Electronic Supplementary Information (ESI)

Stiffness of thermoresponsive gelatin-based dynamic hydrogels affects fibroblast activation

M. Mario Perera[1†], Demetria M. Fischesser[2†], Jeffery Molkentin[2] and Neil Ayres[1*]

[1]Department of Chemistry, The University of Cincinnati, P.O. Box 210172, Cincinnati, Ohio 45221

[2]Cincinnati Children's Hospital Medical Center, Division of Molecular Cardiovascular Biology, Cincinnati, OH, 45229

*Corresponding Author: Phone +01 513 556 9280; Fax +01 513 556 9239; E-mail Neil.Ayres@UC.edu

CONTENTS **PAGE**

Materials

Type B gelatin (Bloom 100), amino-2-propanol, methacrylic anhydride, acryloyl chloride, 2,2-Dihydroxy-1,3-indanedione, and L-cysteine, were purchased from Sigma Aldrich (MO, USA), and used as received. Carbic anhydride (endo-*cis*-5-norbornene-2,3-dicarboxylic anhydride) and 5,5'-dithiobis-(2-nitrobenzoic acid) were purchased from Alfa Aesar (MA, USA), and used as received. Hydrindantin dihydrate, L-methionine, *N*-isopropylacrylamide and 2-aldrithiol were purchased from Oakwood Chemicals (SC, USA) and used as received. GelNB, Hydroxyethylpyridyl disulfide, and 2-hydroxypropyl methacrylamide were synthesized as described in our previous work.[1] *S*-Dodecyl-*S*′-(α,α′-dimethyl-α″-acetic acid)trithiocarbonate (DDMAT) was synthesized following reported procedures.[2] For cell culture studies, CD31- and CD45-conjugated magnetic beads and magnetic depletion LD columns were purchased from Milltenyi Biotech (CA, USA). Collagenase 2 was purchased from Worthington Biochemicals, USA. Dispase II, and αSMA antibody were purchased from Sigma Aldrich (MO, USA) Phenol red-free Dulbecco's Modified Eagle Media (DMEM) containing HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and high glucose as well as Bovine Growth Serum (BGS) were purchased from Thermo Fisher Scientific (MA, USA). The mouse line used in this study was αSMA-RFP mice (JAX B6.FVB-Tg(Acta2-DsRed)1Rkl/J). Equal ratios of male and female animals were used in generating the cells used in this study. Mice were housed and cared for in an AAALAC-accredited animal care facility at Cincinnati Children's Hospital Medical Center. The facility meets or exceeds the requirements of the Office of Laboratory Animal Welfare. Mice were inspected daily by veterinary technicians to ensure healthy conditions. All experimental use of mice was approved by the institutional animal care and use committee (IACUC), protocol number 2018-0069 to Jeffery Molkentin.

Instrumentation

^{1}H and ^{13}C NMR spectroscopy were performed using a Bruker Ultrashield 400 MHz (100 MHz for ^{13}C NMR) instrument and the data were processed using MestReNova 10 software. Fourier-transform infrared (FTIR) spectra were collected on a Nicolet 6700 spectrometer and analyzed with OMNIC32 software. Molecular weights of polymers were determined by gel permeation chromatography (GPC) using an Agilent 1200 series HPLC equipped with a PSS Gram guard column (10 μm) and two PSS Gram columns (10 μm) with filtered DMF with 0.1% LiBr w/v mobile phase at a flow rate of 0.5 mL/min at 70 °C and an Optilab rEX differential refractometer (light source 658 nm) detector calibrated against poly(methyl methacrylate) standards (850 – 2,000,000 Da) and ASTRA software v. 6.1.0 data calculation.

Synthesis of Pyridyl disulfide ethylacrylate (PDSEA)

Pyridyl disulfide ethylacrylate (PDSEA) was synthesized following previous literature reports.[3] Hydroxyethylpyridyl disulfide (HPDS) (8.00 g, 42.7 mmol) and 8.70 g of triethylamine (85.9 mmol) were dissolved in 70 mL of dichloromethane and the mixture cooled in an ice-bath. Acryloyl chloride (4.23 g, 46.7 mmol) in 30 mL of dichloromethane was added over 30 min using an addition funnel. The reaction mixture was stirred at room temperature for 12 h. After the reaction, the solution was filtered to remove solids and the filtrate washed with water (3 x 100 mL), saturated $NaHCO_3$ (3 x 100 mL) and brine (3 x 100 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated using a rotary evaporator at 30 ˚C to obtain the crude product as a brown oil. The crude product was purified using column chromatography with silica gel as the stationary phase and a 30/70 (v/v) mixture of ethyl acetate/*n*-hexane as the mobile phase. The purification was monitored using TLC and the desired fraction was collected with a R_f of 0.35. The solvent was removed using a rotary evaporator and the pure product isolated as a

pale-yellow oil in a 67% yield. ^{1}H NMR ($CDCl_3$, 400MHz, Figure S1): δ [ppm] = 8.47 (aromatic proton *ortho*-N), 7.70−7.61 (aromatic proton *para*-N and *meta*-N), 7.11 (aromatic proton, *ortho* disulfide), 6.45 (vinylic proton), 6.10 (vinylic proton) 5.84 (vinylic proton), 4.40 (−OCH_2CH_2S−), 3.07 (−OCH_2CH_2S−). ^{13}C NMR ($CDCl_3$, 200MHz, Figure S2): δ [ppm] = 165.77 (−C=O), 159.62 (aromatic carbon, *ortho*-disulfide), 149.71 (aromatic carbon *ortho*-N), 137.03 (−$CH=CH_2$), 131.31 (aromatic carbon *para*-N), 127.99 (−CH_2=CH−), 119.77-120.85 (aromatic carbon *meta*-N), 62.25 (−OCH_2CH_2S−), 37.32 (−OCH_2CH_2S−).

Synthesis of poly(NIPAAm-*s*-HPMA-*s*-PDSEA)

A 100 mL round bottom flask was filled with NIPAAm (6.10 g, 54.1 mmol) HPMA (1.21 g, 8.1 mmol) PDSEA (1.95 g, 8.1 mmol) of *S*-Dodecyl-*S*′-(α,α′-dimethyl-α′′-acetic acid)trithiocarbonate (DDMAT) (215 mg, 0.59 mmol), azobisisobutyronitrile (AIBN) (19.4 mg, 0.12 mmol), and 35 mL of 1,4-dioxane. The reaction flask was placed in an ice bath and equipped with a stir bar, sealed with a rubber septum and purged with N_2 gas for 30 min, then placed in a pre-heated oil bath at 70 ˚C for 12 h. The polymerization was quenched by exposing the reaction mixture to air (O_2) and product was obtained by precipitation in ice-cold diethyl ether. The polymer was obtained as a pale-yellow solid. The product was dried in vacuum for 12 h to obtain 6.95 g of pure poly(NIPAAm-*s*-HPMA-*s*-PDSEA) with a yield of 73%.

