Electronic Supplementary Information for

Strength-tunable Printing of Xanthan gum Hydrogel via Enzymatic Polymerization and Amide Bioconjugation

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Contents

Materials: L-Lysine monohydrochloride and *N*-(3-dimethylaminopropyl)-*N*'-ethyl carbodiimide hydrochloride (EDC·HCl) were obtained from Shanghai Baoman Biotechnology Co., Ltd. (Shanghai, China). *N*-Hydroxysulfosuccinimide sodium salt (Sulfo-NHS) and D- (+)-glucose were purchased from Aladdin. glucose oxidase (GOx) obtained from Sigma-Aldrich. Xanthan gum was purchased from Jiuding Chemical (Shanghai, China). All other chemicals were of the highest purity commercially available and were used as received.

Synthesis of *N*-acryloyl-L-lysine: To a solution of L-lysine monohydrochloride (4.08 g, 22.35 mmol), $CuSO_4 \cdot 5H_2O$ (2.79 g, 11.15 mmol), NaOH (1.83 g, 45.8mmol), and Na_2CO_3 (2.37 g, 22.35 mmol) in H_2O (100 mL) were added dropwise to acryloyl chloride (2.5 g, 27.6 mmol) at 0 °C over 15 minutes. The reaction mixture was stirred at RT for 2 h. The blue precipitate was filtered and washed with H_2O (200 mL), ethanol (80 mL), petroleum ether (100 mL), sequentially. After air drying, the violet-blue solid was resuspended in H_2O (100 mL) and CH_2Cl_2 (100 mL). To this solution 8-hydroxyquinoline (6.0g, 41.3 mmol) was added portion wise over 30 min. The reaction mixture was vigorously stirred for 1 h, at which time the green solid was filtered and washed with H_2O (50 mL). The filtrate was separated into two layers, and the aqueous layer was washed with dichloromethane (70 mL ×3) until no yellow color was extracted. Freeze dry the water layer to give a white solid (2.4 g, 46%) (Figure S1). ^{1}H

NMR (D_2O, 400 MHz) δ (ppm) 6.09 (dd, 1H, J = 10.0, 17.2 Hz, CH_2=C), 5.62 (dd, 1H, J = 2.4, 11.6 Hz, CH=CH_2), 3.59 (t, 1H, JS19= 6.0 Hz, α-CH), 3.16 (t, 2H, J = 6.8 Hz, CH_2-N), 1.75 (m, 2H), 1.47 (m,2H), 1.30 (m, 2H).

^{13}C NMR (D_2O, 100 MHz) δ (ppm) 174.71, 168.45, 129.96, 127.00, 54.61, 39.91,30.01, 27.91, 21.72.

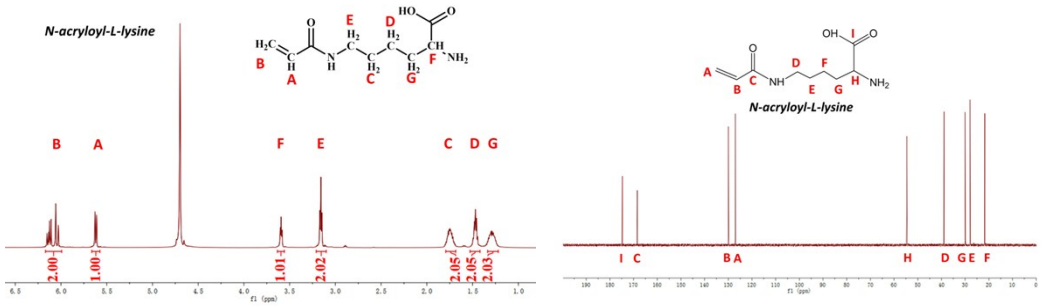


Figure S1. Hydrogen and carbon NMR spectra of monomers *N*-acryloyl-L-lysine

Preparation of poly (*N*-acryloyl-L-lysine) hydrogels: The gel was prepared in the following procedure. 700 μL of *N*-acryloyl-L-lysine (20wt%), 5mg EDC·HCl, 10mg Sulfo-NHS, and 100 μL of Glu (10mg/mL) were added to 100 μL H_2O solution respectively, and were mixed thoroughly on vortex to gain a transparent solution, After the addition of 100 μL of a GOx solution (5 mg/mL), the vial was placed in water bath at 37 °C for 30 minutes to give a self-standing hydrogel.

