Reinforced Hydrogel Network Building by the Rapid Dual-Photo-Coupling Reaction for 3D Printing

Renjie Zhou,†[a,b] Yujie Hua,†[c] Lipeng Yang, [d] Bingkun Bao,*[d] Qiuning Lin*[d] and Linyong Zhu [a,b,d]

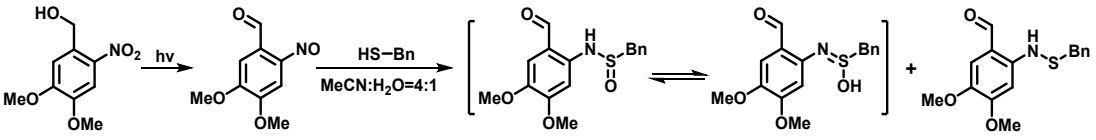
Experimental Section

Materials. Hyaluronic acid (HA; Mw: 340 or 48 kDa), Carboxymethyl chitosan (CMCh; 60% carboxylation degree), Gelatin (GL; from porcine skin) were purchased from Sigma-Aldrich. Methacrylate-modified gelatin (GelMA, 82% substituted degree of methacrylate) and methacrylate-modified alginate (AlgMA, 40% substituted degree of methacrylate) were purchased from EFL (Engineering For Life, China). Thiolated polyethylene glycol (TP) was purchased from JenKem company. 4, 5-dimethoxy-2-nitrobenzyl (d-NB), benzyl mercaptan, 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methyl morpholinium chloride, 4-morpholineethanesulfonic acid, dithiobis(propanoic dihydrazide) (DTP), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl), N-hydroxysuccinimide (NHS), dithiothreitol (DTT), carbohydrazide were purchased from TCI or Aladdin. Dialysis bags (MWCO 3500 Da) were purchased from Aladdin. The syringe filter unit (PES, 0.22 μm pore size) was purchased from Millex. Fetal bovine serum (FBS) was acquired from Sigma-Aldrich and penicillin-streptomycin (Pen-Strep) solution was from HyClone. DMEM (1:1, 1×) cell culture media were purchased from HyClone. CCK-8 assay kit was purchased from DOJINDO. All other chemicals were reagent grade and deionized water was used.

Methods. Proton and carbon magnetic resonance spectra (^{1}H NMR, ^{13}C NMR) were recorded on a Bruker Ascend 600 (600 MHz) spectrometer. Chemical shifts were reported in parts per million (ppm) downfield from the Me_4Si resonance which was used as the internal standard when recording ^{1}H NMR spectra. High resolution mass spectra were recorded on a Waters XEVO G2 TOF mass spectrometer. UV-vis absorption spectra were recorded on a Shimadzu UV-2550 UV-Vis spectrometer. The reversed-phase HPLC was monitored on an Agilent 1200 Series using BetaBasic-18 column. ATR-FTIR spectra were recorded on a NICOLET 5700 FTIR spectrometer. X-ray photoelectron spectroscopy was carried out by an ESCALAB 250Xi XPS system. Dynamic rheology experiments were performed on HAAKE MARS Ⅲ photorheometer with parallel-plate (P20 TiL, 20-

mm diameter) geometry and OmniCure Series 2000 (395 nm, 20 mW/cm^2). Mechanical tests were carried out using GT-TCS-2000 double column apparatus with the capacity of 20 kN. The gels were printed on an EnvisionTEC 3D-Bioplotter. Confocal luminescence imaging was performed with an A1R Nikon confocal microscope with 4× or 10× objective lens.

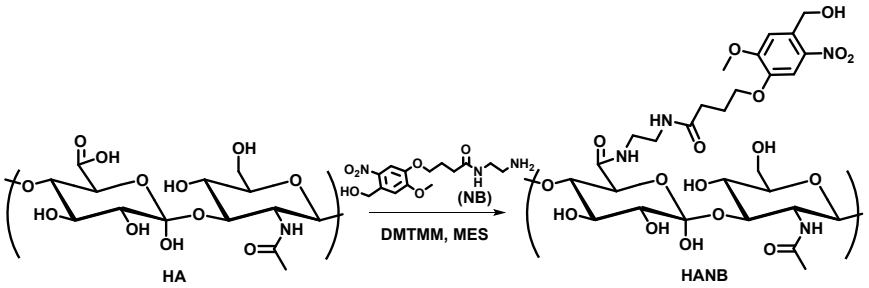
Synthesis of Sulfinanilide adduct (Compound 2) and Sulfenamide adduct (Compound 3). 4, 5-dimethoxy-2-nitrobenzyl (5 mg, 0.024 mmol, 1.0 eq.) was dissolved in 250 mL solvent (acetonitrile:H_2O = 4:1). The above solution was irradiated for 30 min to photolysis completely using 395-nm LED light (20 mW/cm^2). Then, benzyl mercaptan (1.0 ~ 4.0 eq.) was added to the above solution and stirred at room temperature overnight. The acetonitrile was evaporated in vacuum, and the water phase was extracted using ethyl acetate. The crude was purified by column chromatography on silica gel using petroleum ether/ethyl acetate (4:1). **Compound 2**: ^{1}H NMR (600 MHz, DMSO): δ = 9.56 (s, 1H), 9.32 (s, 2H), 8.18 (s, 1H), 7.96 (s, 2H), 7.33 (m, 15H), 6.53 (s, 2H), 6.50 (s, 1H), 6.47 (s, 2H), 6.15 (s, 1H), 4.19 (s, 2H), 4.13 (s, 4H), 3.72 (s, 6H), 3.69 (s, 3H), 3.65 (s, 6H), 3.48 (s, 3H). ^{13}C NMR (600 MHz, DMSO): δ = 168.05, 165.66, 149.68, 149.50, 147.60, 147.41, 141.79, 141.70, 136.76, 136.67, 129.96, 129.09, 129.04, 127.86, 121.89, 120.12, 113.55, 113.04, 101.63, 101.18, 56.79, 56.60, 55.99, 55.96, 43.25, 41.26. ESI-HRMS: [M+Na$^+$] 342.0775 ($C_{16}H_{17}NO_4NaS$: 342.0776). **Compound 3**: ^{1}H NMR (600 MHz, DMSO): δ = 9.66 (s, 1H), 8.84 (s, 1H), 7.34-7.14 (m, 6H), 6.88 (s, 1H), 3.95 (s, 2H), 3.77 (s, 4H), 3.73 (s, 3H). ^{13}C NMR (600 MHz, DMSO): δ = 192.89, 156.42, 148.38, 142.18, 137.49, 129.63, 128.94, 127.45, 117.48, 113.78, 97.38, 56.43, 56.06, 42.92. ESI-HRMS: [M+Na$^+$] 326.0824 ($C_{16}H_{17}NO_3NaS$: 326.0827).