Synthesis of poly(NIPAAm-*s*-HPMA-*s*-MEA)

The pyridyl disulfide groups were reduced using (tris(2-carboxyethyl) phosphine) (TCEP) following literature protocols.[4] Briefly, 1.50 g of poly(NIPAAm-*s*-HPMA-*s*-PDSEA) was dissolved in 10 mL of acetate buffer (pH 5.5) and a five-fold molar excess of TCEP with respect to disulfide groups added. The flask was sealed with a rubber septum and stirred for 16 h in the dark. After 24 h the reaction mixture was dialyzed (MWCO = 3.5 kDa) in a 0.1% HCl solution in

the dark for 2 days and freeze dried to obtain the product as a pale-yellow powder. Yield = 71%. The product was stored under N_2.

LCST determination of poly(NIPAAm-*s*-HPMA-*s*-MEA)

The optical transmittance of a poly(NIPAAm-*s*-HPMA-*s*-MEA) solution (5 mg/mL) in PBS was measured at 600 nm at temperatures from 25 ˚C to 40 ˚C and heated at a rate of 1˚C per 5 min. The LCST was defined as the temperature which resulted in 50% optical transmittance.[4]

Preparation of hydrogels

GelNB (0.25 g) was dissolved in 2 mL of 0.01 M PBS (pH 7.4) at 60 ˚C. The solution was cooled to room temperature after the GelNB completely dissolved. In a separate vial poly(NIPAAm-*s*-HPMA-*s*-MEMA), TCEP (10% weight by copolymer weight), and Irgacure 2959 (1% weight by total polymer weight) were dissolved in 2 mL of 0.1 M PBS. A 1:1, 2:1, or 3:1 thiol to ene ratio was used in the final reaction mixture (Amount of each reagents are mentioned in Table S1). The two solutions were combined in a 20 mm tissue culture plate and irradiated with UV light (λ_{max} = 365 nm) for 5 min. The hydrogels were characterized using swelling ratio, gel fraction, rheology, and FTIR spectroscopy.

Rheology measurements

Rheological analysis was performed using oscillatory frequency sweeps (0.1–100 Hz; 0.1% strain, 37 ˚C), oscillatory time sweeps (3.2 Hz, 0.1 % strain, 37 ˚C), and oscillatory temperature sweeps (20 - 50 ˚C, 3.2 Hz, 0.1 % strain) with a Discovery Series Hybrid Rheometer (DHR) (Model HR-2, TA Instruments) using 20 mm diameter parallel plates and controlled temperature using an advanced Peltier system. Hydrogels swollen in PBS for 12 h were used for rheological analysis.

Swelling ratio and gel fraction

The prepared hydrogels were swollen at room temperature for 24 h in 500 mL of 0.01 M PBS (pH 7.4) with PBS being changed for three times before determining the swelling ratio. After recording the mass of the swollen gel (M_s), the hydrogels were freeze-dried and the dry mass measured (M_d). The swelling ratio was calculated according to Equation 1:

$$\text{Swelling ratio} = \frac{M_s - M_d}{M_d} \qquad \text{Equation 1}$$

Gel fractions of the hydrogels were calculated using Equation 2, where the M_i is the initial mass of polymers in the hydrogel.

$$\text{Gel fraction} = \frac{M_d}{M_i} \qquad \text{Equation 2}$$

Kinetics of disulfide crosslink formation

Six hydrogels of $TE_{2:1}$ and $TE_{3:1}$ were prepared and swollen in PBS as described for the swelling ratio studies. The mechanical properties of the hydrogels were evaluated by time sweep rheology using a hydrogel gel for each experiment every hour for 6 h.

Thiol exchange reaction with L-cysteine or L-methionine

Swollen hydrogels were placed in a 6-well cell culture plate and 10 mL of a 1mM, 2 mM, or 5 mM solution of L-cysteine or L-methionine in 15% bovine growth serum in phenol red free Dulbecco's modified eagle medium (DMEM) added on to the hydrogels. The media was removed and replaced with fresh L-cysteine or L-methionine solutions three times over 24 h. The resultant hydrogels were characterized using swelling ratio and rheology.

Cardiac fibroblast (CF) isolation

Hearts were excised from 6-8-week old αSMA-RFP mice and stored in ice cold PBS. Hearts were finely minced using scissors and the resulting homogenate was put into 2mL of BGS-free DMEM containing 2 mg/mL collagenase IV and 1.2 U/mL dispase II. This was placed on a shaker in a 37 ˚C incubator for 20 min, then removed, triturated, and allowed to settle. Media was removed and placed in DMEM containing 50% BGS. This digestion was repeated twice more. The cell population contained in the 50% DMEM was centrifuged at 10 xg for 5 min at 4 ˚C. The supernatant was then centrifuged at 500 xg for 10 min at 4 ˚C. The resulting pellet was resuspended in 1mL Hank's Buffered Saline Solution (HBSS). The samples were then incubated with 30 mL each of CD31- and CD45-conjugated magnetic beads on a rotator at 4 ˚C for 30 min. The samples were then run through a magnetic depletion LD column. The resulting population was pure cardiac fibroblasts, which were plated at a density of ~180,000 cells per hydrogel in phenol red-free DMEM containing 10% BGS. Each hydrogel covered a 30 mm diameter well of a tissue culture plate. Media was refreshed every 48 h.

Cell Treatment

5 mM cysteine and 5 mM methionine solutions were prepared by dissolving the compounds in phenol red-free DMEM containing HEPES and high glucose containing 10% BGS. After seven days of culture, cells were treated with either the 5 mM cysteine or 5 mM methionine solution, or plain media. All treatment media was changed 4 times in 24 h and then replaced with plain phenol red-free DMEM containing HEPES and high glucose containing 10% BGS. At least 3 hydrogels of each stiffness as well as polystyrene plates were used for each protocol.

Live Cell Imaging and Analysis

Hydrogels were imaged using a Nikon Ti2 Widefield microscope at 7 and 14 days. Area analysis was conducted using the manual area trace tool of NIS Elements Analysis. The αSMA positivity was analyzed by counting red fluorescent protein (RFP) positive cells and comparing to total number of cells using the NIS Elements manual count tool.

