Electronic Supplementary Information for

D-Serine enzymatic metabolism induced formation of powder-remoldable PAAM-CS hydrogel †

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Contents

1. Experimental section

Materials: Horseradish peroxidase (HRP, EC 1.11.1.7, MW = 40 Kda, 300 U/mg), Chondroitin sulfate sodium salt (CS, MW = 5 × 104 Kda), and D-Serine were purchased from Shanghai Baoman Biotechnology Co., Ltd. D-Amino acid oxidase from porcine kidney (DAAO, 5.4U/mg) and Catalase from bovine liver (2000U/mg) were purchased from Sigma-Aldrich. Tris-HCl (pH 9, 1M) was purchased from Beijing Solarbio Science & Technology Co., Ltd. Acrylamide was purchased from Aladdin Industrial Inc. (Shanghai, China). Concentrated hydrochloric acid (HCl, 37.5 wt%), Ethanol and Rhodamine B were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Glycidyl methacrylate was purchased from Energy Chemical (Shanghai, China). O-phenylenediamine was purchased from Qiangshun Chemical Reagent Co., Ltd (Shanghai, China). All materials were used as received without further purification.

Synthesis of methacrylated chondroitin sulfate (CS): The modification of chondroitin sulfate was adapted from the literature.[1] Briefly, chondroitin sulfate (2.08 g) was dissolved in 50 mL of water for 2 h at room temperature to form a homogeneous solution. Subsequently, the pH of the solution was adjusted to 3.5 by adding 0.1 M HCl. Then, glycidyl methacrylate (675 μL, 5.10 mmol) was added to the solution, and the mixture was stirred at 50 °C for 24 h. After that, 100 mL of ethanol was added and white precipitation was produced, followed by filtering three times with ethanol to gain the white product. The product was subsequently dissolved in water, followed by dialysis (MWCO 10000) against deionized H_2O for 5 days. The dialysate was finally lyophilized to give a white powder product and was stored at room temperature prior to use. The ^1H NMR spectra (Bruker, 400 MHz, D_2O) of the product verified the success of methacrylated chondroitin sulfate.

Preparation of gel: The gel was prepared in the following procedure. 200 μL of D-Ser (1 M), 100 μL of modified chondroitin sulfate (10 wt%), and 250 μL of AAM (40 wt%) were added to 370 μL Tris-HCl (pH 9.0) solution respectively, and were mixed thoroughly on vortex to gain a transparent solution, After the addition of 80 μL of a D-AAO solution (2.5 mg/mL), the vial was placed in water bath at 37 °C for several minutes to give a yellow transparent and self-standing hydrogel. The addition of catalase was the same as above procedure except the addition of catalase solution (20 μL, 5 mg/mL) after adding D-AAO solution.

Visualized gelation: The visualized printing was carried out by using a 3D printer (Nano-Plotter NP 2.1, GeSiM, Grosserkmannsdorf, Germany) of Shanghai Institute of Ceramics (Chinese

Academy of Sciences). The procedure was as follows. Typically, 1000 μL of D-Ser (1 M), 500 μL of modified chondroitin sulfate (10 wt%), and 1250 μL of AAM (40 wt%) were added to 1800 μL OPD (pH 9.0, 100 mM) solution respectively, and were mixed thoroughly on vortex, after adding of 400 μL of D-AAO solution (2.5 mg/mL) and 50 μL of HRP solution (0.05 mg/mL), the vial was placed in water bath at 37 °C for several minutes (10-15 minutes), and the color gradually turned into yellow-green. Transferring the mixture into the printing syringe, and fixed the printing syringe to the printer to start printing. The as-obtained pattern was still placed in water bath at 37 °C for further color change.

Self-healing ability: The two colors hydrogels were cut in half respectively and simply putted two pieces of cracked hydrogel plates which were yellow and red (addition of rhodamine B)together and kept contacting for 24 h at 37 °C.

Reprinting of lypholized gel powder: The hydrogels were firstly lyophilized and the soybean milk machine was used to smash them into powder. Then, a certain proportion of water and powder (Since 1 g of the hydrogel obtained 0.13 g of the powder, so the proportion was 870 μL of water per 0.13 g of powder) were directly putted into a printing syringe in succession and mixed thoroughly, the powder could absorb water immediately and supported itself in a few seconds. The printing syringe was loaded into the printer and the pre-designed patterns were printed. The recoverable property was showed by the tensile length before and after the repair with a ruler.