Scheme S1. The reaction of S-nitrosylation to form sulfinanilide adduct and sulfenamide adduct.

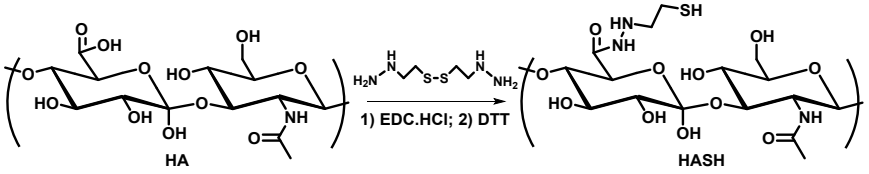
Synthesis of NB-modified hyaluronic acid (HANB). HA (2 g, molecular weight 340 kDa) was dissolved in 100 mL 0.1M MES solution (pH = 5.17), and 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methyl morpholinium chloride (0.4 g, 1.36 mmol, DMTMM) was added. **NB** was synthesized according to a previous reference.[1] Then, **NB** (60 mg, 0.18 mmol) was dissolved in dimethyl

sulfoxide (DMSO) and added into the above solution to stir overnight in the dark at 30 ℃. Then, the solution was dialyzed against deionized water for 3 days and followed by freezing and lyophilizing. ^{1}H NMR analysis was performed to determine the substitution degree of nitrobenzyl group as previously described.[1]



Scheme S2. Synthesis of **NB**-modified hyaluronic acid (**HANB**).

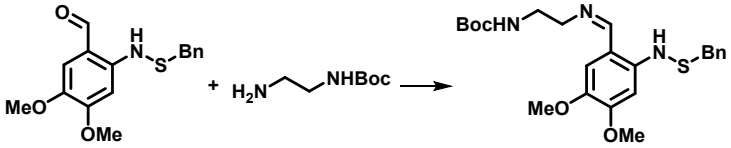
Synthesis of thiolated hyaluronic acid (HASH). HA (2 g, molecular weight 48 kDa) was dissolved in 50 mL of deionized water, and DTP (0.1 g, 0.42 mmol) was added when the solution was stirred. The pH of the reaction mixture was adjusted to 4.75 by the addition of 1.0 M HCl. Next, EDC·HCl (0.1 g, 0.5 mmol) in solid form was added. The pH of the reaction mixture was maintained at 4.75 with aliquots of 1.0 M HCl. The reaction was stopped by addition of 1.0 M NaOH and the pH of reaction mixture was raised to 7.0. Then, DTT (0.3 g, 2 mmol) in solid form was added, and the pH of the solution was raised to 8.5 by addition of 1.0 M NaOH. After the mixture was stirred for 24 h, the pH of reaction mixture was adjusted to pH 3.5 by the addition of 1.0 M HCl. The acidified solution was transferred to dialysis bags (MWCO 3500 Da) and dialyzed exhaustively against dilute HCl (pH 3.5, approximately 0.3 mM) containing 100 mM NaCl, followed by dialysis against dilute HCl (pH 3.5). The solution was then centrifuged, and the supernatant was lyophilized. ^{1}H NMR analysis was performed to determine the substitution degree of thiol group as previously described. [2]



Scheme S3. Synthesis of thiolated hyaluronic acid (**HASH**).

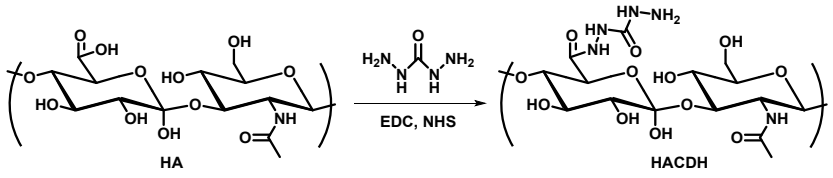
Synthesis of Schiff base compound. The sulfensmide adduct (or sulfinanilide adduct) and N-Boc-

ethylenediamine (1.0 eq.) were dissolved in ethyl acetate and stirred at 40 ℃ for several minutes. Then, the solvent was evaporated in vacuum without any purification to obtain crude product. ^{1}H NMR (600 MHz, DMSO): δ = 9.78 (s, 1H), 8.23 (s, 1H), 7.33-7.14 (m, 5H), 6.96 (s, 2H), 6.81 (m, 1H), 3.86 (s, 2H), 3.74 (s, 3H), 3.69 (s, 3H), 3.44 (t, *J* = 6.7 Hz, 2H), 3.07 (t, *J* = 6.4 Hz, 2H), 1.36 (m, 9H). ^{13}C NMR (600 MHz, DMSO): δ = 165.11, 156.07, 152.38, 145.58, 141.62, 137.79, 129.57, 128.59, 127.25, 117.35, 112.06, 97.90, 78.06, 60.34, 56.64, 55.73, 42.71, 41.61. ESI-HRMS: [M+H^{+}] 446.2115 ($C_{23}H_{32}N_3O_4S$: 446.2114).



Scheme S4. Synthesis of Schiff base compound.

Synthesis of carbohydrazide-modified hyaluronic acid (HACDH). HA (2 g, molecular weight 48 kDa) was dissolved in 50 mL of deionized water, and 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (0.2 g, 1 mmol, EDC·HCl), N-Hydroxysuccinimide (0.12 g, 1 mmol, NHS) in solid form were added. Then, carbohydrazide (0.09 g, 1 mmol) was dissolved in deionized water and added into the above solution to stir overnight. Then, the solution was dialyzed against deionized water for 3 days and followed by freezing and lyophilizing. The degree of hydrazide modifications (10.8%) was determined following literature procedure using trinitrobenzene sulfonic acid (TNBS) assay, which gives a quantitative assessment of free carbohydrazide residues using UV spectroscopy.[3]



Scheme S5. Synthesis of carbohydrazide-modified hyaluronic acid (**HACDH**).

