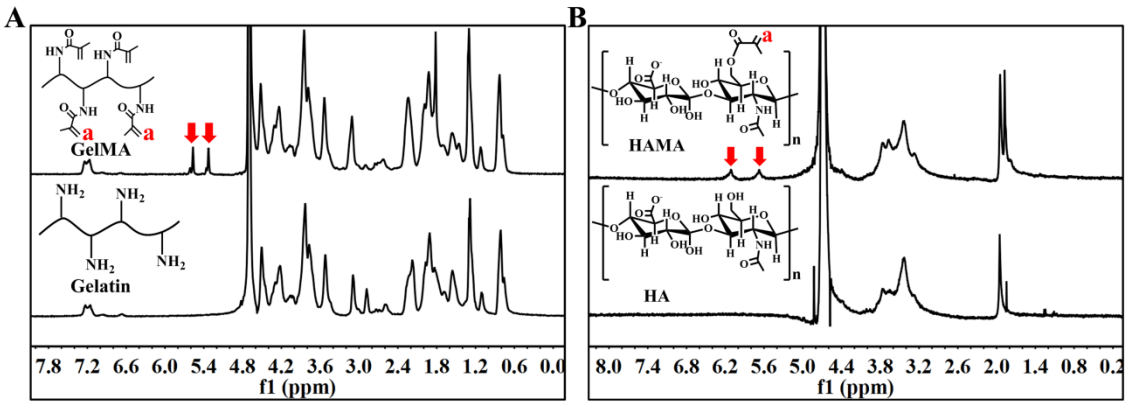

Figure S3. The ^{1}H NMR (400 MHz, D_2O) spectrum of Gelatin, GelMA, hyaluronic acid (HA) and methacrylated hyaluronic acid (HAMe). (A) ^{1}H NMR comparison spectrum of GelMA (up) and Gelatin (down). (B) ^{1}H NMR comparison spectrum of HAMe (up) and HA (down).

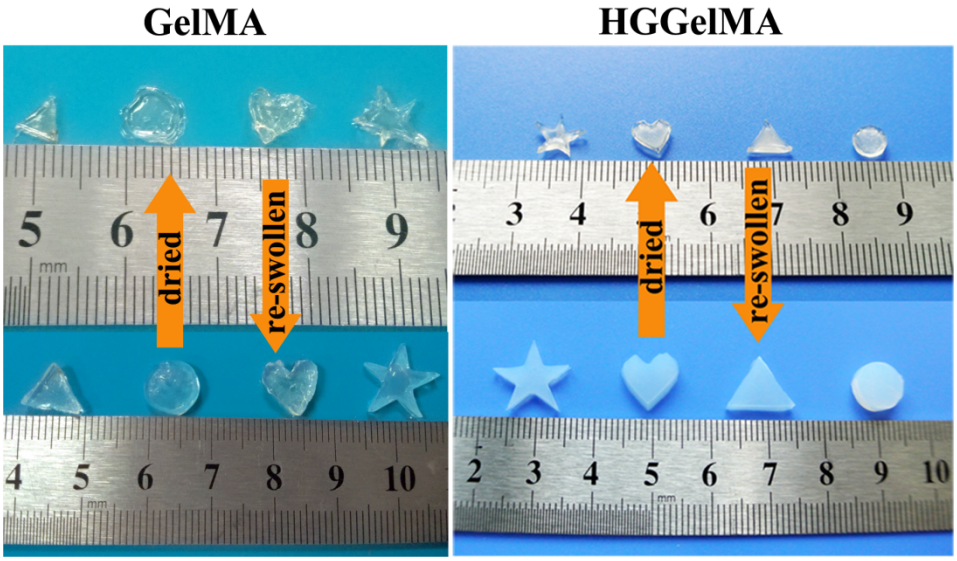

Figure S4. The comparison of hydrogel shapes which swelled after drying-swelling cycles.

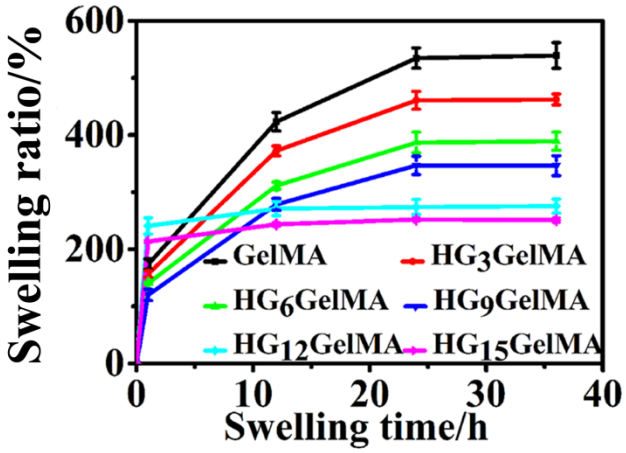

Figure S5. Swelling kinetics curves of HGGelMA and GelMA hydrogels.

