

Electronic Supplementary Materials for

Viscosity-controlled Printing of Supramolecular-Polymeric Hydrogel via Dual-Enzyme Catalysis

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1. Materials

Horseradish peroxidase (HRP, EC 1.11.1.7, MW = 40Kda, 300 U/mg) was purchased from Shanghai Baoman Biotechnology Co., Ltd. Glucose oxidase from *Aspergillus niger* (GOx) and poly (ethylene glycol) methacrylate (PEGMA, average MW = 360 g/mol) were purchased from Sigma-Aldrich. All amino acids used in SPPS were purchased from GL Biochem (Shanghai) Ltd. Glucose, acetylacetone (AcAc), piperidine and other reagents were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All materials were used as received.

2. Preparation of SGel and HGel

2-(Naphthalen-2-yl)acetyl-(L)-Phe-(L)-Phe-(L)-Arg-(L)-Lys-acryloyl (NapFFRK-acryloyl) was synthesized via standard solid phase peptide synthesis (SPPS) on a Rink-amide resin by successive coupling of Fmoc-protected-L-amino acids^[S1, S2]. After completion of the synthesis, peptides were cleaved from the resin with concomitant deprotection of the side chain protecting groups via treatment of TFA/H₂O/triisopropylsilane (TIS) (95:2.5:2.5, v/v/v) for 2 hours. Peptides were then precipitated in cold diethyl ether, and the crude peptides were further purified through semi-preparative reverse-HPLC on a PRC-ODS 20 mm × 25 cm column (Shimadzu, Japan) under a linear gradient from 90:10 to 30:70 of water/acetonitrile (addition of 0.1 % TFA) at 3 mL/min. ESI-TOF m/z calcd for C₄₅H₅₅N₈O₇ 819.4193, found 819.4113.

Preparation of SGel: NapFFRK-acryloyl (5.0 mg) and 2 N NaOH solution were

added to 400 μL distilled water, and the mixture was sonicated for thorough dissolution. Then diluted HCl solution was added carefully until the pH reached 7, the volume was adjusted to 500 μL by adding water. After the mixture was mixed thoroughly on vortex, the colorless transparent solution was kept at 25 $^{\circ}\text{C}$ for 30 min and SGel (1.0 wt% of NapFFRK-acryloyl) was formed

Fabrication of HGel: NapFFRK-acryloyl (5.0 mg) and 2 N NaOH solution were added to 300 μL distilled water, and the mixture was sonicated for thorough dissolution. Then diluted HCl solution was added carefully until the pH reached 10, subsequently, the dual enzyme-catalyzed reagents including 25 μL glucose_(aq) (80 mM), 10 μL HRP_(aq) (42 mg/mL), 10 μL AcAc and 25 μL PEGMA were added and the volume was adjusted to 400 μL by adding water. Then 100 μL GOx_(aq) (10 mg/mL) was added and the mixture was mixed thoroughly on vortex. The final yellow transparent precursor solution was kept at 37 $^{\circ}\text{C}$ for 120 min and HGel (1.0 wt% of NapFFRK-acryloyl) was formed.

3. Pre-treatments and characterizations

Pre-treatment of SEM samples: The silicon wafer coated with samples was freeze-dried in liquid nitrogen, and further dried 24 h in vacuum. The samples were sputter-coated with a thin layer of gold before testing with a field emission scanning electron microscopy (Hitachi S-4800) at a 3 KV voltage.

Pre-treatment of TEM samples: The samples were coated on the carbon-coated copper grids and stained with sodium phosphotungstate (2 wt% in water). The pictures were acquired with a transmission electron microscopy (JEM-2010) at a 80 KV voltage.

Rheological characterization: The rheological properties of hydrogels were tested using a RS6000 rheometer (Thermo Scientific, Karlsruhe, Germany) with parallel plate geometry (25 mm diameter, 0.3 mm gap) at 25 $^{\circ}\text{C}$. The frequency-dependent sweep was taken as a function of angular frequency at fixed strain of 0.03 %. The strain amplitude sweep of gels was carried out at a fixed frequency of 1 Hz. The self-recovering process of HGel in response to applied shear forces was performed using

continuous step strain sweep test with alternate small oscillation force ($\gamma = 0.03\%$) and large one ($\gamma = 50\%$).