UV-Vis Absorption Spectroscopy. d-NB (10^{-4} mol L^{-1}) was dissolved in 4:1 CH_3CN/PBS (v/v, pH=7.4) solution using a cuvette. UV-vis absorption spectroscopy was used to detect the change in the absorption profiles of d-NB under 395-nm LED irradiation (20 mW/cm^2) and followed by the addition of benzyl mercaptan (1.0 eq.).

HPLC-MS. d-NB (10^{-4} mol L^{-1}) was dissolved in 4:1 CH_3CN/PBS (v/v, pH = 7.4) solution. The solution was irradiated with 395-nm LED (20 mW/cm^2) and followed by the addition of benzyl mercaptan (1.0 eq.). Then, a small aliquot (100 μL) of the solution was removed and analyzed by reversed-phase HPLC using a BetaBasic-18 column eluted with 70% acetonitrile and 30% water at a flow rate of 0.5 mL/min. The chromatogram was plotted using absorbance detection at 254 nm.

ATR-FTIR and XPS experiments. The gel precursor samples were dried in the air for 12 h. The hydrogel samples were formed under 395-nm LED (20 mW/cm^2) for 3 min and dried in the air for 12 h. Then, the gel precursor and hydrogel samples were dried at 40 ℃ for 12 h. ATR-FTIR spectra were recorded on a Nicolet 6700 FTIR spectrometer. Film characterization using X-ray photoelectron spectroscopy was carried out in an ultrahigh vacuum chamber by an ESCALAB 250Xi XPS system. Then, XPS spectra were analyzed by XPSPEAK software to conduct peak separation.

Rheological analysis. Dynamic rheology experiments were performed on HAAKE MARS Ⅲ photorheometer with parallel-plate (P20 TiL, 20-mm diameter) geometry and OmniCure Series 2000 (395 nm, 20 mW/cm^2) at 25 °C. Time sweep oscillatory tests were performed at a 10% strain (CD mode), 1 Hz frequency and a 0.5 mm gap for 180 s. Strain sweeps were performed to verify the linear response. The gel point was determined as the time when the storage modulus (G') surpassed the loss modulus (G''). The final modulus was determined as the storage modulus (G') reaching to the complete gelation.

Mechanical properties tests. Mechanical tests were carried out on as-prepared hydrogels using GT-TCS-2000 double column apparatus with the capacity of 20 kN. For compression tests, hydrogel samples were prepared to have cylindrical shape with 10-mm diameter and 3-mm length and the speed was set at 1 mm/min. The cyclic compression tests were conducted for ten times at the strain of 92%. The hydrogels after complete gelation under light irradiation (395 nm, 20 mW/cm^2) were subjected to compression tests.

3D printing of hydrogels. The polymers (**HANB/TP/CMCh** or **HANB/TP/GL**) were dissolved into D-

PBS (pH 7.4) to get certain concentration solution. Then, the gel precursor solution was loaded into extrusion cartridges, which were placed on the printing carriage of 3D printer and extruded through a needle. Then, the gel precursors were printed on an EnvisionTEC 3D-Bioplotter, whose printing pressures and speeds were altered depending on ink flow properties. Gel precursor solutions were typically printed by applying a pressure of 0.5-1 bar at a printing speed of 2 mm s^{-1} onto culture dish and followed by *in situ* light irradiation (395 nm, 20 mW/cm^2). After printing, the 3D shape was stabilized as the process of immediate photocrosslinking.

CCK-8 test. The cytotoxicity was assessed by the CCK-8 method. Briefly, NIH 3T3 fibroblast cells were seeded in 96-well plates at a concentration of 5,000 cells per well for 24 hrs culture. The tested materials were dissolved in D-PBS and sterilized by a filtration membrane (0.22 μm). Then, every well was added a certain volume of the above solutions to get 0.5%, 0.1% or 0.05% w/v final concentration. The culture solution was removed after 24 hrs of incubation, and 10 μL CCK-8 reagent and 100 μL culture medium were added to each well and incubated for 2 hrs at 37 °C. The absorbance was measured using a microplate reader (Synergy H1, BioTek) at 450 nm.

Live/dead staining. The printing procedure with cells can be realized when mixing NIH 3T3 fibroblast cells with precursor solution to get desired cells density (1×10^6 cells mL^{-1}). For observing cell viability, live/dead staining was performed to differentiate living cells stained with calcein-AM (green) versus the dead cells stained with PI (red). Encapsulated cells were imaged on an A1R Nikon confocal microscope with 4× or 10× objective lens.

Quantification of precursor materials in hydrogels. The precursor materials (e.g., HANB, TP, and CMCh) were weighted together and recorded M_0. These materials were dissolved in deionized water and then crosslinked to form a hydrogel, which was further immersed in deionized water for 24h to remove the materials that present in the sol network. The resultant hydrogel was freeze-dried and weighted and recorded M_1. The amount of precursor materials that remained in the hydrogel network were calculated as $(M_0–M_1)/M_0$.

The magic-angle spinning (MAS) NMR Characterization. ^{1}H MAS NMR spectra were collected on a Bruker 600 MHz AVANCE NEO WB spectrometer (Bruker, Switherland) at a 14.09 T magnetic

field, using a 3.2 mm HXY probe at a Lamor frequency of 600.43 MHz. A 3.2 mm zirconia rotor with a spinning rate of 15 KHz was used. The excitation pulse length (90°) was 2 μs, and 32 cans were acquired with relaxation delay of 1 s.

Data Analysis: All experiments were carried out independently in triplicate. Data were presented as mean ± standard deviation (s. d.). Differences between the values were evaluated using One-Way analysis of variance (ANOVA).

Figure S1-S20

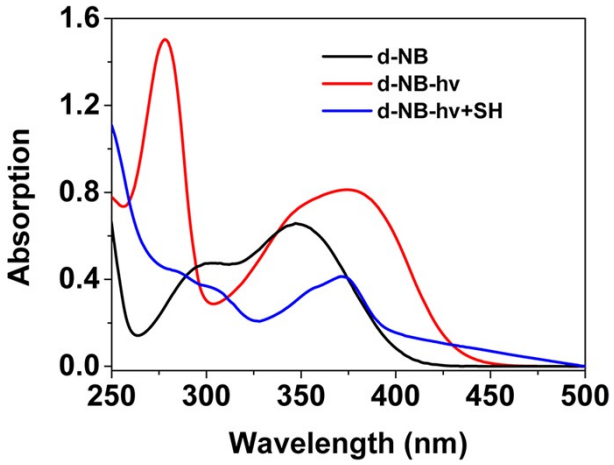


Fig. S1. UV-Vis spectra displayed the changes from 350 nm to 375 nm of absorption maximum (λ_{max}) of **d-NB** before and after 395-nm irradiation. Upon addition of benzyl mercaptan, the decay of the absorption maximum (λ_{max} = 375 nm) was observed.