Statistics

GraphPad Prism was used to conduct 1-way ANOVA for cell area and %RFP positivity data at 7 days with a Tukey's post hoc analysis. The cell area and %RFP positivity data at 14 days were analyzed using 2-way ANOVA and Sidek's Multiple Comparison test (between non-treated, cysteine treated, and methionine treated groups) or Tukey's test (to compare means between 5 kPa, 12 kPa, 20 kPa, and plastic groups). In all cases significance is determined as $p<0.05$.

Supplementary Figures

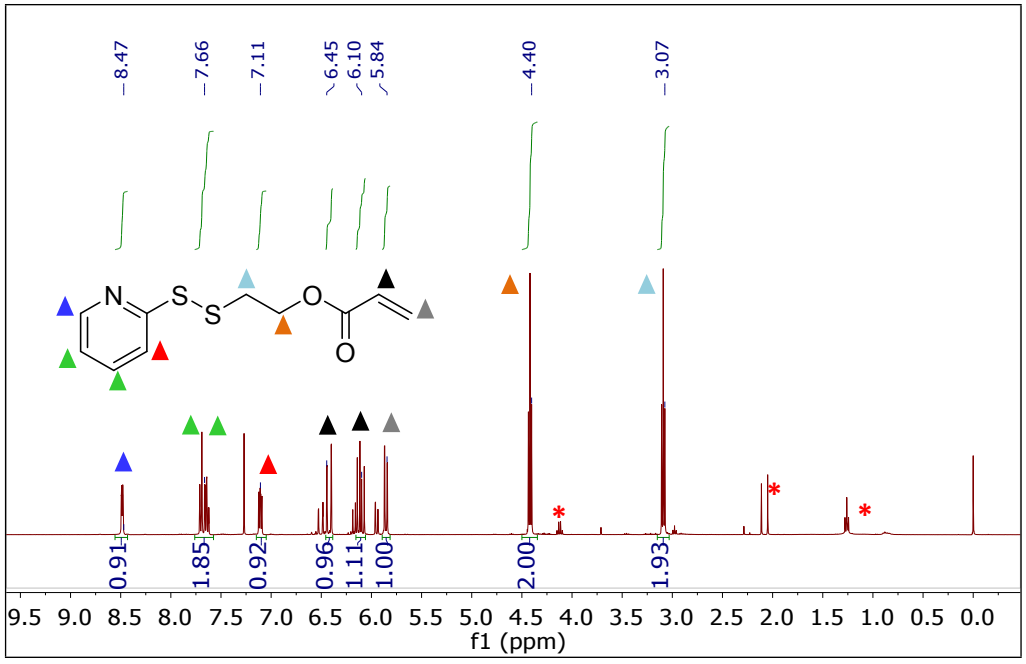

Figure S1. ^{1}H NMR spectrum of pyridyl disulfide ethylacrylate (PDSEA). (* indicates ethyl acetate solvent).

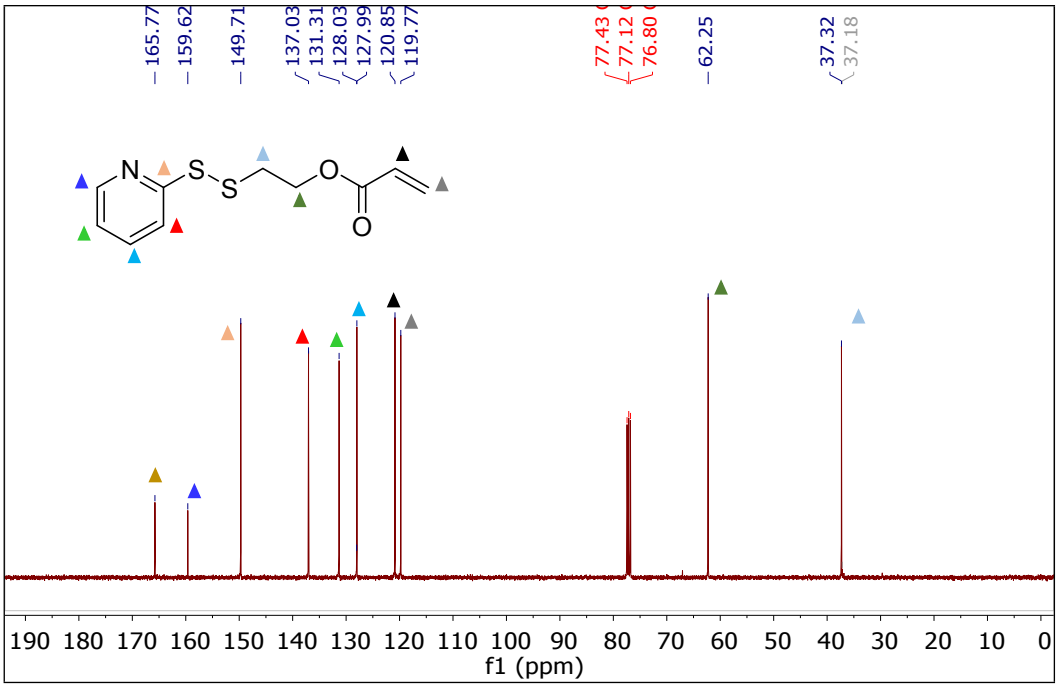

Figure S2. ^{13}C NMR spectrum of PDSEA

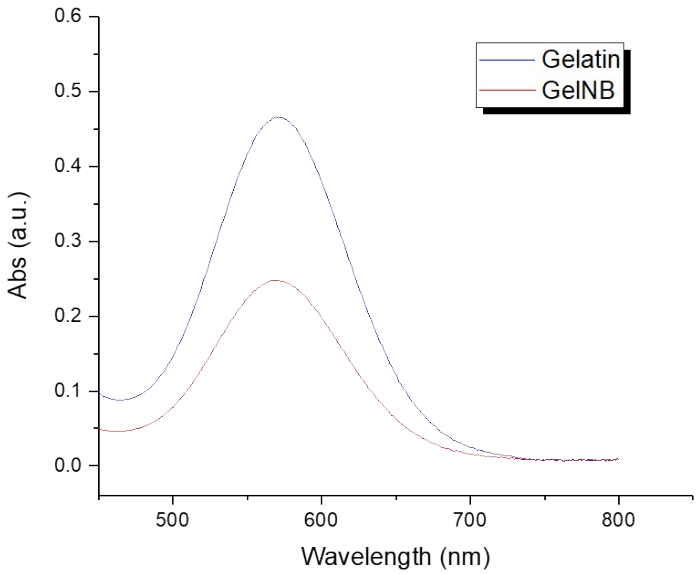

Figure S3. UV-VIS absorbance curves for pure gelatin and GelNB (Amine concentrations were determined using a calibration plot where L-Glycine was used as the primary standard[1]).