Mechanical analysis: The compressive test of hydrogels was taken on a FR-108B testing machine (Farui Co., China) at a crosshead speed of $1\text{ mm}\cdot\text{min}^{-1}$. The diameter of the gels is about 13 mm and the thickness is 3-4 mm. The compressive stress (σ) was approximately calculated as $\sigma = F_{\text{load}}/\pi R^2$, where R is the original radius of the sample. The compressive strain (ϵ) is defined as the change of the thickness relative to the original thickness. The stress and strain between $\epsilon = 5$ and 15 % were used to calculate the Young's modulus with at least 3 parallel tests for each hydrogel. And the tensile property was taken on the same machine by using cylindrical HGels (length 50 mm, diameter 0.47 mm).

MTT cytotoxicity assay: NIH-3T3 cells were seeded homogeneously in 96-well tissue culture plates at a density of 50,000 per well and cultured for 24 h. After changing medium, cubic HGel ($1.5\text{ mm} \times 1.5\text{ mm} \times 1.5\text{ mm}$) was added to the well and the cells were cultured for another 24 h. Then MTT stock solution was added and incubated in a humidified cell culture incubator at 37°C for 4 h. Formazan salt solution in isopropanol was added, and the plate was covered with aluminum foil and placed on an orbital shaker (150 rpm) at room temperature for 1 h. 200 μL of the formazan salt solution was transferred to a new 96-well plate. Absorbance was recorded at a 580 wavelength on a plate reader. Results were calculated by simply dividing the value of each sample with the value of the control (no cell addition).

3D printing: The pre-gel solution of HGel was kept at 37°C for 30 min then loaded into the 3D printer (Ingenovo, Shanghai in-G information Science & Technology Co., Ltd. China). Different arrays and patterns were designed and printed.

4. Test of the gelator conversion ratio

In a typical control test, 5 mg NapFFRK-acryloyl was dissolved in d6-DMSO with acetonitrile as internal standard substance. Then HGel (containing 5 mg NapFFRK-acryloyl) was crushed and dispersed in NaOH(aq) ($\text{pH} = 10$). The resulting mixture was stirred violently for 2 hours and filtered. The filtrate was centrifuged at 10000 rpm for 20 min and the supernatant was acidized by 1M HCl solution to pH 2-3. The

white precipitate was obtained by centrifuging and dried, then it was dissolved in d6-DMSO with the same amount of acetonitrile as internal standard substance compared to the control. Three parallel experiments were taken and the results were presented as mean values $\pm$ Standard Deviation. The conversion ratio of gelators was $80.2 \% \pm 2.4 \%$.

5. 3D cell culture

NIH-3T3 cells (1×10^7 cells mL^{-1} , 20 μL) were added to the pre-gel solution (1.0 wt% of NapFFRK-acryloyl, 500 μL) in a vial and mixed thoroughly. The resulting mixture was incubated at 37 $^{\circ}\text{C}$ for 30 min, and then transferred to serum-free Dulbecco's Modified Eagle's Medium (DMEM) containing 10 % (v/v) of foetal bovine serum and 1 % (v/v) penicillin-streptomycin in a glass coverslip. After culturing for 24 h, the cells were stained with 40 $\mu\text{g}/\text{mL}$ fluorescein diacetate (FDA) and 10 $\mu\text{g}/\text{mL}$ propidium iodide (PI) in DMEM media and incubated at 37 $^{\circ}\text{C}$ for 30 min. Images were acquired using a Olympus 2000 confocal laser scanning microscope. A live/dead staining assay was used to calculate the viability of the cells. And the viability was calculated via the Imaris Spot Detection function in order to determine the relative proportion of live (green, FDA stained) or dead (red, PI stained) cells in different areas of view. The data are presented as mean values $\pm$ Standard Deviation.

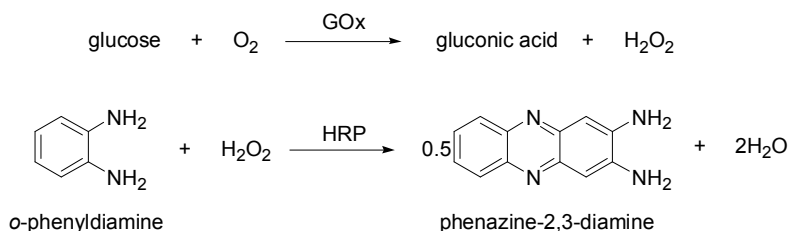
6. 3D cell printing

3T3 cells (1×10^7 cells mL^{-1} , 20 μL) were added to the pre-gel solution (1.0 wt% of NapFFRK-acryloyl, 500 μL) and mixed thoroughly. After incubated at 37 $^{\circ}\text{C}$ for 30 min, the mixture was loaded into the printer. Grids were printed with 3 layers. And further, the grids were stained with 40 $\mu\text{g}/\text{mL}$ FDA and 10 $\mu\text{g}/\text{mL}$ PI in DMEM media and incubated at 37 $^{\circ}\text{C}$ for 30 min, and imaged using a Olympus 2000 confocal laser scanning microscope.