Cytotoxicity assay: NIH-3T3 cell (obtained from Wuhan Biofavor Biothch Service Co., Ltd) was chosen as model cell to evaluate the cytotoxicity of samples by using CCK-8 assay. The powder was sterilized under UV irradiation for 120 mins before the assay. NIH-3T3 cells were cultured in DMEM. The culture media were supplemented with 10% fetal bovine serum and 1 % penicillin/streptomycin. The NIH-3T3 cells were seeded into 96-well culture plates (Corning, USA) with a density of 5.0×10^3 cells/well and cultured overnight at 37 °C before test. Then the culture medium was removed and fresh medium without antibiotics was added into the plates, after that the samples with various final concentration of 1 μg/mL, 5 μg/mL, 10 μg/mL and 20 μg/mL were put into culture plates and co-cultured for 24 h and 48 h at 37 °C in 5 % CO_2, respectively. A blank control (no sample) was also conducted for each time point. At each time point, the samples and culture mediums were removed and fresh medium with 10 μL CCK-8 solution was added into plates, then incubated for 4 h at 37 °C. The absorbance values of

resulting solutions were recorded at 450 nm with a microplate reader (MULTISKAN MK3, Thermo). The results were indicated as percentage of cell viability relative to the blank control at each time point. The experiment was repeated twice, and each concentration of the sample was performed in three and six times, respectively.

Rapid hemostatic and wound healing: All experiments involving animals were ethically and scientifically approved by the University and complied with Practice for Laboratory Animals in China. The adult Sprague-Dawley (SD) rats (180–200 g, 8 weeks old) were supplied by Experimental Animal Research Center of Hubei. Adult SD rats were randomly divided into three groups to assess the rapid hemostatic and wound healing behavior of powder and regenerated hydrogels. All rats were anesthetized by intraperitoneal injection of 10 % chloral hydrate at a dose of 0.33 mL/100 g body weight before experiment. The hair on the light leg of each rat was shaved and the skin was sterilized with medical alcohol. A full-thickness skin wound of 0.7 cm × 0.7 cm area was created on the light leg of each rat. Then, the artery exposed at each skin wound was poked a little hole by using a syringe needle to make a hemostatic model. The bleeding wounds were scattered and covered by the powder and regenerated hydrogels respectively, the hemostatic time was recorded. The wounds were further covered by surgical tape and fixed with sterile gauze. The control group was just covered by surgical tape and fixed with sterile gauze without adding powder or regenerated hydrogel. The rats were kept in separate cages with free access to food and water in a temperature controlled room at 25 °C. The surgical tape and sterile gauze were replaced every second day and the wound area was measured by a ruler along its side and photographed to record its healing condition. In order to further investigate the degree of wound healing, all the SD rats were sacrificed after 10 days of surgery by excess chloral hydrate. The wound regions were quickly removed, and they were fixed in buffered 10% formalin solution for 24 h, washed and dehydrated by serial dilutions of alcohol, the tissue samples were cleared in xylene and embedded in paraffin, then sectioned in 4 μm (Leica RM 2016, Germany). Sections were stained by Hematoxylin (Sigma, America) & Eosin (H & E) (Solarbio, Beijing, China). The bright-field optical microscope (Olympus BX 53, Tokyo, Japan) was used to measured the stained sections for histological examination. Three separate sections from each wound were examined by light microscopy.

2. Characterization

Rheological analysis: The rheological properties of hydrogels were measured using a Thermo Haake RS6000 rheometer (Thermo Scientific) with parallel plate geometry (diameter: 20 mm, 0.5 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. The rheology of the visualized injecting process was also measured as above. All the tests were repeated at least three times.

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 1 mm/min and compressed to 90 %. The diameter of the samples was 14 mm and the thickness was 6-8 mm. The stress and strain at strain between 0 to 10 % were adopted to calculate the modulus of the hydrogels. The tensile tests of hydrogels were taken at an extension speed of 10 mm/min, the tests were performed at room temperature with the shape of rectangle. All the tests were repeated at least three times.

Scanning electron microscopy (SEM) measurement: The hydrogel samples were soaked in water for 4 h to gain the swelled hydrogel samples before freezing in -20 °C refrigerator for several hours. The freezed samples were further dried in vacuum for ca. 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 3 KV.

Electron Paramagnetic Resonance (EPR) measurement: The EPR results were performed on an EPR Spectrometer (A300, Bruker) at 9.873 GHz. To ascertain the initiating carbon radicals in this system, the mixture of D-AAO, D-Ser and modified chondroitin sulfate 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, 5, 9 and 30 min reaction. To determine the propagating radical, 10 wt% of the AAM was added to the above initiation system in the absence of DMPO. The spectrum was recorded after 1, 5, 9 and 30 min reaction.