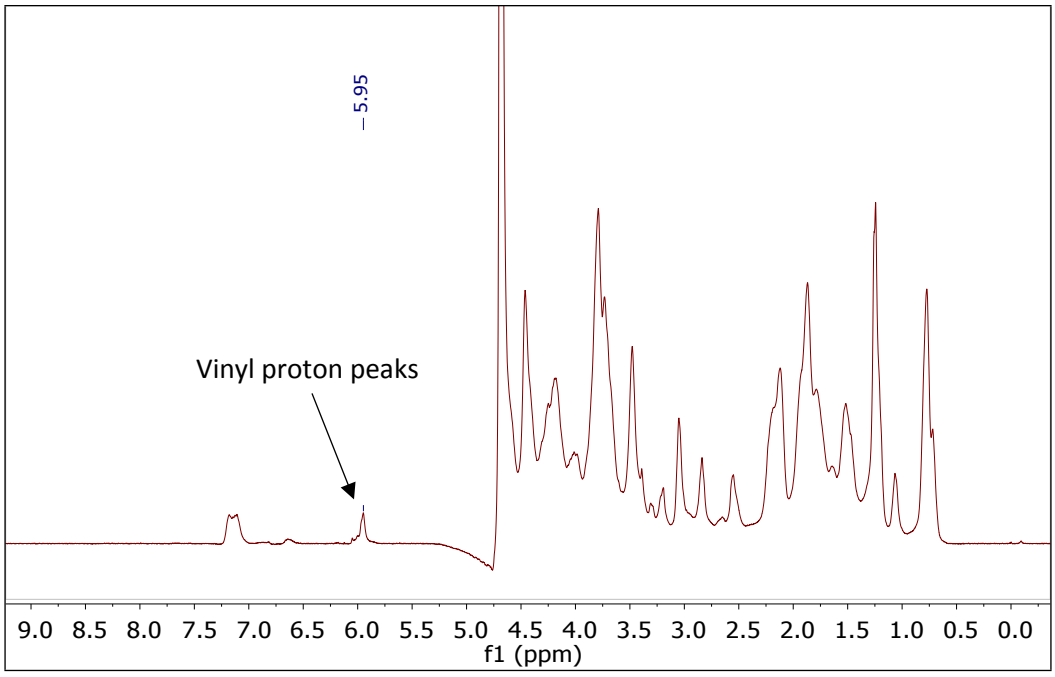

Figure S4. [1]H NMR spectrum of norbornene modified gelatin (GelNB).

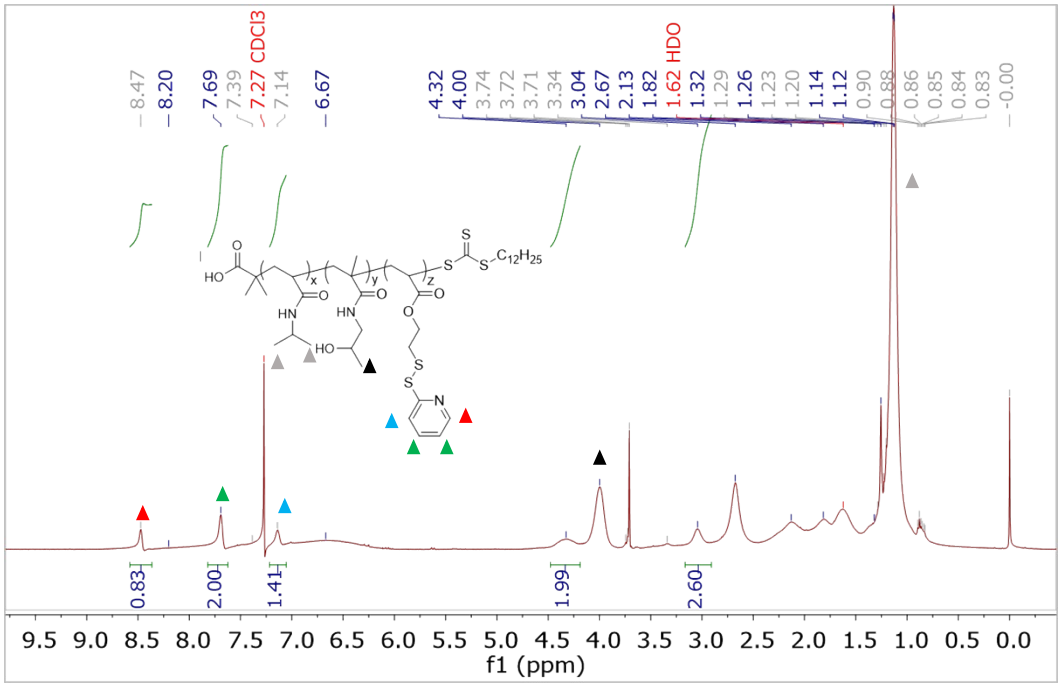

Figure S5. [1]H NMR spectrum of Poly(NIPAAm-*s*-HPMA-*s*-PDSEA)

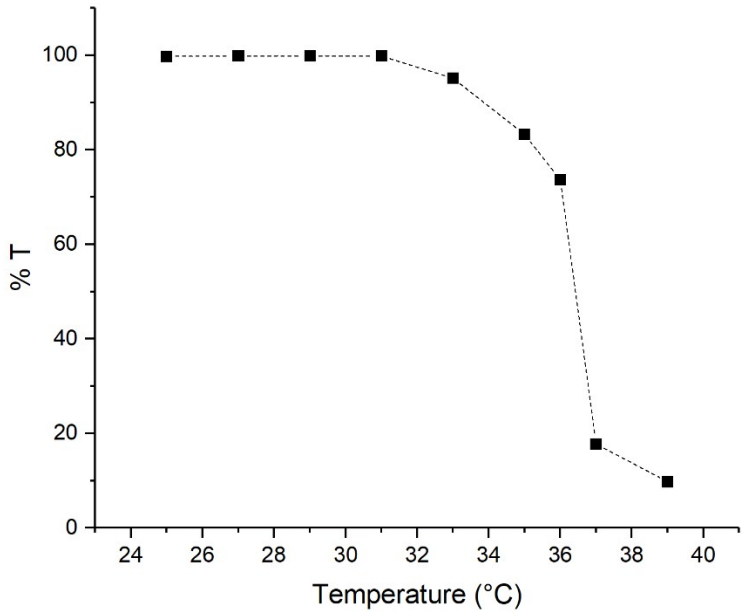

Figure S6. % T (Transmittance) of an aqueous solution of Poly(NIPAAm-*s*-HPMA-*s*-PDSEA) at different temperatures (LCST determination of Poly(NIPAAm-*s*-HPMA-*s*-PDSEA))