Preparation of hydrogels with natural polymer: The gel was prepared in the following procedure. 200 μL of *N*-acryloyl-L-lysine (20wt%), 5mg EDC·HCl, 10mg Sulfo-NHS, and 100 μL of Glu(10mg/mL) were added to 600 μL Protein or polysaccharide solution respectively, and were mixed thoroughly on vortex to gain a transparent solution, After the addition of 100 μL of a GOx solution (5 mg/mL), the vial was placed in water bath at 37 °C for 30 minutes to give a self-standing hydrogel.

Preparation of hydrogels with xanthan gum: The gel was prepared in the following procedure. 400μL of *N*-acryloyl-L-lysine (20wt%), 10mg EDC·HCl, 20mg Sulfo-NHS and 400μL of Glu (10mg/mL) were added to 1000μL xanthan gum (10wt%) solution respectively, and were mixed thoroughly by glass rod stirring to gain a precursor, After the addition of 200 μL of a GOx solution (5 mg/mL), the vial was placed in water bath at 37 °C for 30 minutes to give a self-standing composite hydrogel.

3D Printing Process: Ink A: Dissolve EDC·HCl (25mg)/sulfo-NHS (12.5mg) in 3ml of water, mixed with 0.25g xanthan gum, and add 1mL glucose oxidase (5mg/mL), 1ml *N*-acryloyl-L-lysine (20wt%), stirred well. Ink B: Dissolve EDC·HCl (25mg)/sulfo-NHS (12.5mg) in 3ml of water, mixed with 0.25g xanthan gum, and add 1mL glucose (5mg/mL), 1ml *N*-acryloyl-L-lysine (20wt%), stirred well. The 3D printing was performed by an extrusion 3D printer (Bioscaffolder 3.2, GeSiM, Germany). Fill the ink into two small tubes, and ink A prints the pattern. After printing a layer of ink, ink B prints the same pattern on the previous pattern. Every two layer adhered to the underlying layer perpendicularly to form a 0°/90° strut structure, nozzle size was chosen to be 300 μm diameter, and the spacing between each strand was set at 2.3 mm, the printed pressure is 35 kPa, the printed speed is 12 mm/s

Cytotoxicity assay: NIH-3T3 cells were chosen as model cell to evaluate the cytotoxicity of samples by using CCK-8 assay. The powder was sterilized under UV irradiation for 30mins before the assay. Initially, the NIH-3T3 cells were seeded into 96-well plates (5.0×10^3 cells per well) and cultured in culture medium (containing DMEM medium, 10% fetal bovine serum, and 1% penicillin/streptomycin) in cell incubator at 37 °C in the presence of 5% CO_2 for 24 h. Subsequently, the previous medium was replaced by the fresh medium without antibiotics, different concentrations of hydrogel powder (final concentration 1 μg/mL 10μg/mL 100μg/mL) were added, and the cells were cultured in the cell incubator for 24h/48h. Then the old medium was replaced with fresh medium, and 10μL of CCK-8 was added, the cells were incubated

for another 2h in the cell incubator. Ultimately, the absorbance was measured at 450nm wavelength by a microplate reader, and the relative survival rates were calculated by comparing the absorbance values of the test group and the control group.

Fluorescence imaging: NIT-3T3 cells were cultured in a laser confocal culture dish ($5x10^4$ cells/well), containing 1 mL of medium, and cultured in a 37°C 5% CO_2 incubator for 24 h, adding different concentrations of hydrogel powder (1 μg / mL, 10 μg / mL, 100 μg / mL). The culture was continued for 24 hours, and the living and dead cells were stained with 500 μL of staining working solution containing Calein AM and PI (Calein AM 4μM; PI 4.5μM). The AM excitation/emission wavelength was 488/535 nm, and the PI excitation/emission wavelength was 543/620 nm.