Gel Permeation Chromatography (GPC) measurement: The molecular weight of PAAM formed in the presence of D-AAO and D-Ser was got by Gel Permeation Chromatography (GPC, Waters 1525) measurement equipped with 2410 refractive index detector and Empower workstation. Typically, AAM (10 %), D-AAO (0.02 %), D-Ser (2 %) and H_2O (88 %) were

mixed thoroughly and the mixture was placed at room temperature for 24 h to complete the polymerization. The obtained liquid was tested by the GPC measurement. The mixture of D-AAO, D-Ser and H_2O which was the same ratio with the above solution was also tested by GPC in order to deduct the interference of D-AAO.

^{1}H NMR measurement: The gelation process was monitored by using a Bruker 400 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 (AAM) in the D_2O-substituted precursor. Briefly, 21.08 mg of D-Ser, 100 mg of AAM, and 10 mg of modified chondroitin sulfate were added to 983.35 μL of D_2O (pH 9.0, adjusted by adding NaOH) and shocked vigorously to form a homogeneous solution. Then, 16.65 μL of DMSO and 0.2 mg of D-AAO 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.

UV - Vis spectroscopy: The UV-Vis spectroscopy was tested by using a UV-Vis spectrometer (UV-2700, Shimadzu) with interval of 0.1 minute. Firstly, 160 μL of D-AAO solution (2.5 mg/mL) and 3840 μL of Tris-HCl (pH 9.0) solution were added to cuvettes as blank sample to determine the baseline at 450 nm. Then, 500 μL of AAM (40 wt%) solution, 400 μL of D-Ser (1M), 2000 μL of Tris-HCl (pH 9.0) solution and 200 μL of modified chondroitin sulfate (10 wt%) were added to cuvette and mixed evenly, subsequently, 160 μL of a D-AAO solution (2.5 mg mL-1), 20 μL of a HRP solution (0.05 mg/mL), and 720 μL of a OPD solution (pH 9.0, 100 mM) were also added to the cuvette to start the color reaction. The increase in absorbance at 450 nm could be measured with an interval of 0.1 min.

3. Figures and table

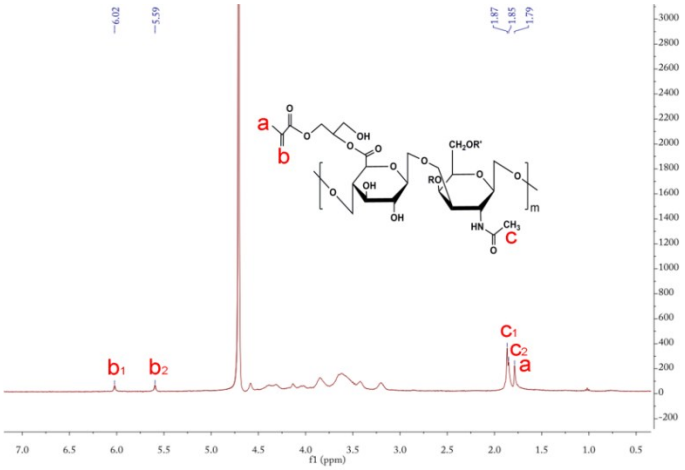


Figure S1. The ^{1}H NMR (400 MHz, D_2O, 20 °C) spectra of methacrylated chondroitin sulfate. The degree of substitution was ca. 0.35. The chemical shifts at 6.02(b_1) and 5.59 (b_2) belong to the hydrogens on the double bond(b), the chemical shift at 1.87(c_1) belongs to the methylic hydrogens in the unmodified CS(c), the chemical shift at 1.85(c_2) belongs to the methylic hydrogens in the modified CS(c), the chemical shift at1.79(a) belongs to the methylic hydrogens on the methyl(a).