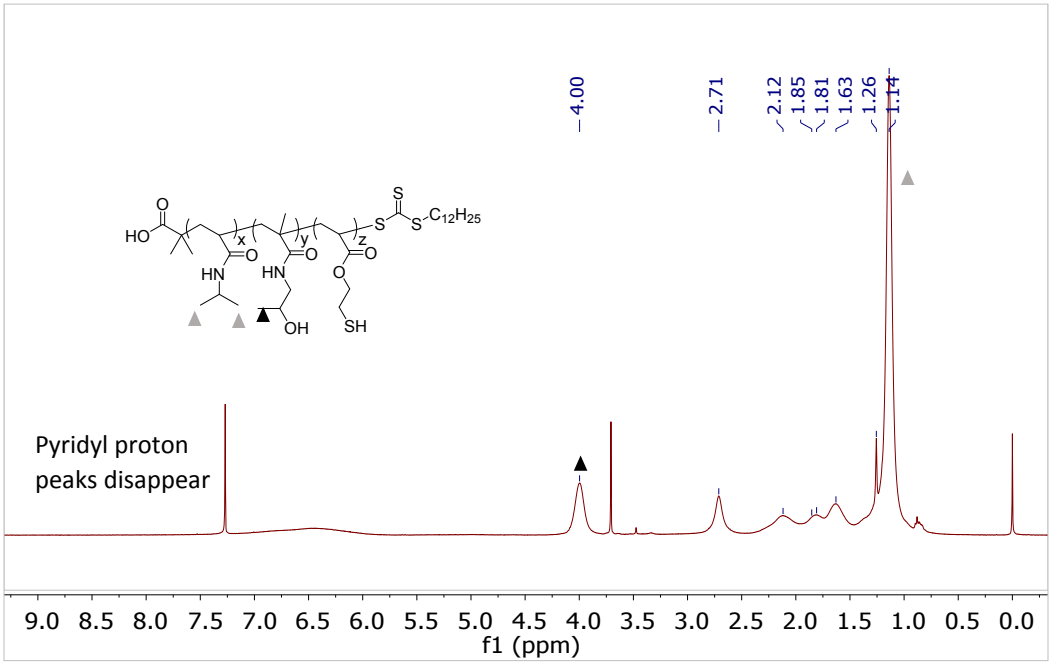

Figure S7. ^{1}H NMR spectrum of Poly(NIPAAm-*s*-HPMA-*s*-MEA)

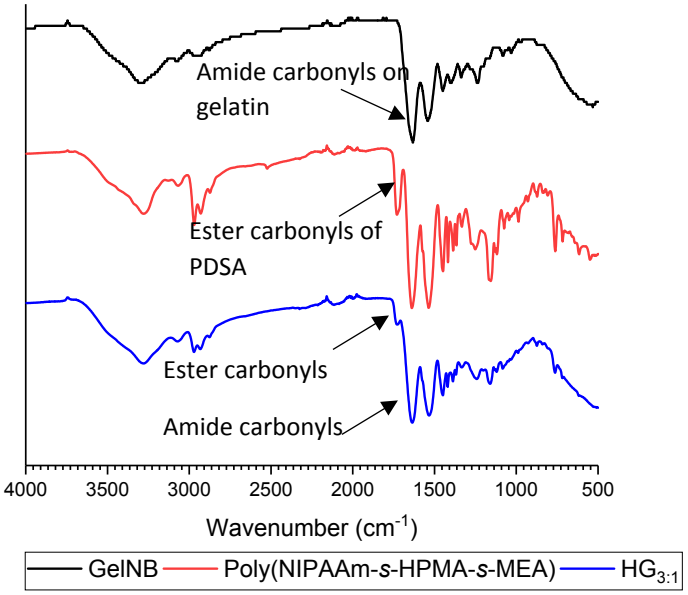

Figure S8. FTIR spectra of GelNB, poly(NIPAAm-s-HPMA-s-MEA) and a dried hydrogel $TE_{3:1}$.

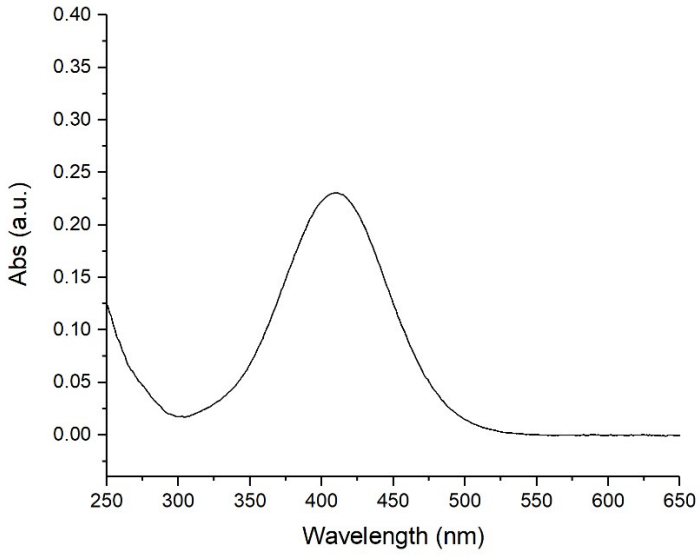

Figure S9. UV-VIS absorbance curve for poly(NIPAAm-*s*-HPMA-*s*-MEA) (Amine concentrations were determined using a calibration plot where L-Glycine was used as the primary standard[1]).