Confocal Microscopical Analysis: The hydrogel and cells were co-cultured for 12h, then it stained with 500 μL of staining working solution containing Calein AM and PI (Calein AM 4μM; PI 4.5 μM). Images were acquired using an OLYMPUS FV1000 confocal laser scanning microscope.

Electron Paramagnetic Resonance (EPR) measurement: The EPR results were performed on an EPR Spectrometer (A300, Bruker) at 9.873 GHz. To ascertain the initiating nitrogen radicals in this system, the mixture of GOx, Glu and Sulfo-NHS which were at the same proportion of hydrogel was rapidly transferred to a standard capillary and placed into the EPR spectrometer with DMPO (dimethyl pyridine N-oxide, Electron capture agent) as the spin trap. The spectrum was recorded after 1, 2, 3, 5, 10 min reaction. To determine the propagating radical, 10 wt% of the *N*-acryloyl-L-lysine was added to the above initiation system. The spectrum was recorded after 1, 5, and 9 min reaction.

ATR-FTIR spectroscopy: The infrared spectroscopy was tested by using a

Fourier transform attenuated total reflection infrared spectroscopy (ATR-FTIR) (Thermo Scientific Nicolet IS10). 200 μL of *N*-acryloyl-L-lysine (20wt%), 5mg EDC·HCl and 10mg Sulfo-NHS, 0.05g Xanthan gum and 100 μL of Glu(10mg/mL) were added to 100 μL H_2O solution respectively, and were mixed thoroughly on vortex to gain a solution, After the addition of 100 μL of a GOx solution (5 mg/mL). Test infrared spectra at different times

Rheological analysis: The rheological properties of hydrogels were measured using a Thermo Haake RS6000 rheometer (Thermo Scientific) with parallel plate geometry (diameter: 20 mm, 0.3 mm gap) at 37 °C. The storage modulus (G') and loss modulus (G'') were measured as a function of time to accomplish dynamic time sweep measurements at a fixed frequency of 1.00 Hz and a fixed stress of 1.00 Pa, the change of system viscosity over time can be tested simultaneously.

Mechanical analysis: The mechanical analysis of the hydrogels was carried out on a FR-108B (Farui Co., China) testing machine. The compressive tests were measured at a speed of 3 mm/min and compressed to 40 %. The diameter of the samples was 16 mm and the thickness was 9-10 mm. The compressive modulus was calculated by the average slope of stress-strain curve in the strain range of 5%-15%.

Scanning electron microscopy (SEM) measurement: The hydrogel sample was soaked in water for 4 h to gain a swollen hydrogel sample, and then frozen in a refrigerator at -20 ° C for several hours. The frozen samples were dried in a freeze dryer for 3 days. The freeze-dried samples were sputtered with a layer of gold before the test with a field emission scanning electron microscopy (Hitachi S-4800) at a voltage of 10 kV.

1H NMR measurement: The gelation process was monitored by using a Bruker

600 MHz NMR spectrometer at 37 °C. In our conversion calculation experiments, dimethyl sulfoxide (DMSO) was selected as an internal standard to calculate the conversion of vinyl double bonds (*N*-acryloyl-L-lysine) in the D_2O-substituted precursor. Briefly, 5 mg of EDC·HCl, 140 mg of *N*-acryloyl-L-lysine, 1 mg Glu and 10 mg of Sulfo-NHS were added to 983.35 μL of D_2O and stir well to form a homogeneous solution. Then, 16.65 μL of DMSO and 0.5 mg of GOx were added to the above solution in sequence to gain the final detection solution. The detection solution was transferred to a NMR tube and placed into the probe of the spectrometer as quickly as possible and started the timer simultaneously. The gelation process was recorded at different time.