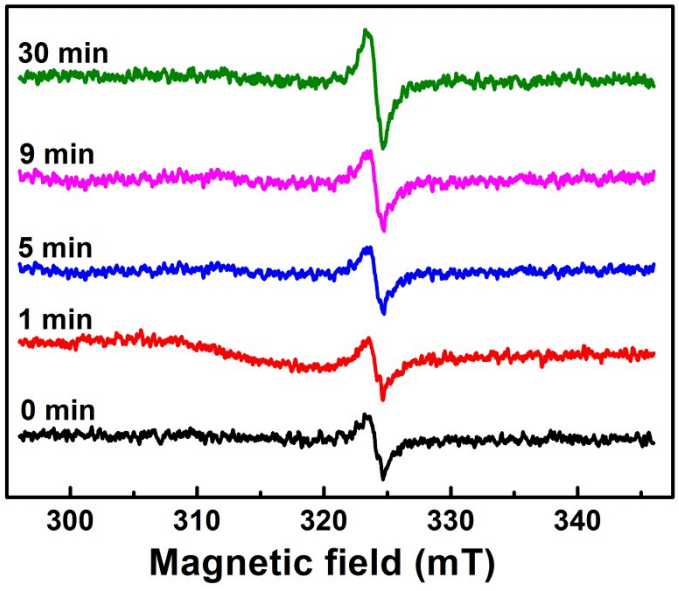


Figure S2. The EPR spectrum of the D-AAO-mediated redox initiation system with the addition of AAM, but without the addition of radical adduct (The signals of the propagating carbon radical).

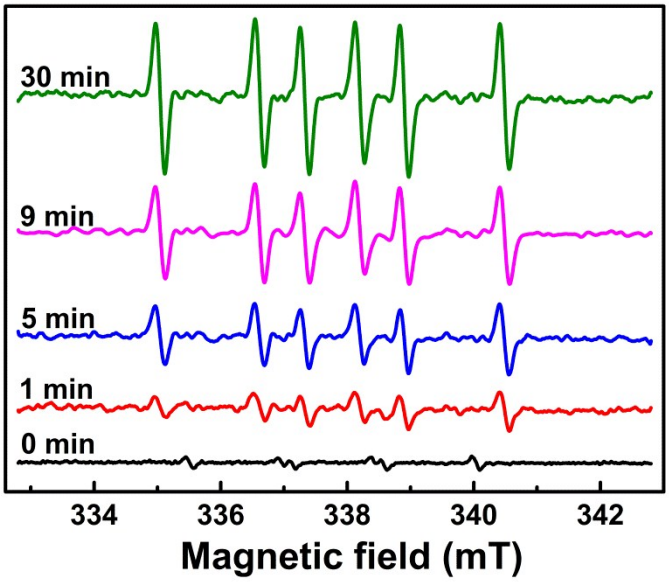


Figure S3. EPR spectrum of the DMPO radical adduct formed in the H_2O_2 and pyruvic acid system without the addition of AAM.

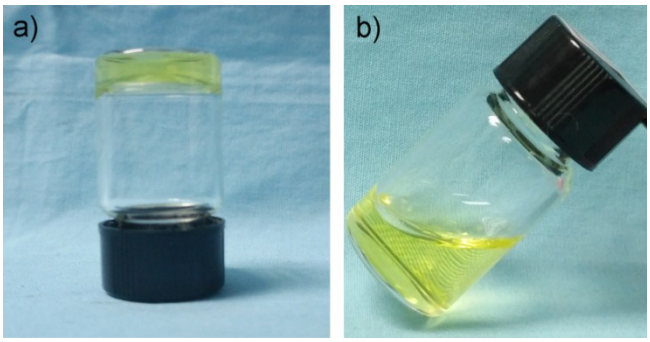


Figure S4. (a) Hydrogel system without catalase. (b) Hydrogel system with catalase.