7. Test of the catalytic activity

The oxidation of *o*-phenylenediamine (OPD) by H_2O_2 was selected as a model reaction with glucose and OPD as substrates to test the catalytic activity of free dual

enzymes and immobilized dual enzymes. Typically, 10 μg GOx and 4.2 μg HRP (or a piece of HGel or given volume of P-Solution containing 10 μg GOx and 4.2 μg HRP) were added into a solution containing OPD (fixed 10 mM) and glucose (a series of concentrations 10 mM, 7.5 mM, 5 mM, 3 mM, 2 mM and 1 mM) in phosphate buffer (pH = 7.0) for catalyzing the reaction.



The increase in absorbance at 450 nm for the oxidation product (phenazine-2,3-diamine) was measured by a UV-Vis spectrometer (UV-2700, Shimadzu) at 0.1-min intervals. The concentrations of the colorful product were corrected according to the molar extinction coefficient ($\epsilon_{450} = 16300 \text{ M}^{-1}\cdot\text{cm}^{-1}$) in aqueous buffer. Then the initial reaction rate was obtained by linear fitting the product concentration with time. We obtained the Lineweaver-Burk plot according to a series of initial reaction rates to estimate the kinetic constant values. The typical characterization of enzymatic reaction is the Michaelis-Menten equation $V_0 = V_{\max}[\text{S}]/([\text{S}] + K_m)$, including the maximum reaction rate $V_{\max}$, Michaelis-Menten constant K_m , and turnover number $k_{\text{cat}} = V_{\max}/[\text{Enzyme}]$, can represent the activity of the enzyme.

The catalytic activity of enzymes (GOx/HRP) immobilized in gel matrix was investigated and another system defined as P-Solution (polymer solution) was prepared as the control using the similar protocol without hydrogelators. As shown in Figure S5a, the initial reaction rate of the immobilized GOx/HRP in HGel at 5 mM OPD and 5 mM glucose is greater than other systems. The kinetic parameters (Table S1) of GOx/HRP are calculated from the Lineweaver-Burk plots (Figure S5b) of the initial rates at different concentrations indicating that the immobilized GOx/HRP catalytic system has the highest k_{cat} value (turnover number, 7.743 s^{-1}). On account of the closely immobilized dual enzyme, the K_m value for GOx/HRP in HGel is lower than free GOx/HRP.

8. Test of the reusability

The fresh and recovered Gel II were used to test the reusability via the oxidation of OPD (10 mM) at a fixed glucose concentration (3 mM) in 2 mL aqueous buffer. The total amount of phenazine-2,3-diamine was measured by UV-Vis spectrometer after 15 min reaction. The recovered Gel II was washed with ethanol and buffer, respectively in order to remove the product. Then the recycled Gel II was mixed with a fresh reaction mixture again to catalyze the reaction.

9. *In vivo* hemostatic test

The hemostatic capability of HGels was evaluated via a mouse (ICR, ten weeks, male) hemorrhaging liver model^[S3]. All experiments involving animals were ethically and scientifically approved by the University and Tongji Hospital, and complied with Practice for Laboratory Animals in China. Rats were anesthetized using zoletil/rompun mixture and then abdominal incision was taken. The bleeding was induced using a 27 G needle. The hemostatic time was measured as a negative control without any treatment. Another control group was used by replacing NapFFRK-acryloyl with NapFFK-acryloyl. Simply, after the precursor solution was incubated at 37 °C for 3600 s, hybrid hydrogel (500 µL) was applied on the bleeding site. The hemostatic time was recorded and all experiments were performed for three times.

10. Test of the hydrolysis of NapFFRK-acryloyl

2 mg NapFFRK-acryloyl was incubated with 20 U proteinase K in 10 mL HEPES (0.167 M) buffer at 37 °C and 20 µL of the solution was tested each time to detect the amount of NapFFRK-acryloyl remained by HPLC.