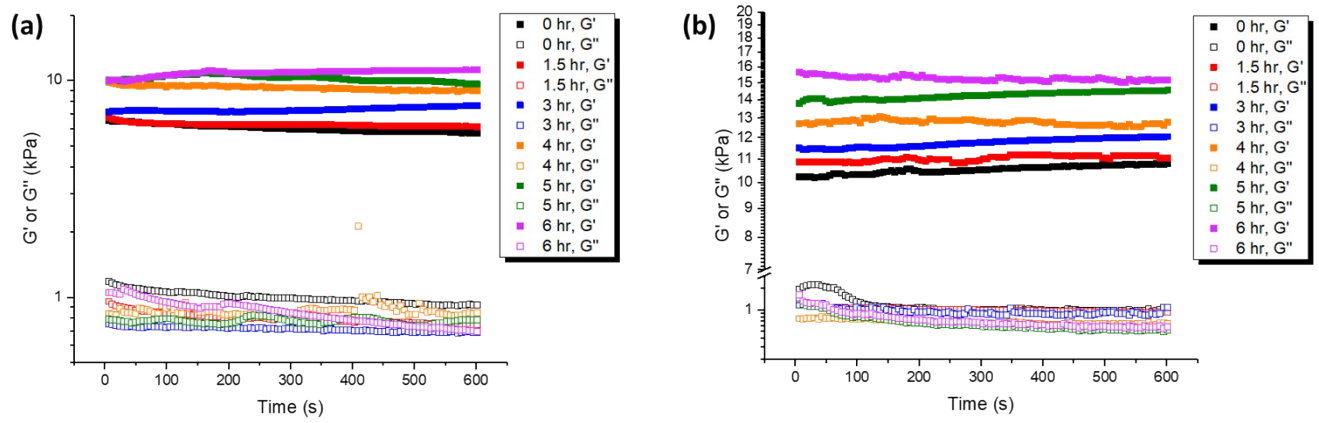

Figure S10. Time sweeps of (a) $TE_{2:1}$ and (b) $TE_{3:1}$ hydrogels after initial synthesis and swelling at room temperature in PBS for different times. Closed symbols = Storage modulus (G'); Open symbols = Loss modulus (G").

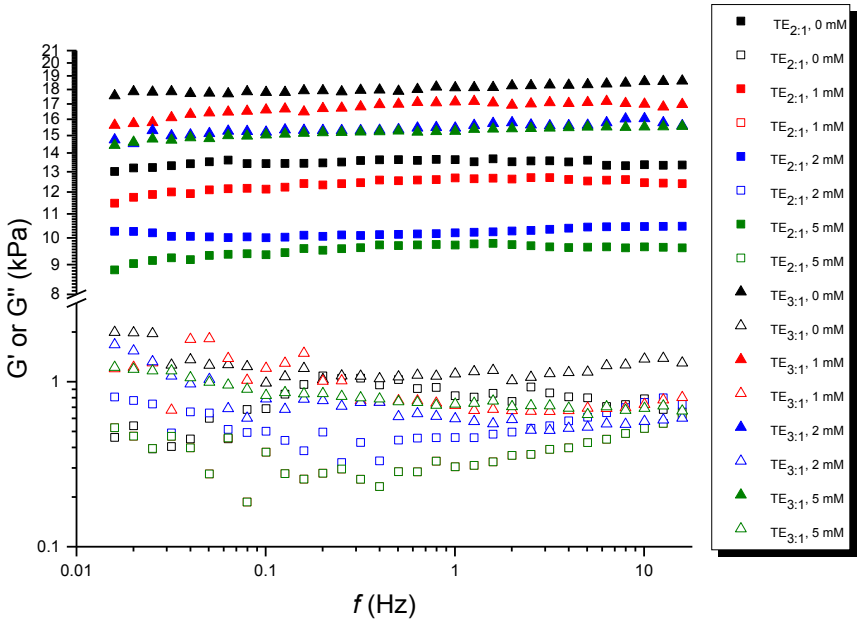

Figure S11. Frequency sweeps of hydrogels softened using different concentrations of L-cysteine. Closed symbols = Storage modulus (G'); Open symbols = Loss modulus (G").

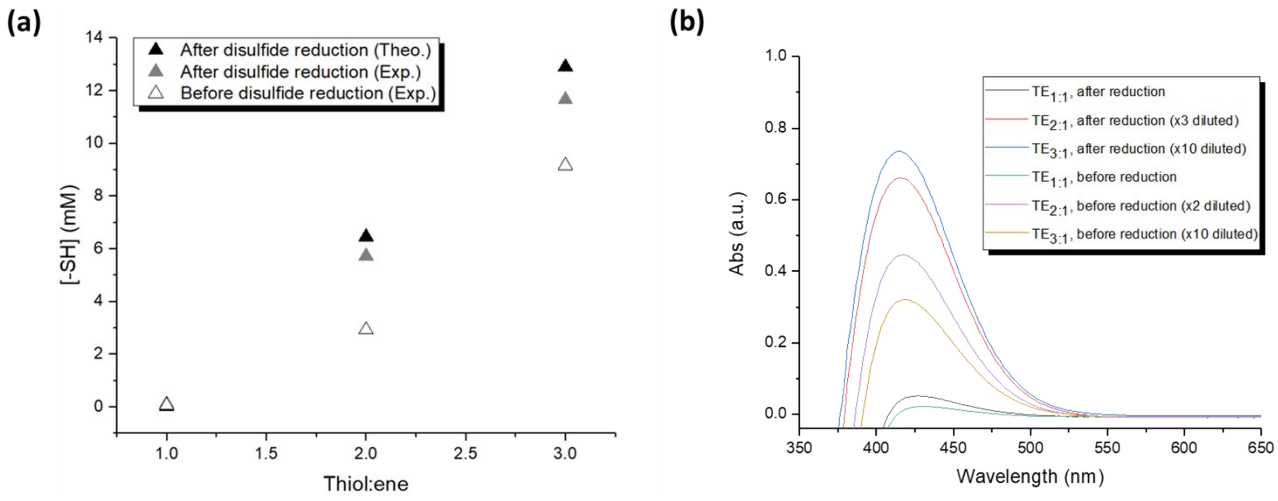

Figure S12. (a) Thiol concentration in hydrogels with disulfide crosslinks and in hydrogels after reducing the disulfide crosslinks with TCEP. (b) UV-VIS absorbance curves for non-injected hydrogel films

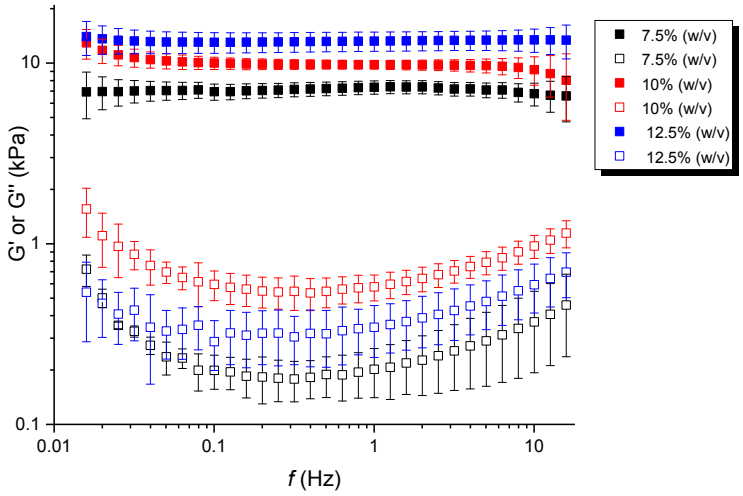

Figure S13. Rheological characterization of the 1:1 thiol:ene hydrogels with different total solid contents using frequency (f) sweep measurements at 38˚C. Closed symbols = G' and open symbols = G" (Loss modulus) of the hydrogels. Three replicates were used for statistical analysis.