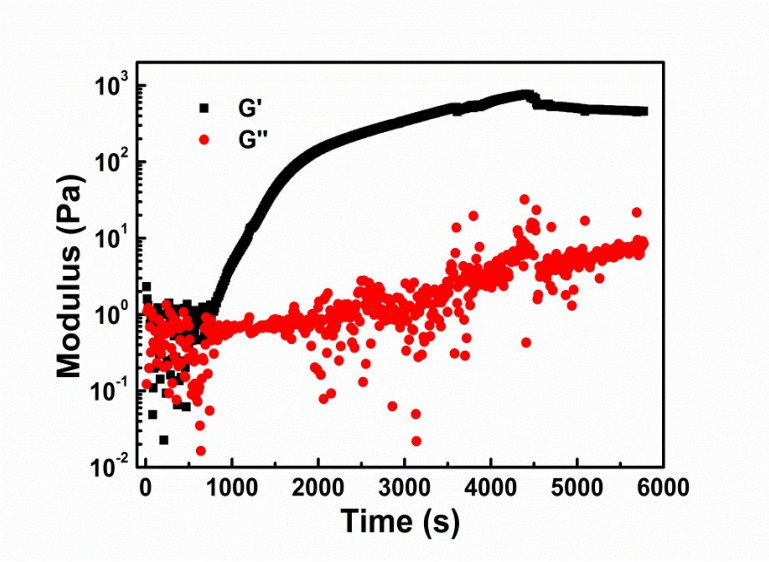
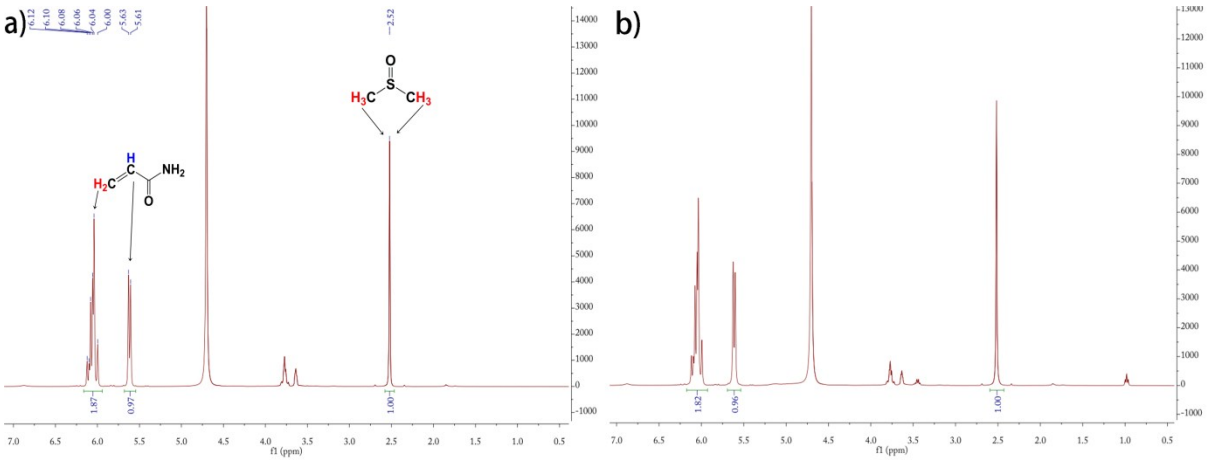
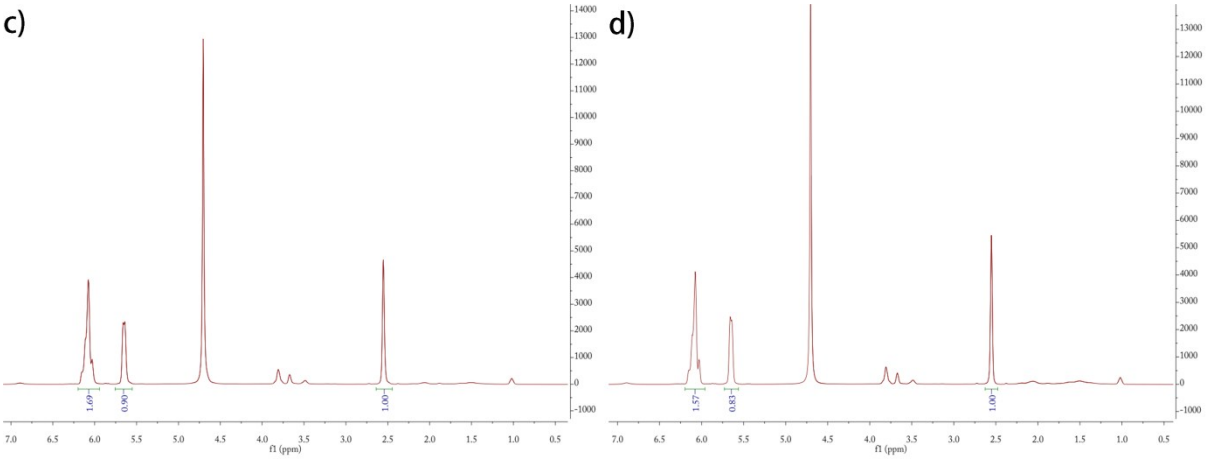
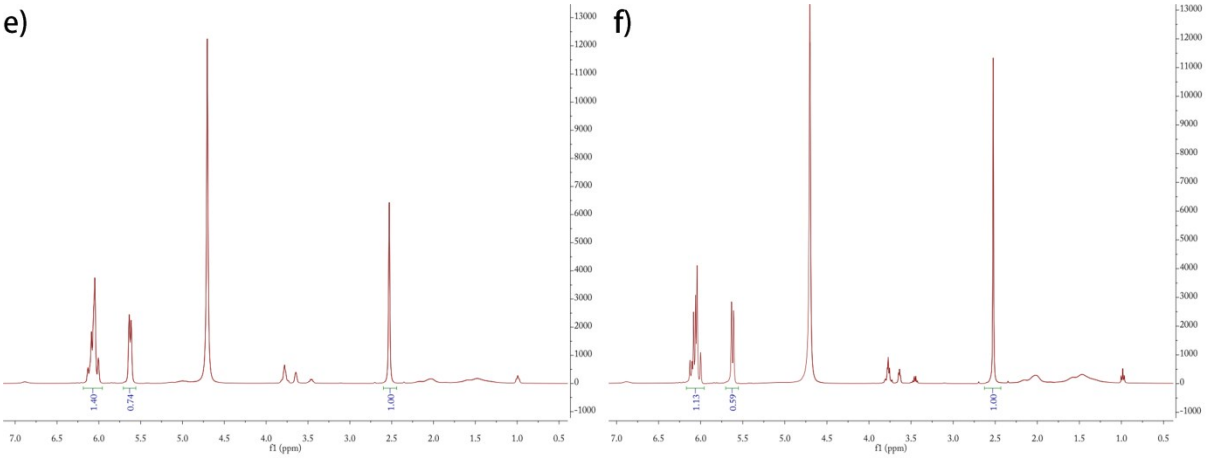