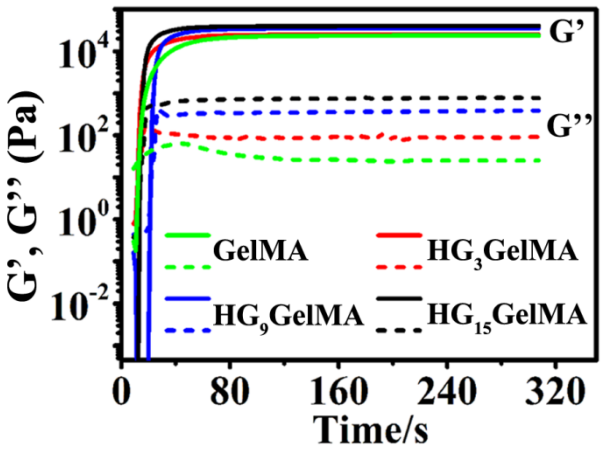

Figure S6. Oscillatory frequency sweeps showing the UV initiating polymerization process of hydrogels (Storage modulus, G'; Loss modulus, G").

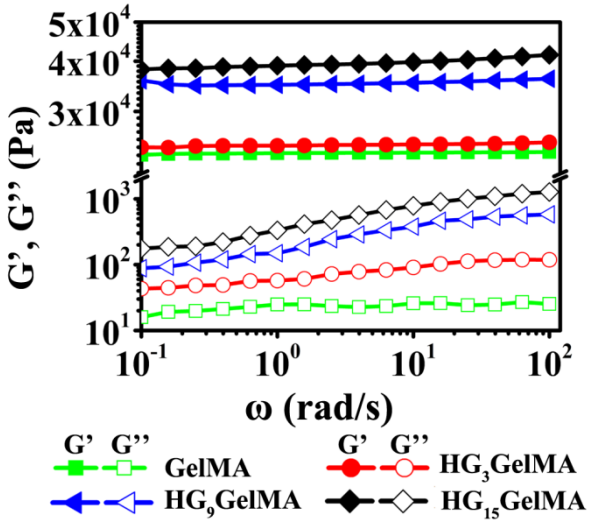

Figure S7. Frequency sweep measurements of GelMA and HGGelMA hydrogels.

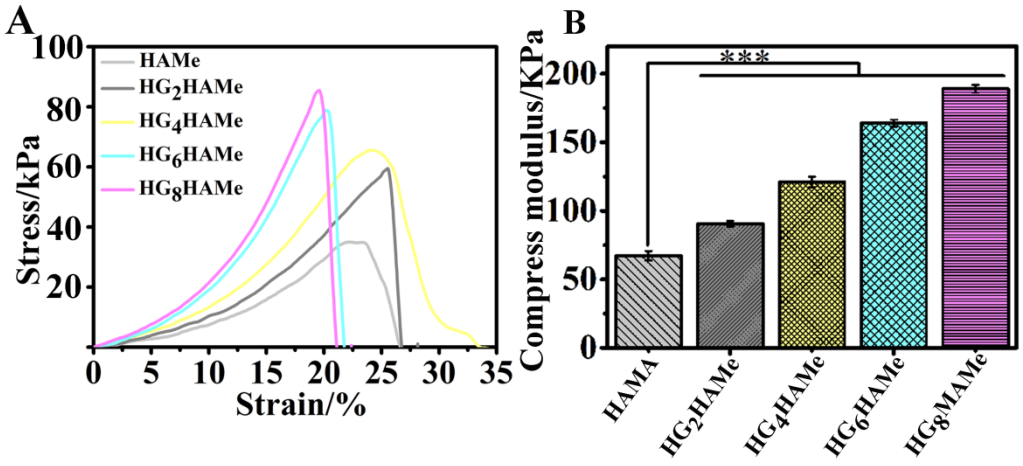

Figure S8. The mechanical characterization illustration of methacrylated hyaluronic acid (HAMe) and HGSM reinforced HAMe hydrogel (HGHAMe). (A) The stress-strain profiles of the HAMe hydrogels undergoing compression (HGXHAMe where x means the concentration of HGSM (w/v %). This

concentration of HAMe in HGHAMe was fixed at 2% (w/v)). (B) The compression modulus comparison beween different hydrogels.