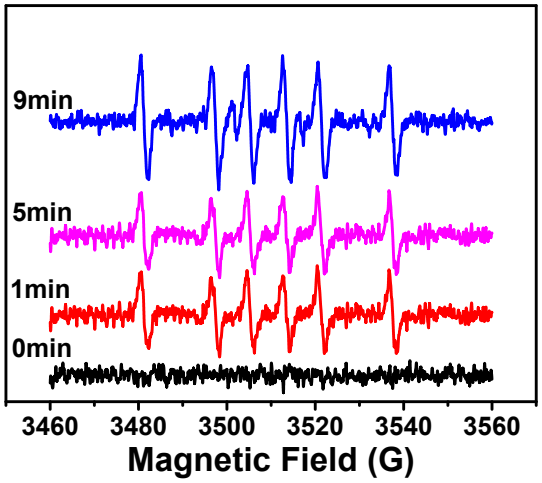
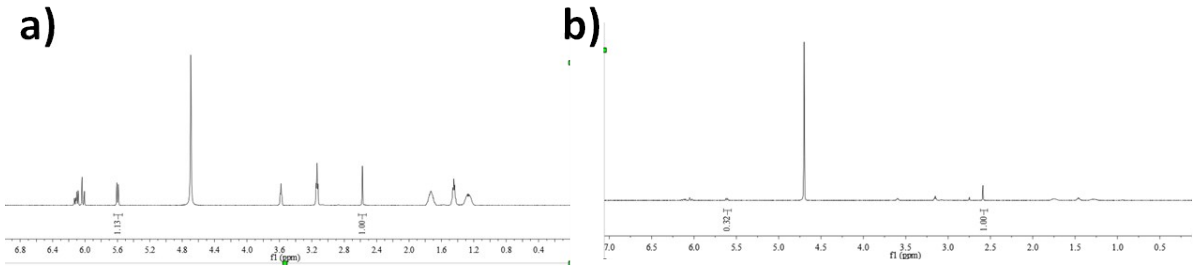
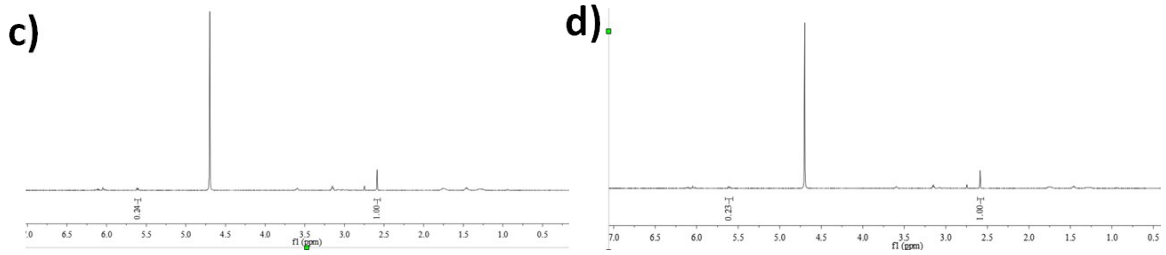


Figure S2. The EPR spectrum of the GOx-mediated redox initiation system with the addition of *N*-acryloyl-L-lysine.





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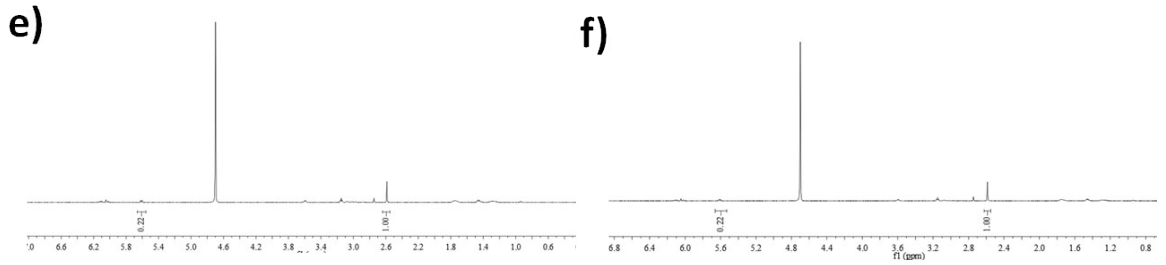