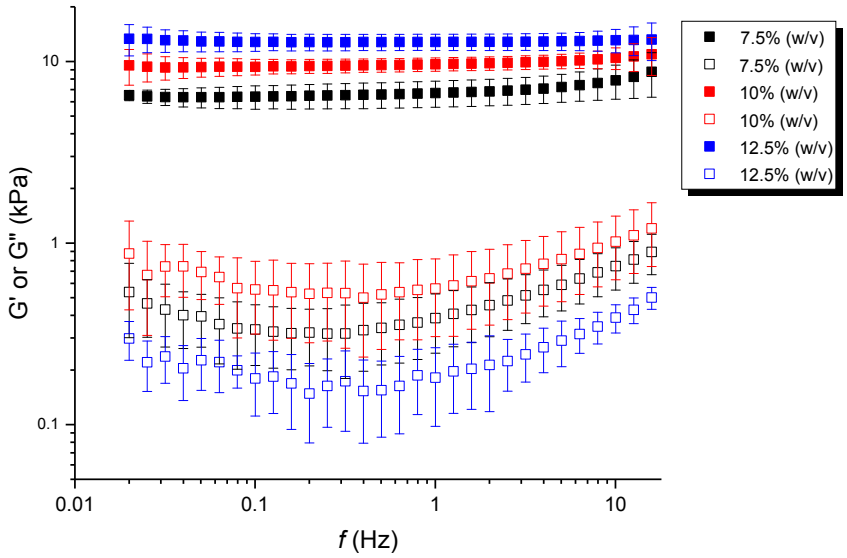

Figure S14. Frequency sweep (f) rheological characterization of the 1:1 thiol:ene hydrogels with different total solid contents after swelling against 5 mM L-cysteine. Closed symbols = G' and open symbols = G" (Loss modulus) of the hydrogels. Rheology was performed at 38˚C. Three replicates were used for statistical analysis.

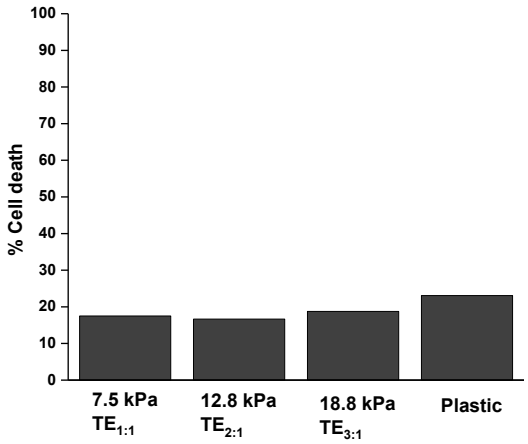

Figure S15. Viability of cells cultured on hydrogels and plastic after 7 days of culture.

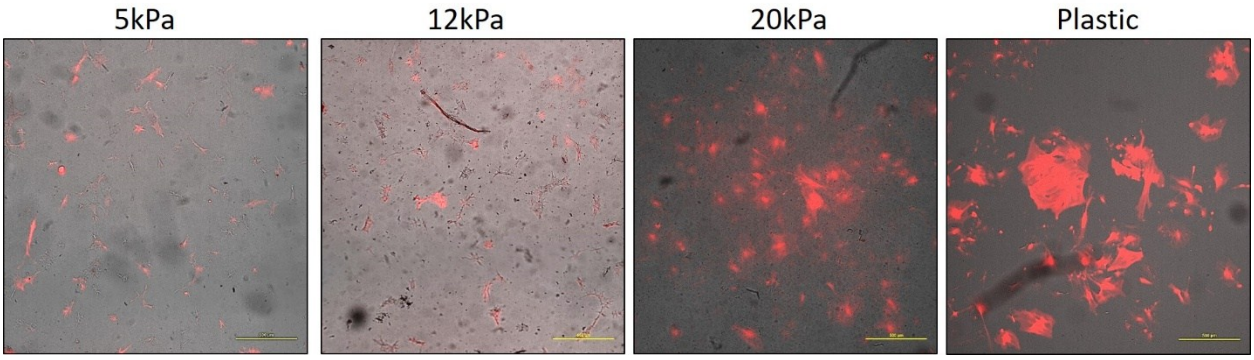

Figure S16. Cell morphologies of αSMA-RFP CFs following 7 days of culture on varying plating substrates. αSMA expression level indicated by red fluorescence.

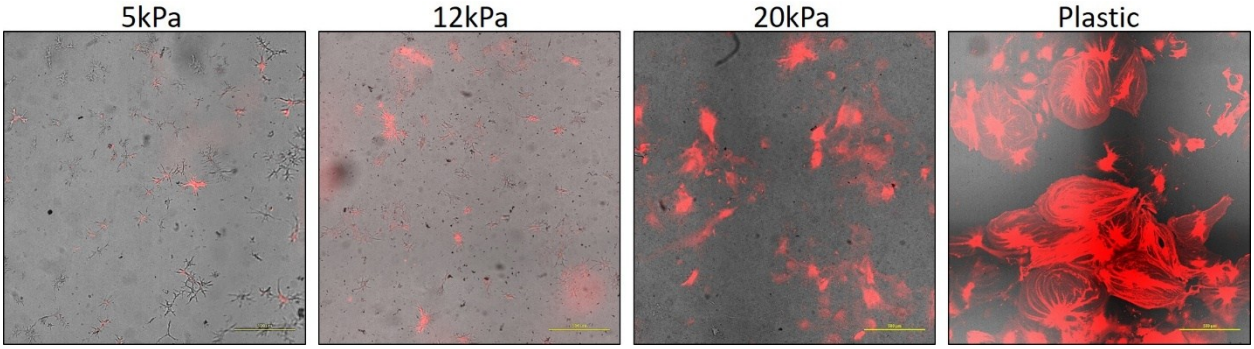

Figure S17. Representative images of αSMA-RFP CFs at 14 days of culture without treatment. Red cells indicate αSMA expression.