11. Figures and Tables

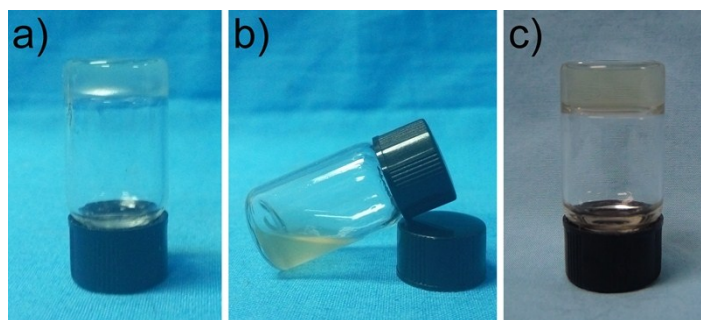


Figure S1 Optical images of SGel (a), pre-gel solution (b) and HGel (c).

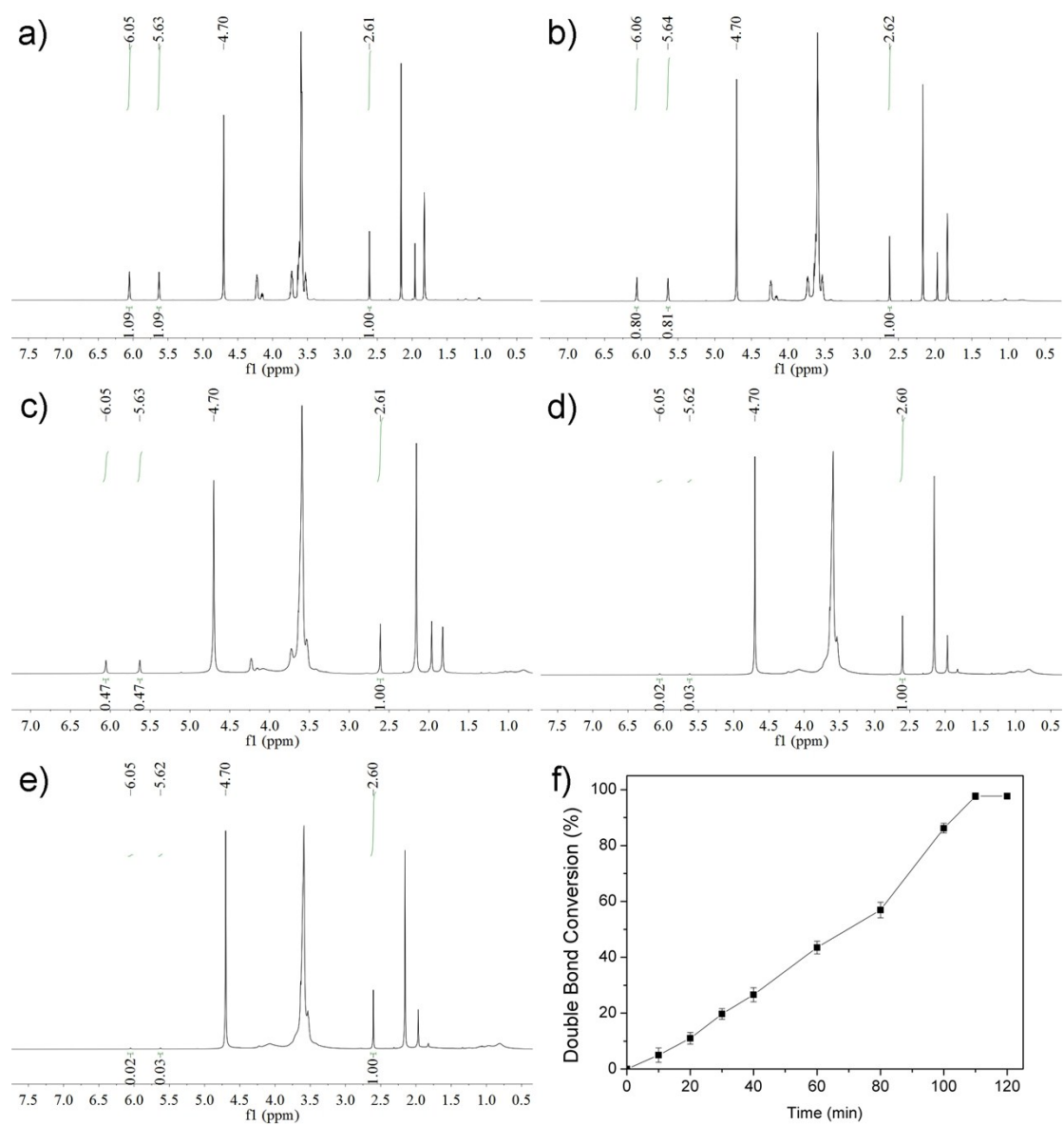


Figure S2 The ^1H NMR spectra of a D_2O -substituted pre-gel solution at different

reaction times: 0 min (a), 40 min (b), 80 min (c); 110 min (d); 120 min (e). The conversion of vinyl double bonds (PEGMA) in the Gel II is calculated using dimethyl sulfoxide as internal standard (f).