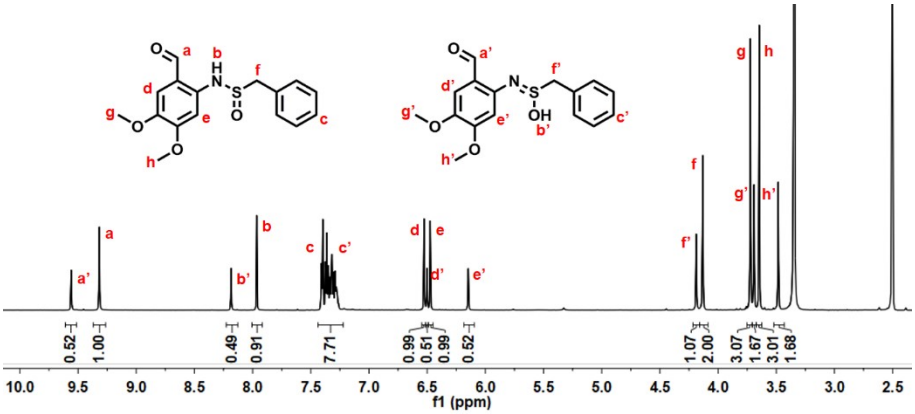


Fig. S2. ^{1}H NMR spectrum of sulfinanilide-S/N-enol tautomerism (molar ratio = 2:1) in DMSO.

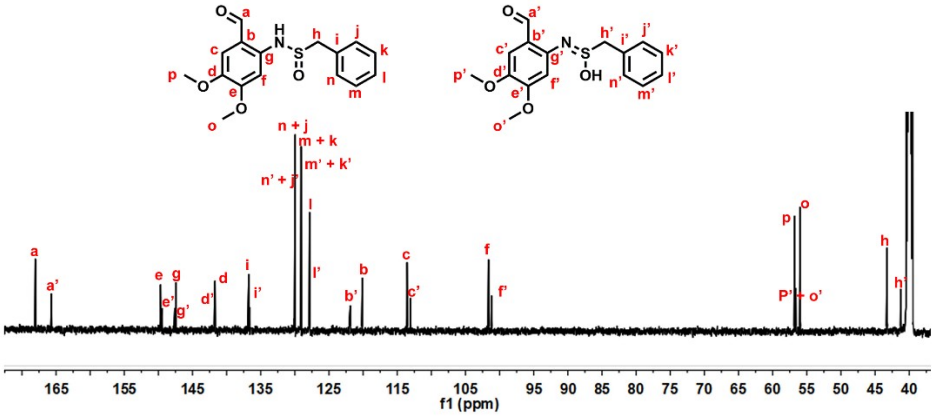


Fig. S3. ^{13}C NMR spectrum of sulfinanilide-S/N-enol tautomerism (molar ratio = 2:1) in DMSO.

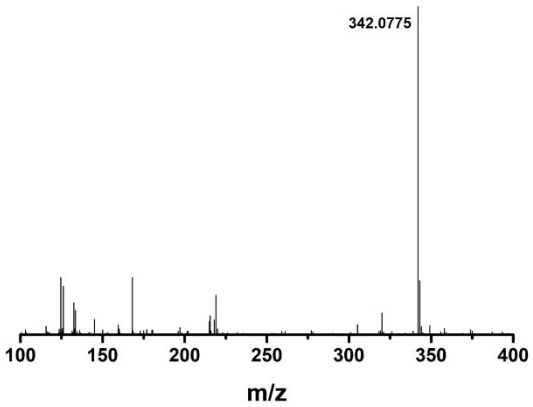


Fig. S4. ESI-HRMS spectrum of sulfinanilide-S/N-enol tautomerism. ($C_{16}H_{17}NO_4NaS$: 342.0776)

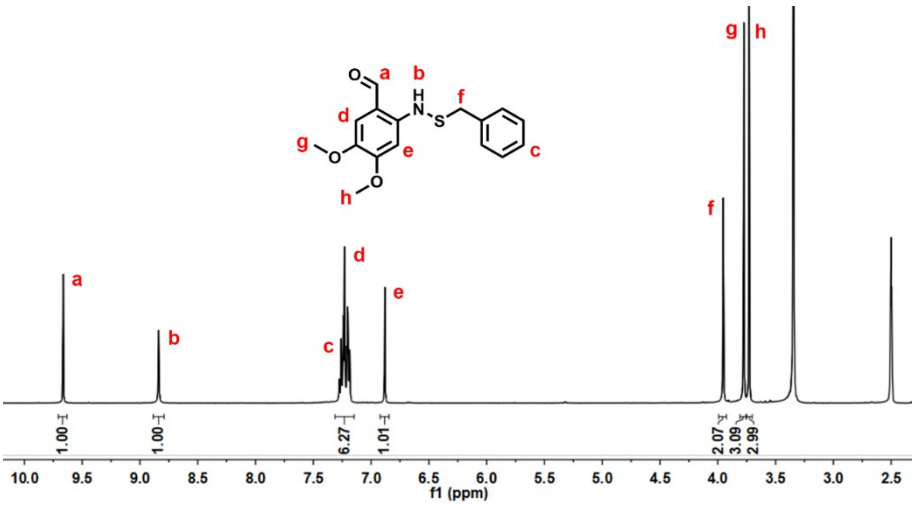


Fig. S5. ^{1}H NMR spectrum of sulfenamide adduct in DMSO.