Figure S5. Dynamic time sweep test of the visualized gelation system.

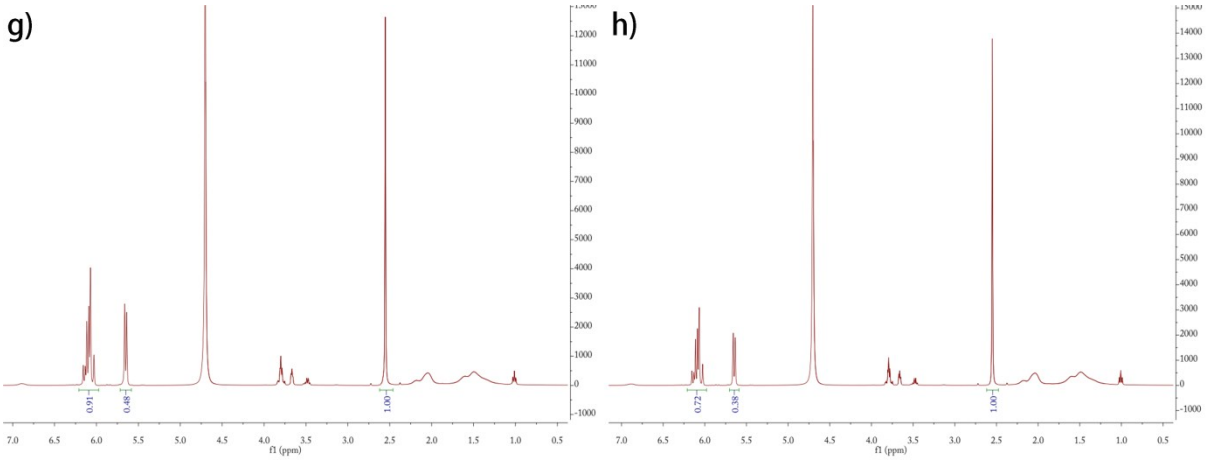



c)
d)
1.69
0.90
1.00
1.57
0.83
1.00
f1 (ppm)

1


e)
f)
1.40
0.74
1.00
1.13
0.59
1.00
f1 (ppm)

2


g)
h)
0.91
0.48
1.00
0.72
0.38
1.00
f1 (ppm)