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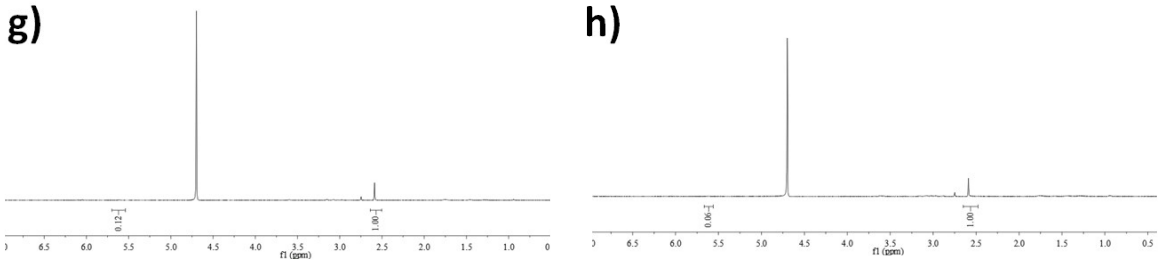

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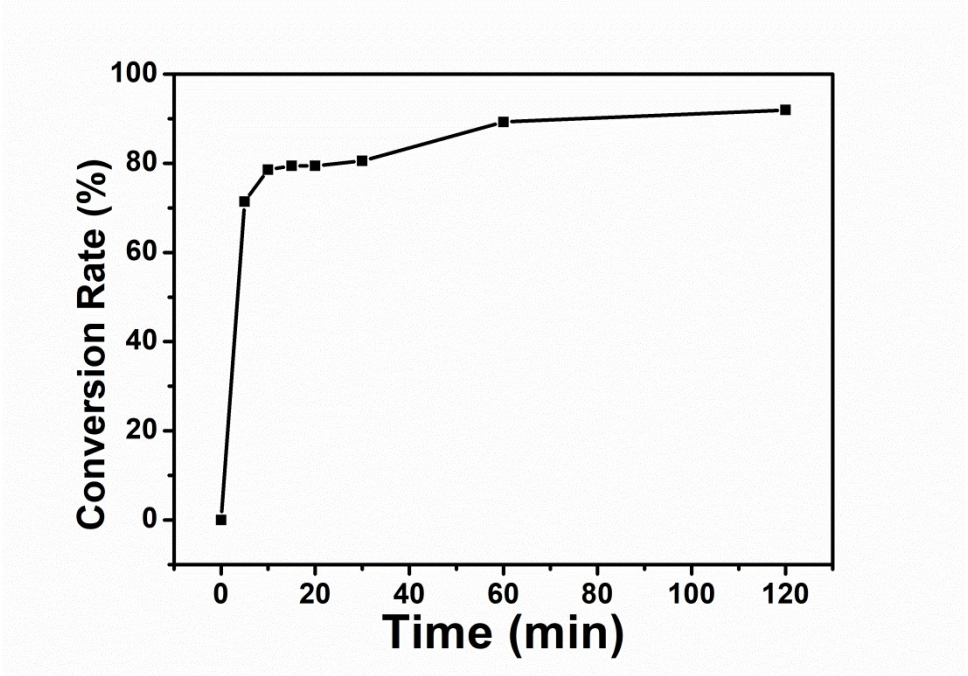

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Figure S3. The 1 H NMR spectra of a D_2O-substituted precursor at different reaction times: 0 min (a); 5 min (b); 10 min (c); 15 min (d); 20 min (e); 30 min (f); 60 min (g); 120min (h); (i). Monomer conversion rate of acryloyl groups.