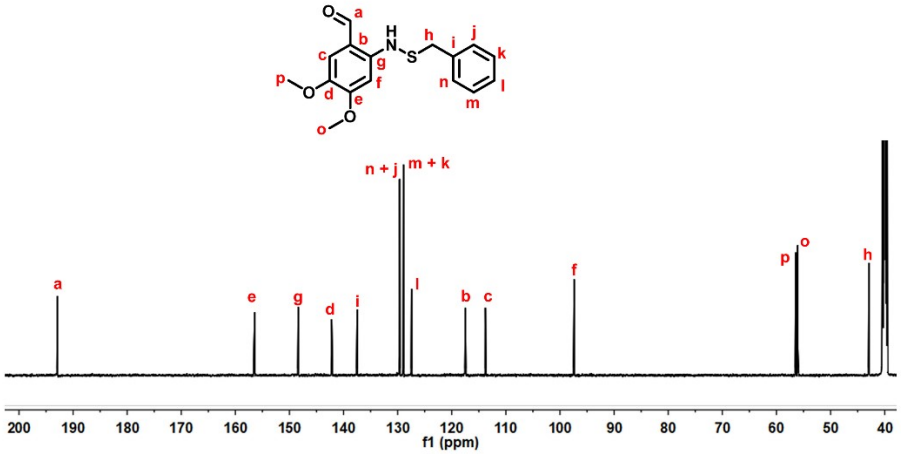


Fig. S6. ^{13}C NMR spectrum of sulfenamide adduct in DMSO.

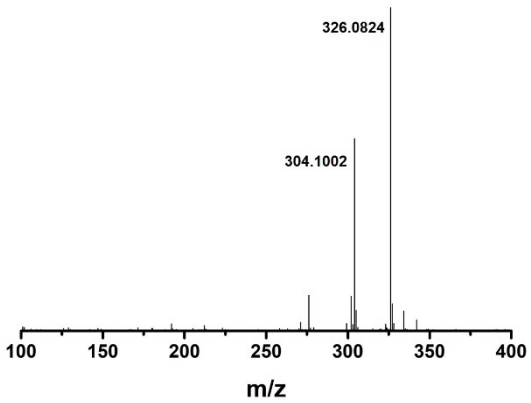


Fig. S7. ESI-HRMS spectrum of sulfenamide adduct. ($C_{16}H_{17}NO_3NaS$: 326.0827)

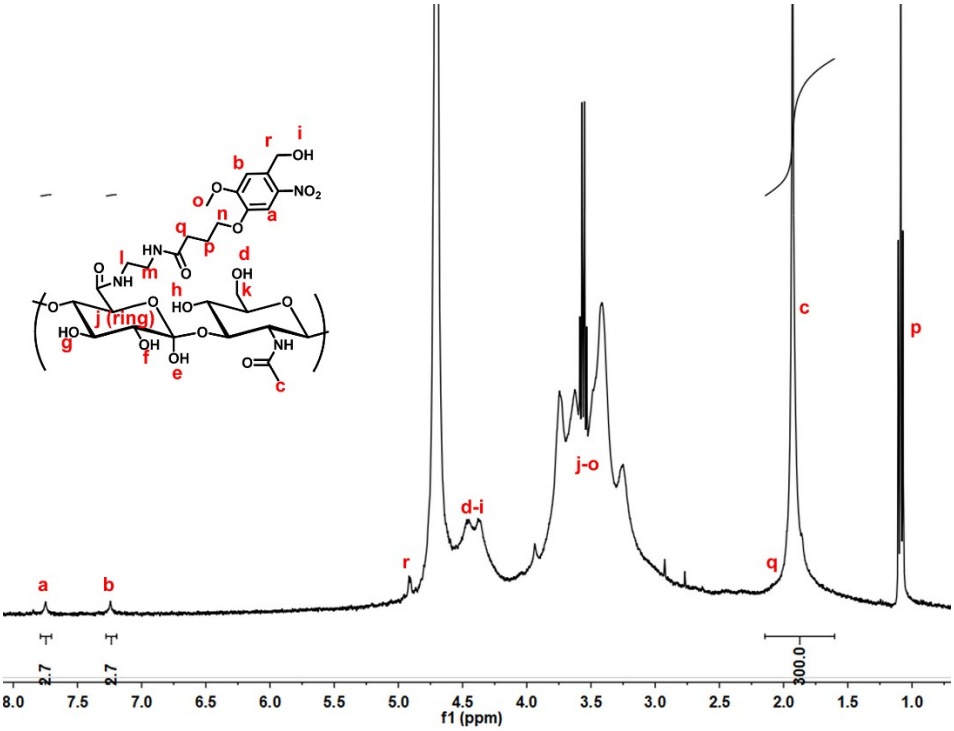


Fig. S8. ^{1}H NMR spectrum of **HANB** (**NB** modified hyaluronic acid). The signal peaks of 7.7 and 7.2 ppm (the characteristic peak of benzene) indicted the successful graft of **NB**. The substitution degree was determined by the integral ratio of the proton peaks at 7.7 and 7.2 ppm to the peak at 1.9 ppm (N-acetyl glucosamine of HA).

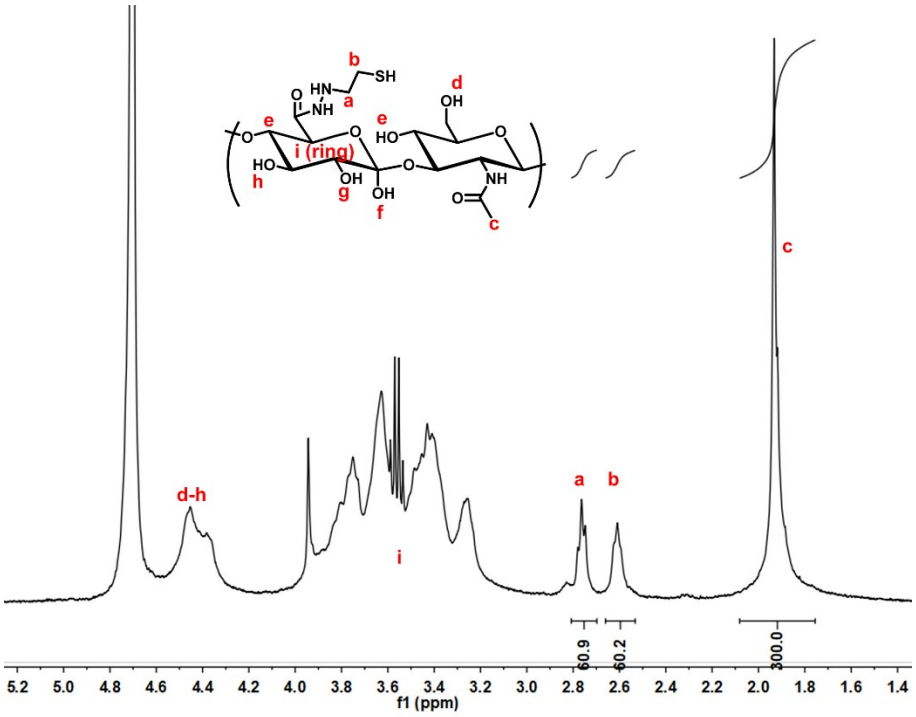


Fig. S9. ^{1}H NMR spectrum of **HASH** (thiolated hyaluronic acid). The signal peaks of 2.6 and 2.8 ppm

(the characteristic peak of ethyl group) indicted the successful graft of thiol groups. The substitution degree was determined by the integral ratio of the proton peaks at 2.6 and 2.8 ppm to the peak at 1.9 ppm (N-acetyl glucosamine of HA).