3

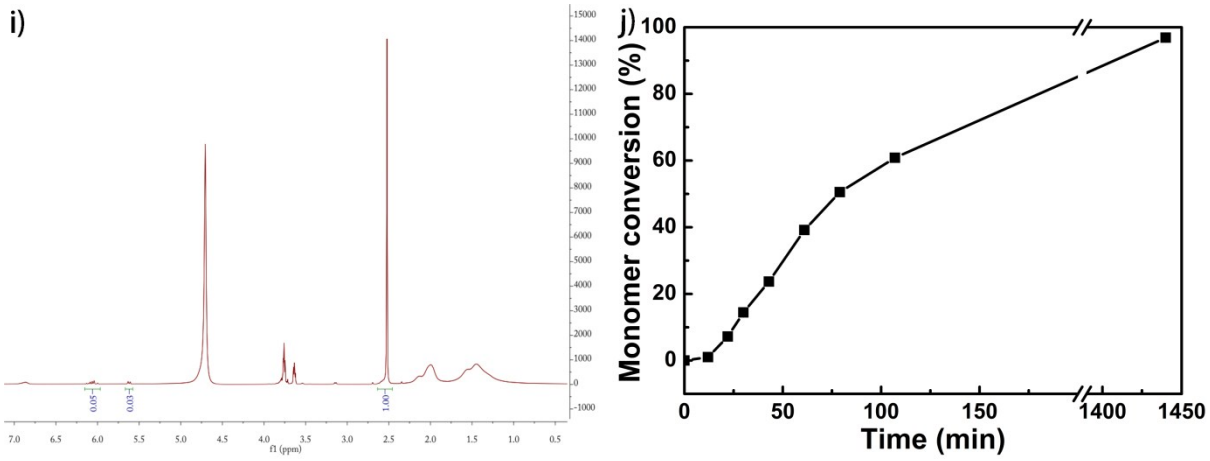


Figure S6. The ^{1}H NMR spectra of a D_2O-substituted precursor at different reaction times: 0 min (a); 12 min (b); 22 min (c); 30 min (d); 43 min (e); 61 min (f); 79 min (g); 107 min (h); 24 h (i). The conversion of vinyl double bonds (AAM) in the hydrogel is calculated using dimethyl sulfoxide as an internal standard (j). (The integral area of DMSO (δ=2.52) was set as 1.0, and thus gained the corresponding integral area of vinyl double bonds (δ=5.63, 5.61) in AAM at each time point. The conversion rate were indicated as percentage of the difference between the integral value in each time point and initial integral value relative to the initial integral value.)

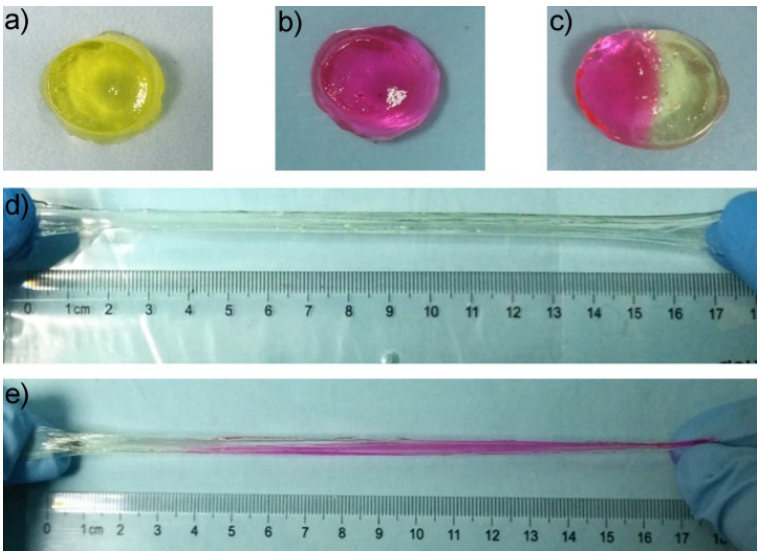


Figure S7. The self-healing property of the hydrogel. (a) The hydrogel. (b) The hydrogel with rhodamine B. (c) The hydrogel after self-healing. (d) The tensile of hydrogel. (e) The tensile of self-healing hydrogel.

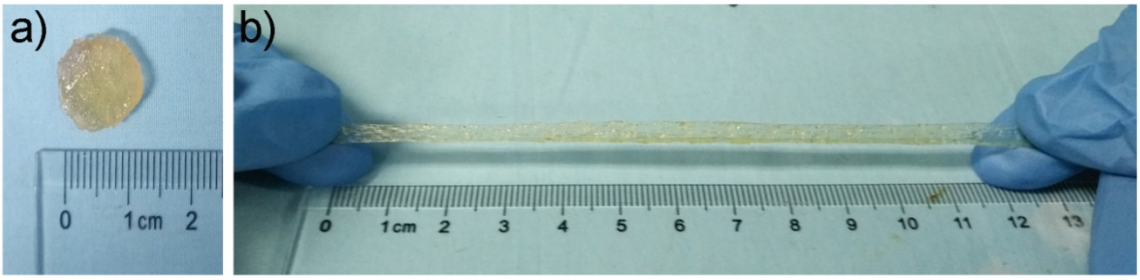


Figure S8. The tensile property of the regenerated hydrogels.