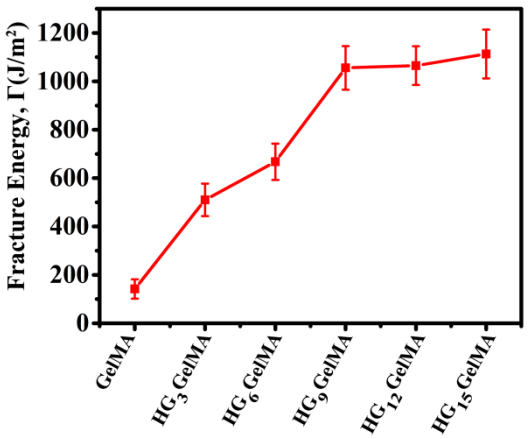

Figure S9. Fracture energy varies with the concentration of host-guest supramolecule.

Figure S10. Digital images of GelMA and HGGelMA after cyclic tensile test.

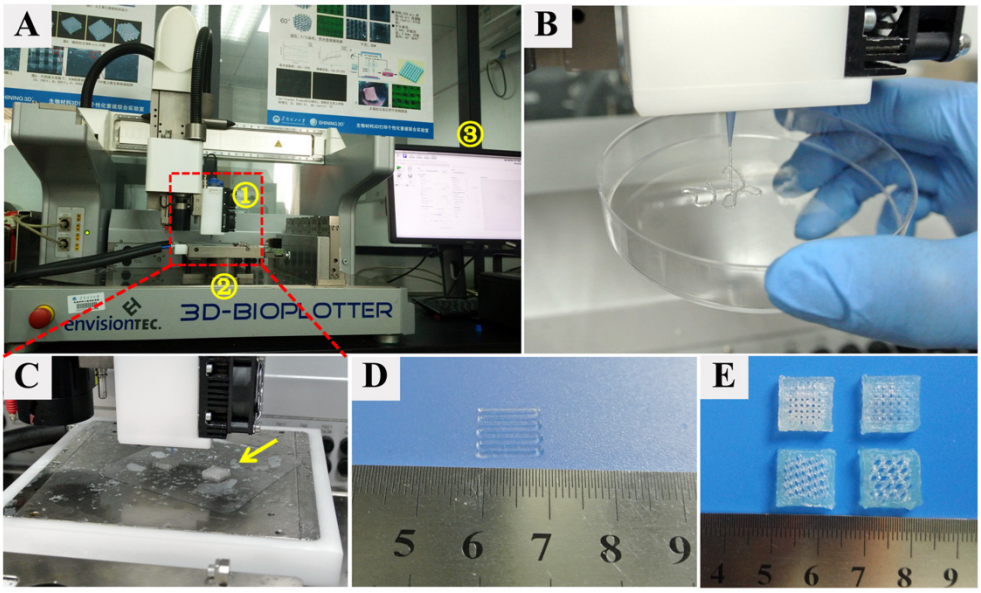

Figure S11. The digital images of 3D-Bioplotter™ under the printing process and the photographs of filament and scaffolds. (A) Photographs of 3D-Biopoltter™ during the 3D printing process (① Low-Temp-Viscous Dispensing-Head. ② Low-Temperature Platform. ③ Computer control system). (B) The images of fibers during printing process. (C) The enlarged photograph of Low-Temp-Viscous Dispensing-Head during the printing process. (D) The images of one layer of HG_9GelMA scaffold. (E) The digital images of 3D scaffolds with two types of pores.