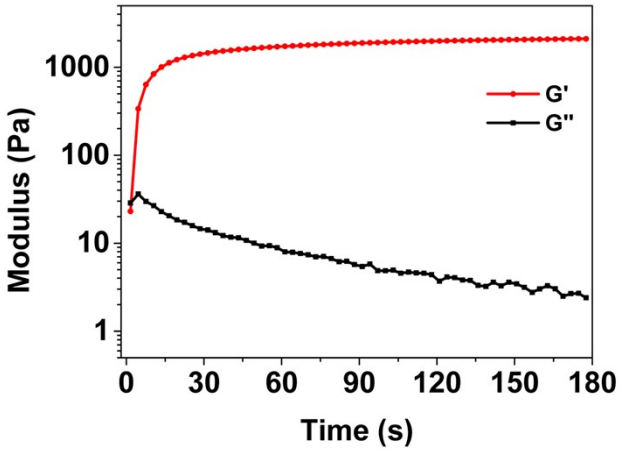


Fig. S10. Rheological test of **HANB/HASH** gel (5% w/v, 2:3).

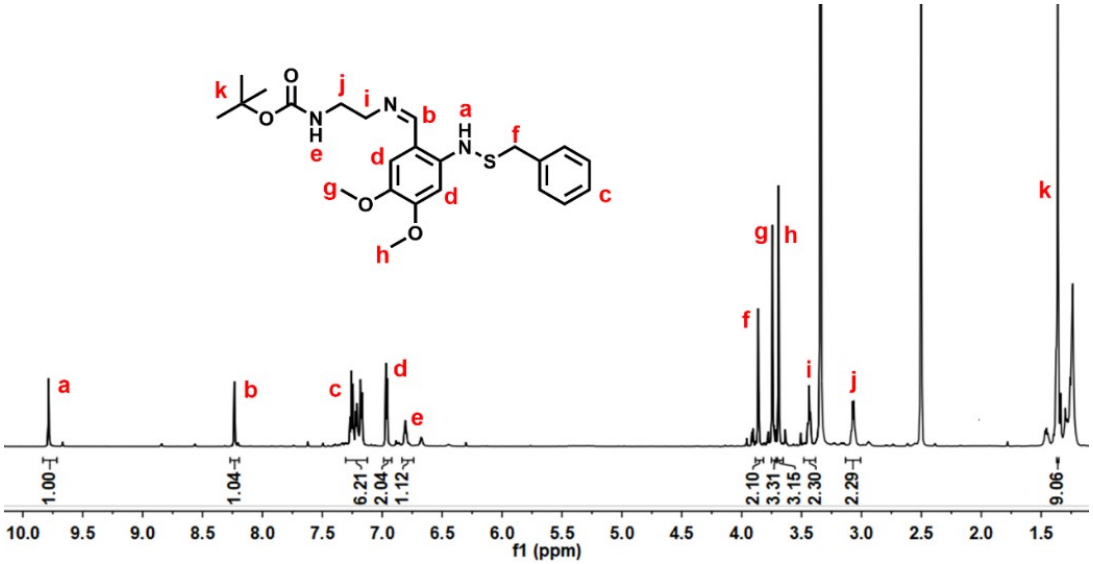


Fig. S11. ^{1}H NMR spectrum of Schiff base compound in DMSO.

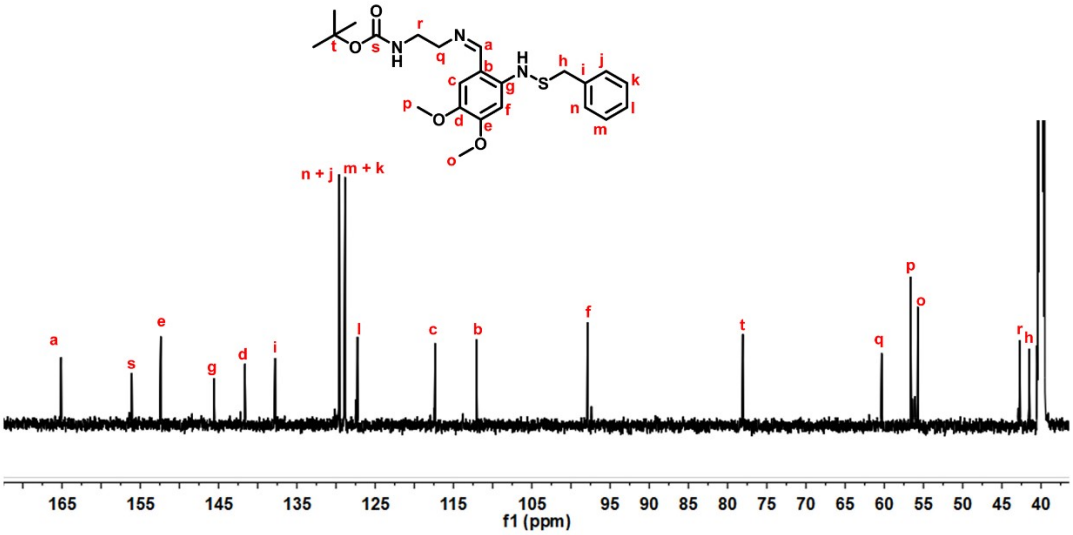


Fig. S12. ^{13}C NMR spectrum of Schiff base compound in DMSO.