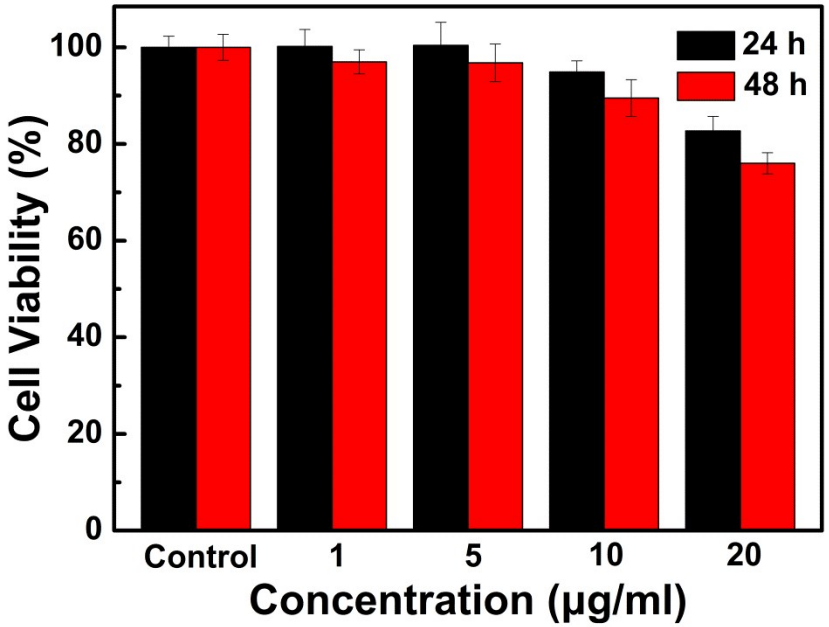


Figure S9. Cytotoxicity of the powder against NIH-3T3 cells (The CCK-8 assay indicated that 82% to 100% and 75% to 97% cells remain viable at various powder concentrations after 24 h and 48 h when co-cultured with NIH-3T3 cells).

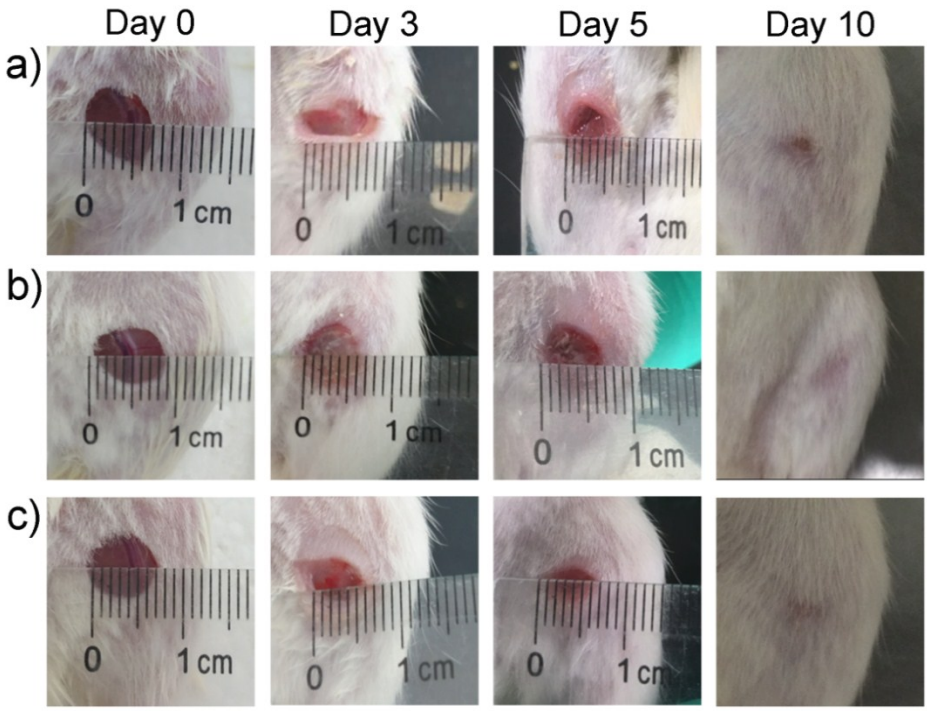


Figure S10. Photograph of wound treated with (a) control, (b) powder and (c) remolded hydrogels within 10 days.

Table S1. The M_n, Mw, PDI and Y_p of the PAAM.

t_R (min)	M_n (g mol^{-1})	M_w (g mol^{-1})	PDI	Y_p (%)
13.7	319187	1665655	5.2	100

4. References

1. A. R. Fajardo, S. L. Fávaro, A. F. Rubira, E. C. Muniz, *React. Funct. Polym.* **2013**, *73*, 1662-1671.