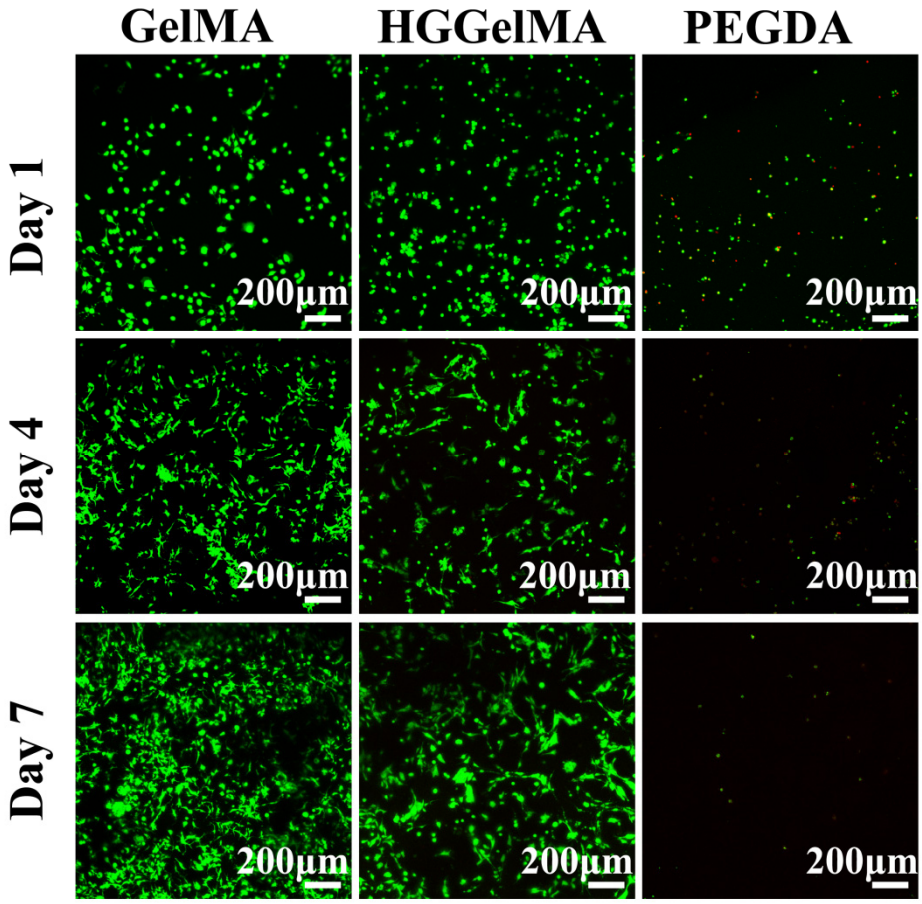

Figure S12. Live/dead fluorescence images of the encapsulated cells on GelMA, HGGelMA and PEGDA hydrogels.

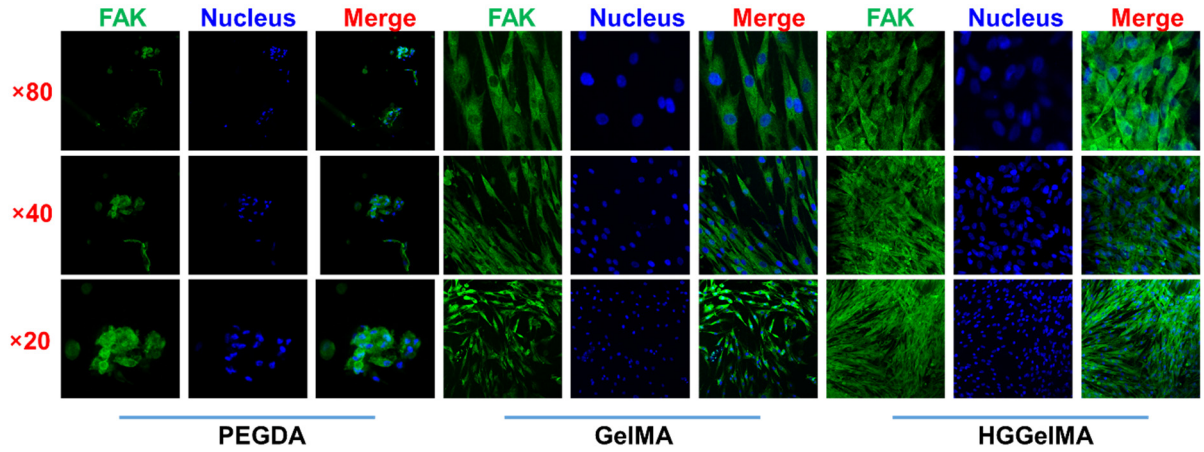

Figure S13. FAK protein fluorescence images showing adhesion morphology of mBMSCs after 3 days of culture on GelMA, HGGelMA and PEGDA hydrogels, respectively.