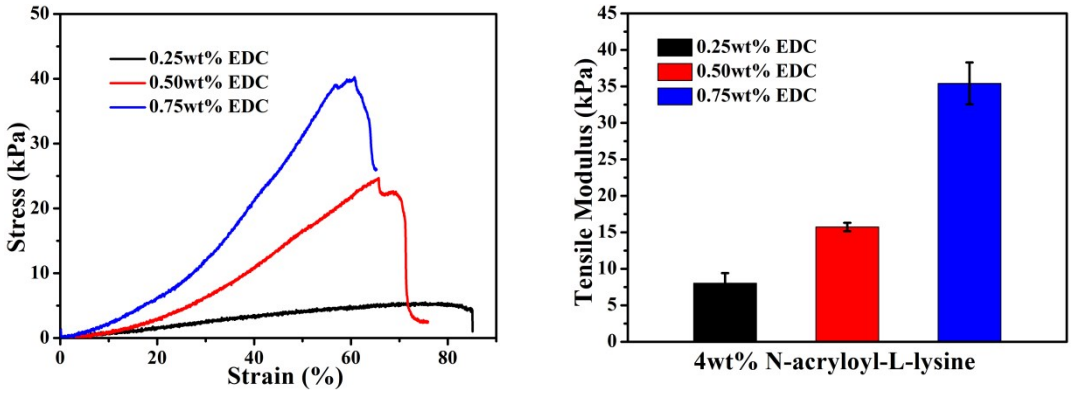

Figure S4. Tensile curves with different EDC·HCl amounts and tensile modulus of hydrogels with different EDC·HCl amounts

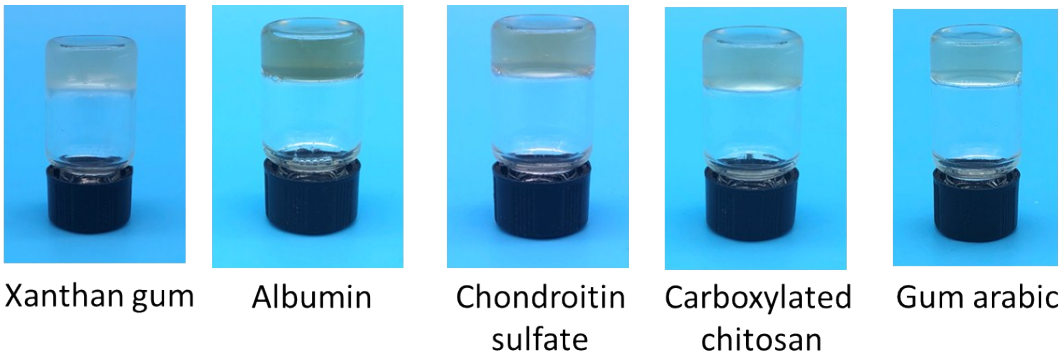

Figure S5. Hydrogels with different natural polymers, Xanthan gum, Albumin, Carboxylated chitosan, Chondroitin sulfate and Gum arabic.

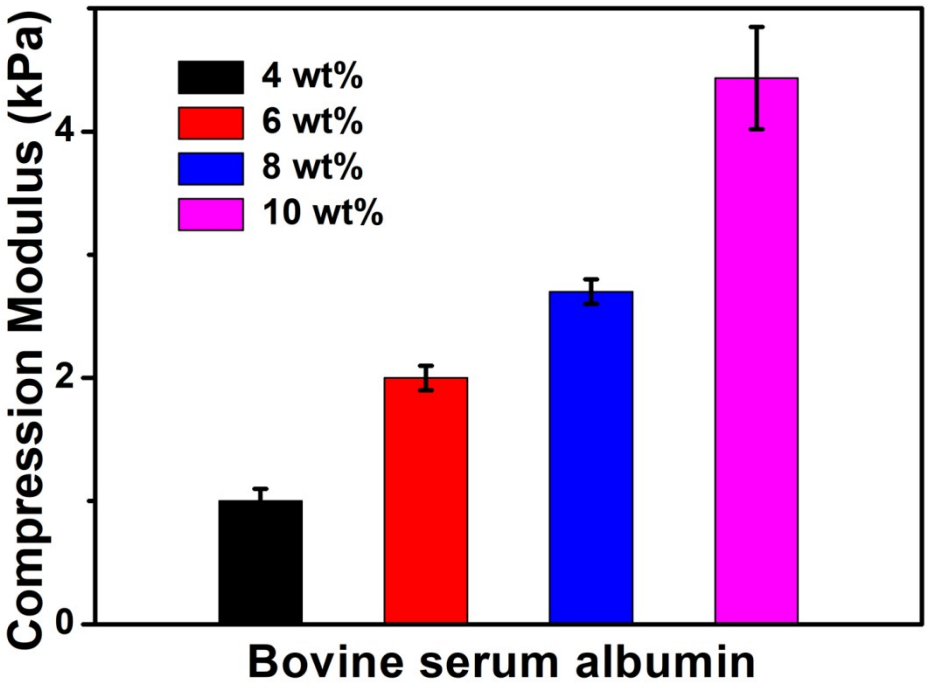

Figure S6. Compressive test of composite hydrogels with different BSA amounts.