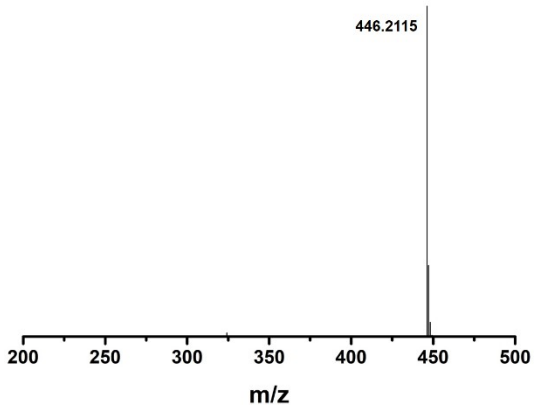


Fig. S13. ESI-HRMS spectrum of Schiff base compound. ($C_{23}H_{32}N_3O_4S$: 446.2114)

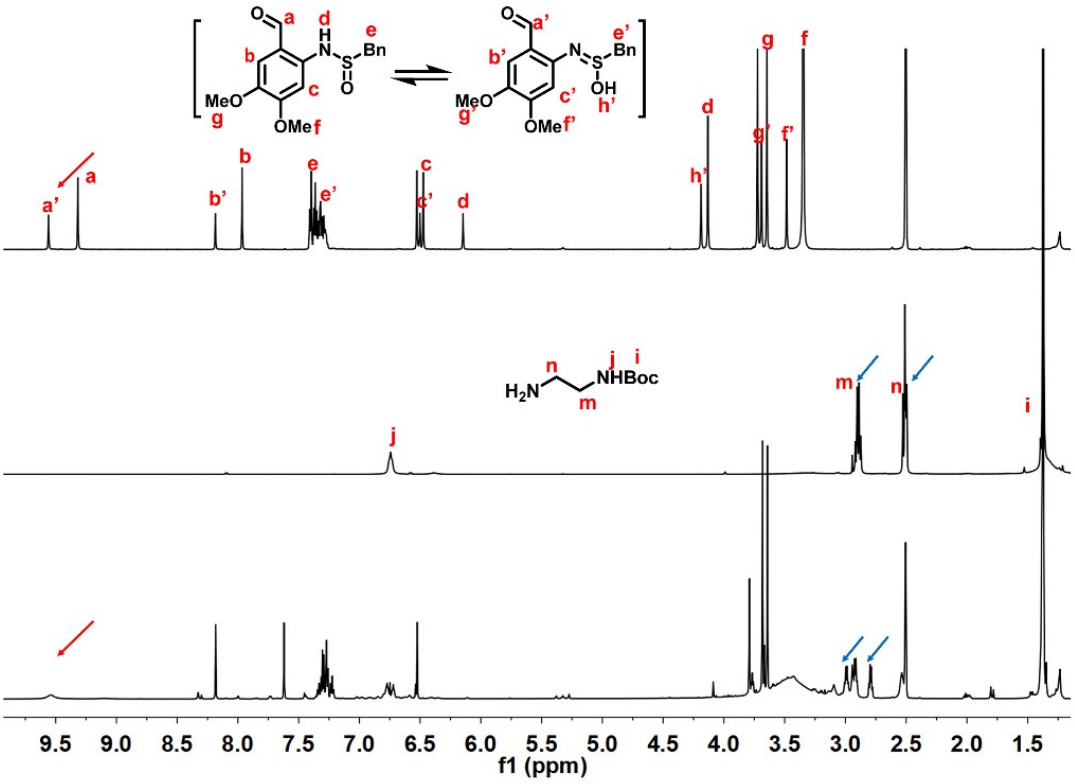


Fig. S14. ^{1}H NMR spectrum of the reaction between sulfinanilide adduct and N-Boc-ethylenediamine. Red arrows represent the disappearance of aldehyde groups; Blue arrows represent the peak shift of ethyl groups.

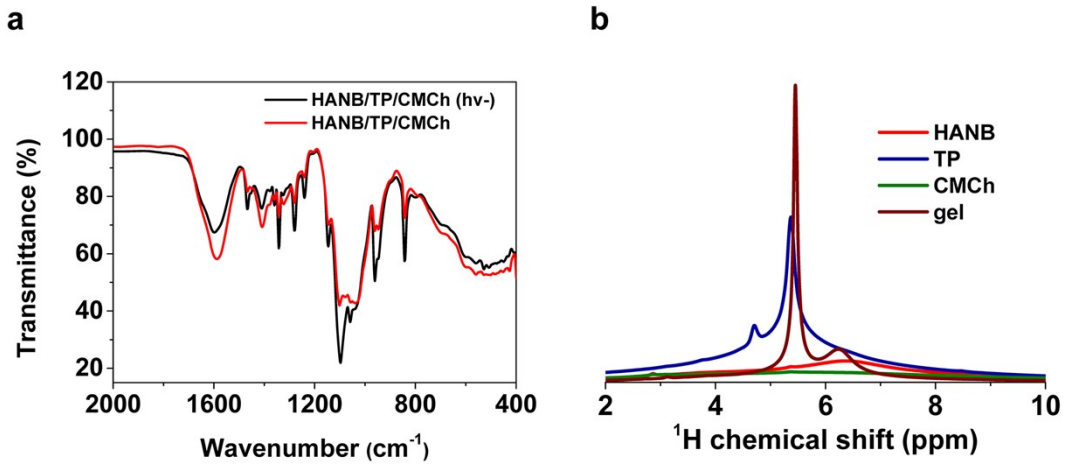


Fig. S15. (a) ATR-FTIR spectra of the gel precursor (**HANB/TP/CMCh** (hv-)) and the corresponding hydrogel (**HANB/TP/CMCh**) before and after light irradiation. (b) ^{1}H MAS NMR spectroscopy of the raw materials (*i.e.*, HANB, TP, and CMCh) and the obtained gel (freeze-dried). The PEG segment in the TP showed different chemical ^{1}H shifts with that in the gel, indicating the changes of local

environments caused by network crosslinking. In addition, the ^{1}H peak width of the HA became narrow after network crosslinking. This should attribute to the conjugation of the polyethylene glycol to the hyaluronic acid, and thereby a reduced rigidity of the hyaluronic acid.

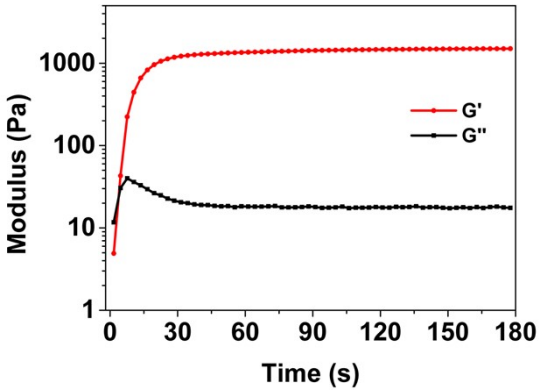


Fig. S16. Rheological test of **HANB/TP/CMCh** gel (7% w/v, 2:3:2).