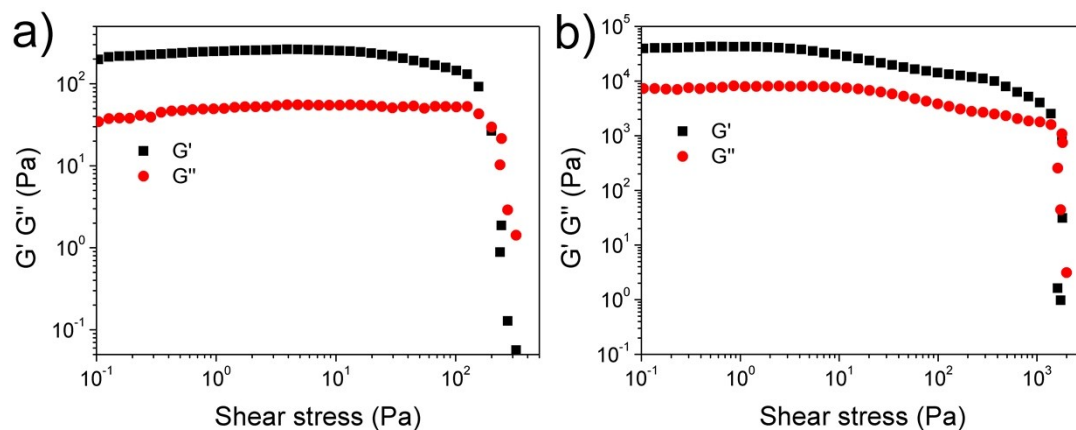


Figure S3 The oscillatory stress sweep tests of SGel (a) and HGel (b).

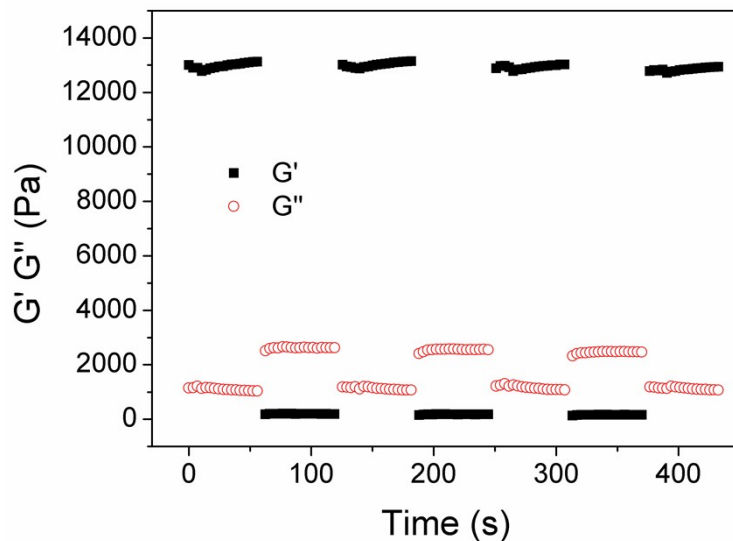


Figure S4 The self-recovering nature of HGel after large amplitude oscillatory breakdown.

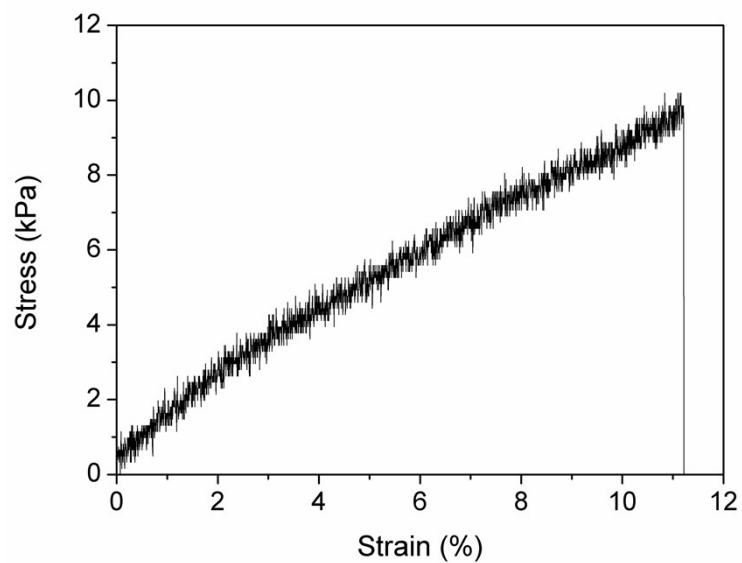


Figure S5 Representative tensile stress–strain plot for HGel (cylinder, length 50 mm, diameter 0.47 mm).