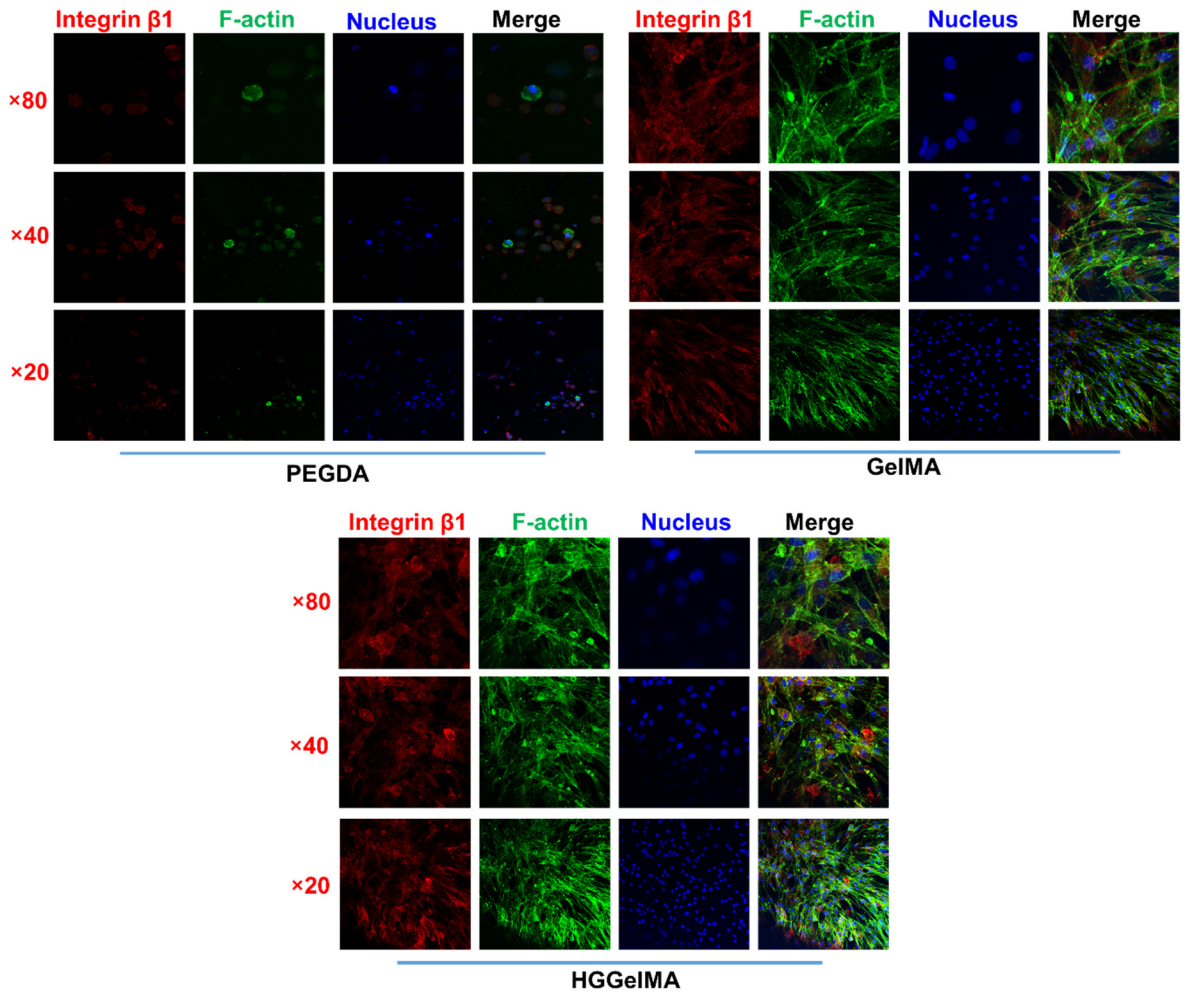

Figure S14. Integrin β1 protein fluorescence images showing adhesion morphology of mBMSCs after 3 days of culture on GelMA, HGGelMA and PEGDA hydrogels, respectively.

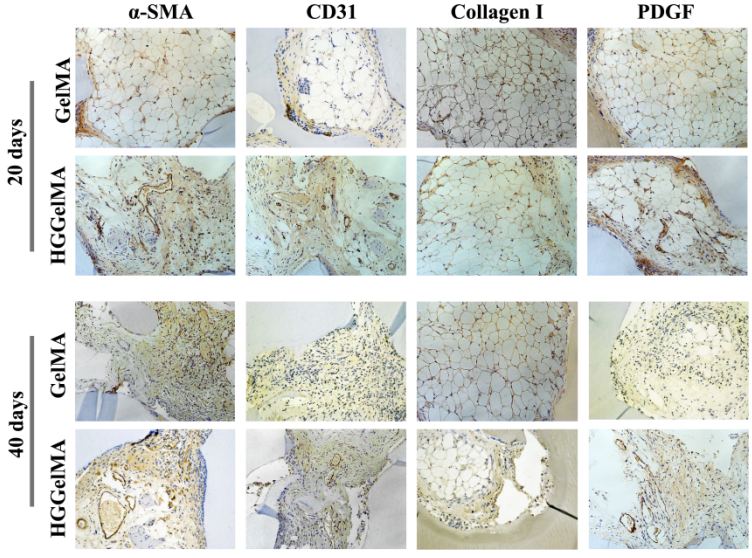

Figure S15. The immunohistological images of α-SMA, CD31, collagen I and PDGF proteins in implanted scaffolds.

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