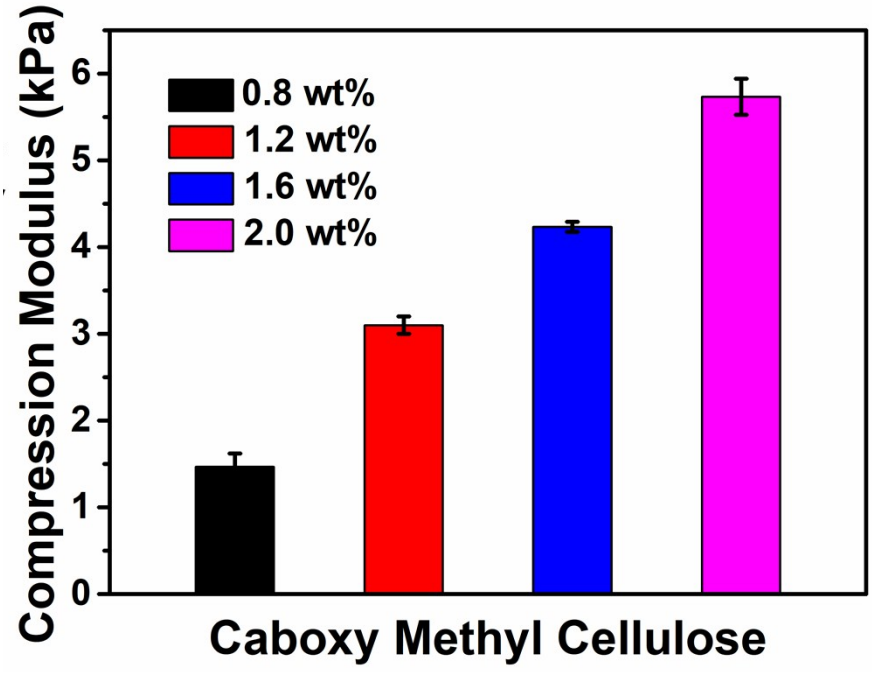

Figure S7. Compressive test of composite hydrogels with different CMC amounts.

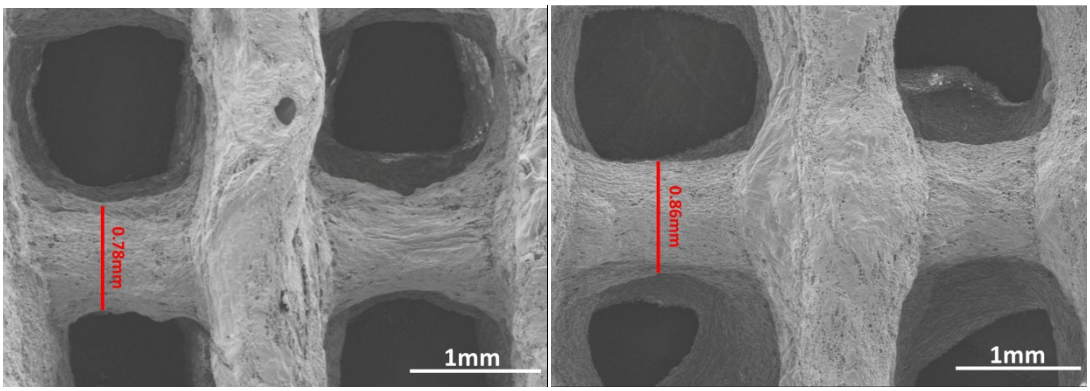

Figure S8. SEM images of printing structure. Using single ink (left), and using ink A and ink B (right).

References.

1. N. Cui, J. Qian, W. Xu, M. Xu, N. Zhao, T. Liu and H. Wang, *Carbohydrate Polymers*, 2016, **136**, 1017-1026.