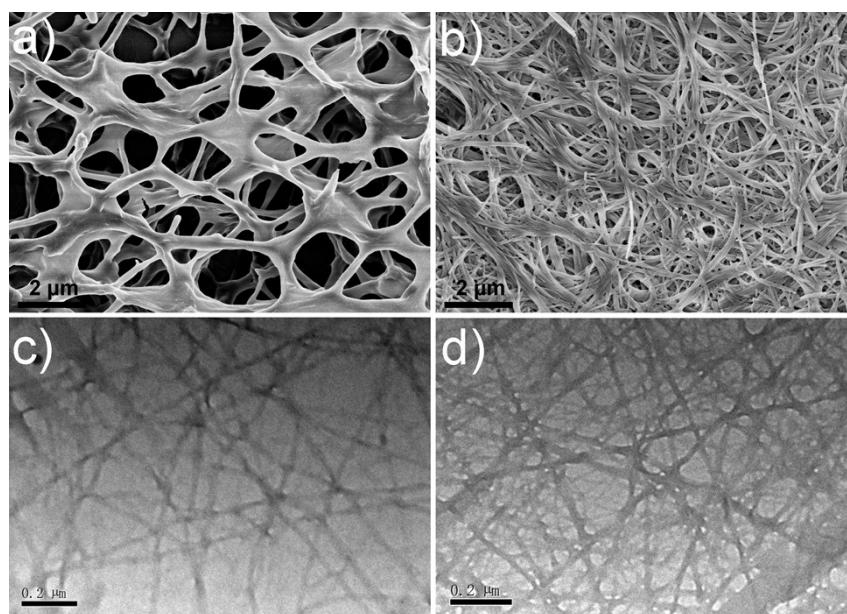


Figure S6 SEM images (scale bar 2 μm) of cryo-dried SGel (a) and HGel (b). TEM images (scale bar 0.2 μm) of negatively stained fibrils of SGel (c) and HGel (d).

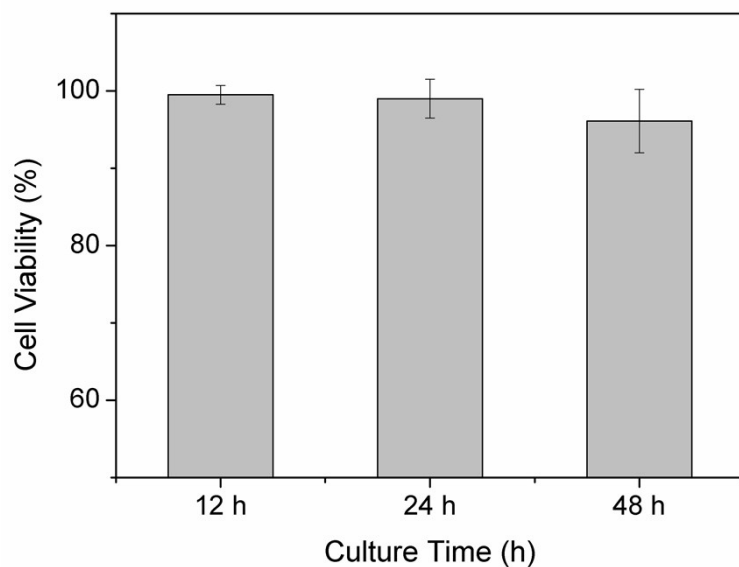


Figure S7 The cell viability versus different culture times.