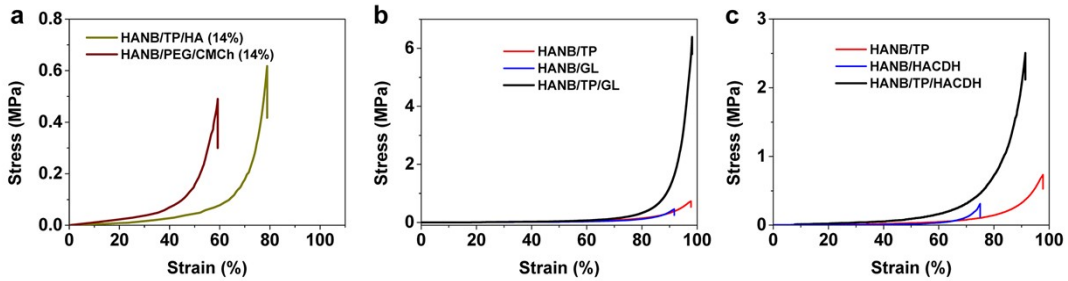


Fig. S17. (a) The compression tests of **HANB/TP/HA** gel (14% w/v, 2:3:2) and HANB/PEG/CMCh gel (14% w/v, 2:3:2). The molecular weight of HA was 48 kDa. PEG was abbreviation of Four-arm-PEG (Mw = 40 kDa). To avoid the effect of solid contents on mechanical properties, the total solid content of hydrogels was kept constant for comparison. We thus synthesized HANB/TP/HA gels (14% w/v, 2:3:2) that comprised 4% solid of HA and HANB/PEG/CMCh gels (14% w/v, 2:3:2) that comprised 6% solid of PEG. The similar outcomes were achieved when comparing these gels with HANB/TP/CMCh gel (14% w/v, 2:3:2), demonstrating that the compressive strength of the hydrogels was largely depended on the underlying reaction mechanism. (b) **HANB/TP/GL** gel (14% w/v, 2:3:2) compared with **HANB/TP** gel (10% w/v, 2:3) and **HANB/GL** gel (8% w/v, 2:2). (c) **HANB/TP/HACDH** gel (14% w/v, 2:3:2) compared with **HANB/TP** gel (10% w/v, 2:3) and **HANB/HACDH** gel (8% w/v, 2:2).

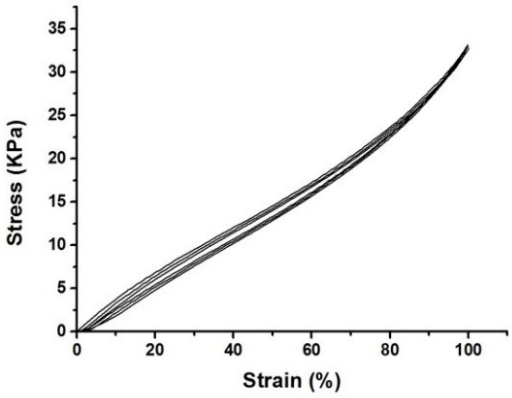


Fig. S18. Cyclic extension test of **HANB/TP/CMCh** gel (14% w/v, 2:3:2) to 100% strain.

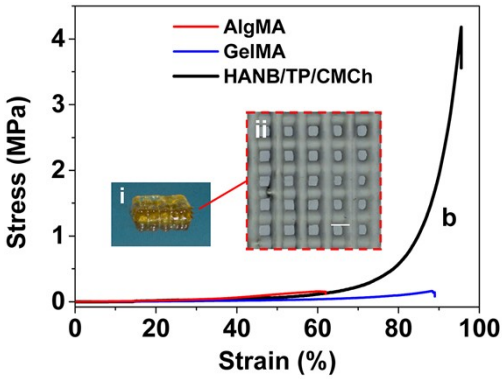


Fig. S19. Representative compression curves of the printed lattice construct of HANB/TP/CMCh gel, GelMA gel (10% w/v, 0.5% w/v LAP), and AlgMA gel (5% w/v, 0.5% w/v LAP). Inset are the (i) photograph and (ii) micrograph (scale bar: 500 μm) of the printed mesh structure.

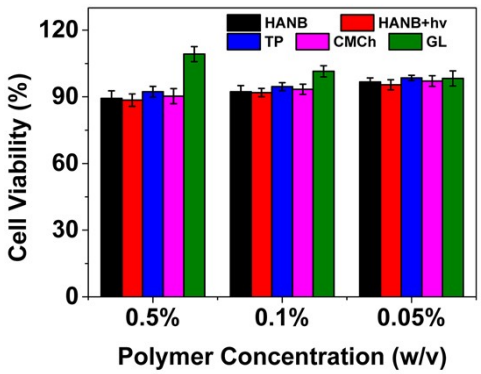


Fig. S20. The cell viability of the hydrogel precursor **HANB**, **HANB+hv** (**HANB** product upon light irradiation), **TP**, **CMCh** and **GL** on the NIH 3T3 fibroblast cells at different concentrations (0.5%, 0.1%, 0.05% w/v) after culturing for 24 h was evaluated by CCK-8 assay.

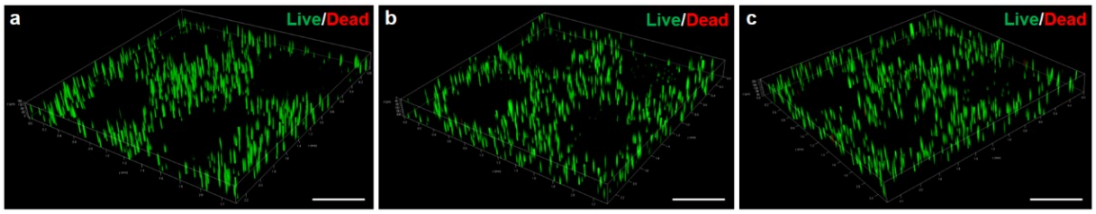


Fig. S21. Confocal images of NIH 3T3 fibroblast cells in 3D printed mesh of **HANB/TP/GL** gel (14% w/v, 2:3:2) stained with a live/dead reagent at day 1 (a), day 4 (b) and day 7 (c). Live cells fluoresce green, whereas dead cells emit red. Scale bar: 500 µm.

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