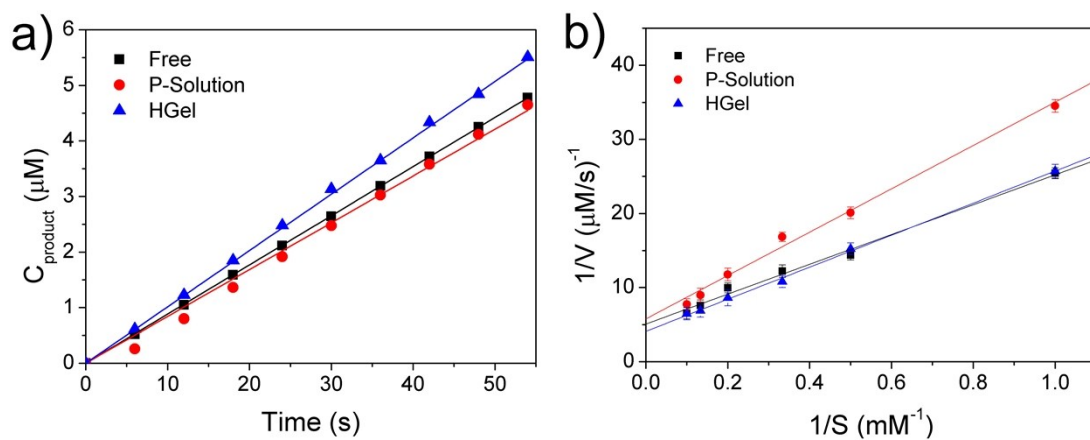


Figure S8 (a) The initial reaction course of o-phenylenediamine (OPD) and glucose in phosphate buffer (pH = 7.0) catalyzed by various enzymatic systems. (b) Lineweaver-Burk plots of four systems in phosphate buffer (pH = 7.0).

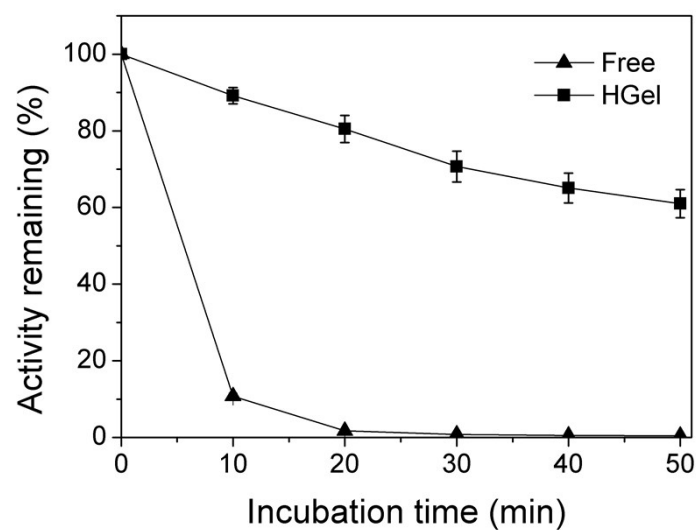


Figure S9 The remaining activity of free enzymes and immobilized enzymes (in HGel) in phosphate buffer (pH = 7.0) after incubation at 60 °C for different periods of time.

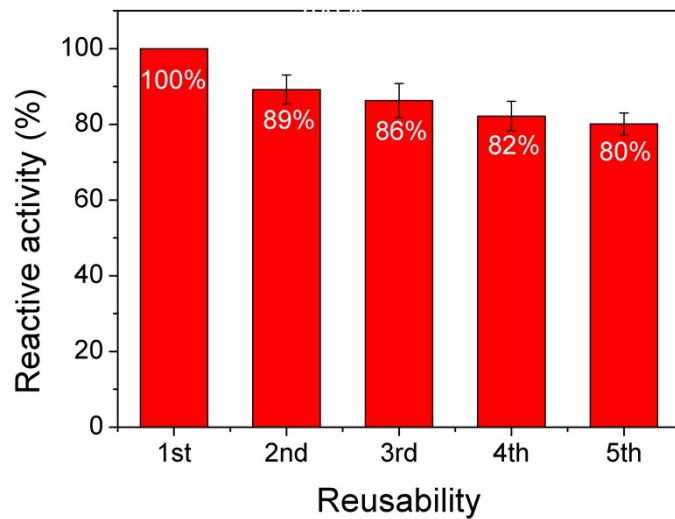


Figure S10 The reusability of HGel in phosphate buffer (pH = 7.0).

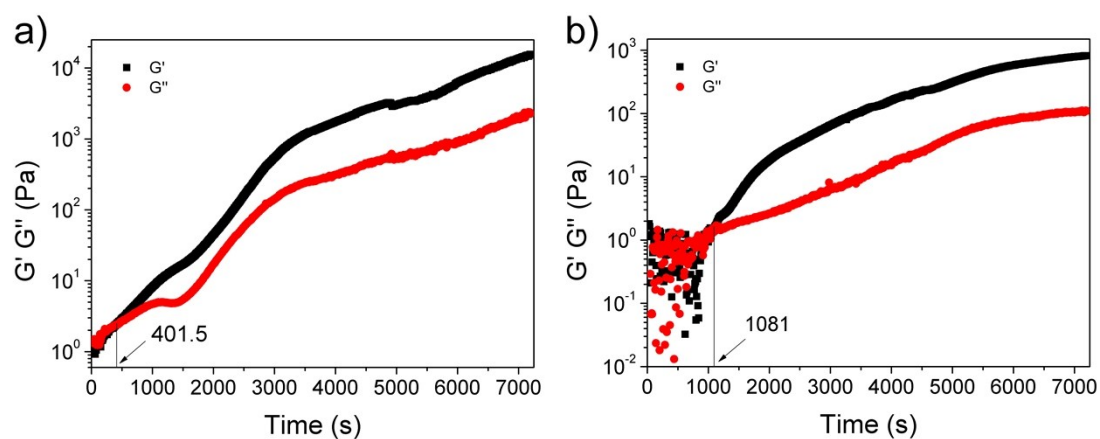


Figure S11 Dynamic time sweep measurements (at a constant strain of 0.03 % and a constant frequency of 1 Hz) of blood hybrid hydrogels containing NapFFRK-acryloyl (a) and NapFFK-acryloyl (b) by mixing isopyknic pre-gel solution and blood.

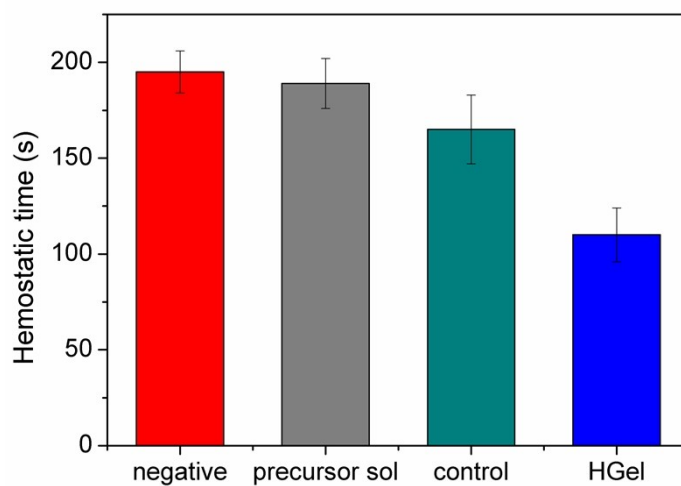


Figure S12 The hemostatic times for different mouse liver bleeding groups.

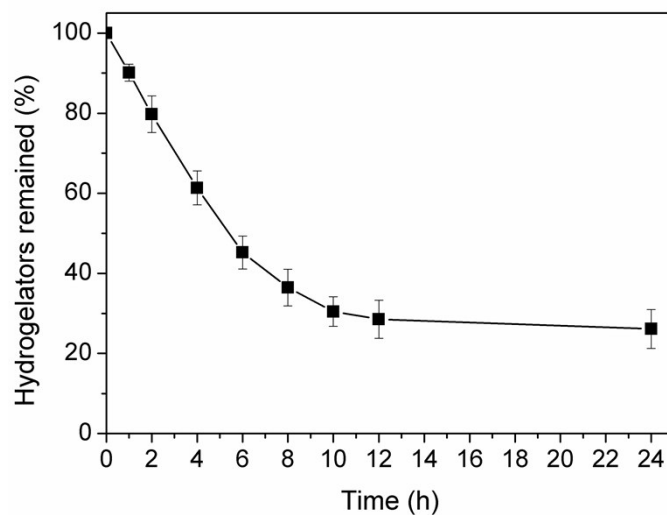


Figure S13 The digestion profile of NapFFRK-acryloyl via proteinase K catalyzed hydrolysis.

Table S1. Reaction kinetic constants of different GOx/HRP systems.

	$V_{\max}$ ($\mu\text{M/s}$)	k_{cat} (s^{-1})	K_m (mM)
Free	0.198	6.340	4.010
P-Solution	0.178	5.71	5.223
HGel	0.242	7.743	5.224

12. References

- S1. H. Rink. *Tetrahedron Lett.* **1987**, 28, 3787.
- S2. K. Feichtinger, H. L. Sings, T. J. Baker, K. Mattheews, M. Goodman. *J. Org. Chem.* **1998**, 63, 8432.
- S3. Y. Murakami, M. Yokoyama, H. Nishida, Y. Tomizawa, H. Kurosawa, *J. Biomed. Mater. Res. B* **2009**, 91, 102.