Supplementary Tables

Table S1. Formulations of the hydrogels

$TE_{X:Y}$[a]	Mass of copolymer (mg)	Mass of GelNB/ (mg)	Mass of PI[b]/ (mg)	Mass of TCEP/ (mg)
$TE_{1:1}$	74	250	3.3	10.2
$TE_{2:1}$	151	250	4.2	19.8
$TE_{3:1}$	217	250	4.7	30.2

[a]$TE_{X:Y}$= Thiol:Ene, ratio in the reaction mixture, [b]Photoinitiator (Irgacure 2959)

Table S2. Statistical analysis of cardiac fibroblast area after 7 days of culture on static stiffness hydrogel using one way ANOVA and Tukey's post hoc.

Stiffness comparison	Mean Diff.	95.00% CI[a] of difference	Adjusted P Value
5kPa vs. 12kPa	-14659	-24063 to -5256	0.0009
5kPa vs. 20kPa	-32475	-41219 to -23731	<0.0001
5kPa vs. Plastic	-44484	-54132 to -34836	<0.0001
12kPa vs. 20kPa	-17816	-26290 to -9342	<0.0001
12kPa vs. Plastic	-29825	-39229 to -20422	<0.0001
20kPa vs. Plastic	-12010	-20754 to -3265	0.0038

[a] Confidence Interval

Table S3. Statistical analysis of cardiac fibroblast %αSMA positivity after 7 days of culture on static stiffness hydrogel using one way ANOVA and Tukey's post hoc.

Stiffness comparison	Mean Diff.	95.00% CI of difference	Adjusted P Value
5kPa vs. 12kPa	-11.58	-20.47 to -2.694	0.0064
5kPa vs. 20kPa	-18.23	-25.70 to -10.76	<0.0001
5kPa vs. Plastic	-23.19	-31.48 to -14.91	<0.0001
12kPa vs. 20kPa	-6.647	-15.00 to 1.701	0.1586
12kPa vs. Plastic	-11.61	-20.70 to -2.523	0.0077
20kPa vs. Plastic	-4.964	-12.67 to 2.741	0.3209

Table S4. Statistical analysis of cardiac fibroblast area after 14 days of culture on static stiffness hydrogel using one way ANOVA and Tukey's post hoc.

Stiffness comparison	Mean Diff.	95.00% CI of difference	Adjusted P Value
5kPa vs. 12kPa	-16634	-58316 to 25048	0.6159
5kPa vs. 20kPa	-88223	-127213 to -49233	0.0003
5kPa vs. Plastic	-123520	-165202 to -81838	<0.0001
12kPa vs. 20kPa	-71589	-110580 to -32599	0.0013
12kPa vs. Plastic	-106886	-148568 to -65204	0.0001
20kPa vs. Plastic	-35296	-74286 to 3694	0.078

Table S5. Statistical analysis of cardiac fibroblast %αSMA positivity after 14 days of culture on static stiffness hydrogel using one way ANOVA and Tukey's post hoc.

Stiffness comparison	Mean Diff.	95.00% CI of difference	Adjusted P Value
5kPa vs. 12kPa	-7.802	-34.13 to 18.53	0.7927
5kPa vs. 20kPa	-8.676	-33.31 to 15.96	0.6986
5kPa vs. Plastic	-19.33	-45.67 to 6.998	0.171
12kPa vs. 20kPa	-0.8735	-25.51 to 23.76	0.9995
12kPa vs. Plastic	-11.53	-37.87 to 14.80	0.5476
20kPa vs. Plastic	-10.66	-35.29 to 13.97	0.5567

Table S6. Statistical analyses of interactions between hydrogel stiffness and treatment conditions using Two way ANOVA with Sidak's Multiple Comparisons post hoc.

(a) Cell area

Source of Variation	P value	P value summary	Significant?
Interaction	0.0203	*	Yes
Stiffness	<0.0001	****	Yes
Treatment	0.0018	**	Yes

(b) αSMA

Source of Variation	P value	P value summary	Significant?
Interaction	0.9137	ns	No
Stiffness	<0.0001	****	Yes
Treatment	0.3454	ns	No

Table S7. Statistical analysis of cardiac fibroblast area after 14 days on hydrogel, with treatment at 7 days, using a two way ANOVA with Sidak's Multiple Comparison's post hoc.

Methionine vs. Cysteine

Hydrogel stiffness	Mean Diff.	95.00% CI of difference	Adjusted P Value
5kPa	3167	-25884 to 32218	0.9973
12kPa	4827	-24224 to 33878	0.9863
20kPa	39933	15876 to 63989	0.0007
Plastic	23201	-5850 to 52252	0.1565

Untreated vs. Cysteine

Hydrogel stiffness	Mean Diff.	95.00% CI of difference	Adjusted P Value
5kPa	7882	-18157 to 33921	0.8848
12kPa	-5941	-33778 to 21896	0.9645
20kPa	51593	28723 to 74464	<0.0001
Plastic	25768	-2069 to 53605	0.0769

Untreated vs. Methionine

Hydrogel stiffness	Mean Diff.	95.00% CI of difference	Adjusted P Value
5kPa	4715	-33695 to 43125	0.9955
12kPa	-10768	-46697 to 25161	0.8896
20kPa	11661	-19896 to 43217	0.7934
Plastic	2567	-33362 to 38496	0.9995

Table S8. Statistical analysis of cardiac fibroblast %αSMA positivity after 14 days on hydrogel, with treatment at 7 days, using two way ANOVA with Sidak's Multiple Comparison's post hoc.

Untreated vs. Methionine

Hydrogel stiffness	Mean Diff.	95.00% CI of difference	Adjusted P Value
5kPa	4.47	-14.59 to 23.53	0.9498
12kPa	-0.384	-19.44 to 18.67	>0.9999
20kPa	-4.598	-20.25 to 11.06	0.894
Plastic	-0.4825	-19.54 to 18.57	>0.9999

Untreated vs. Cysteine

Stiffness	Mean Diff.	95.00% CI of diff.	Adjusted P Value
5kPa	7.765	-13.73 to 29.27	0.8022
12kPa	5.332	-16.17 to 26.83	0.9392
20kPa	4.409	-13.26 to 22.07	0.9379
Plastic	-0.817	-22.32 to 20.68	>0.9999

Methionine vs. Cysteine

Stiffness	Mean Diff.	95.00% CI of diff.	Adjusted P Value
5kPa	3.295	-13.94 to 20.53	0.976
12kPa	5.716	-11.51 to 22.95	0.8473
20kPa	9.007	-4.340 to 22.35	0.2828
Plastic	-0.3345	-17.57 to 16.90	>0.9999

References

1. Perera, M. M.; Ayres, N., *Polym. Chem.* **2017,** *8* (44), 6741-6749.
2. Lai, J. T.; Filla, D.; Shea, R., *Macromolecules* **2002,** *35* (18), 6754-6756.
3. Remant Bahadur, K. C.; Thapa, B.; Xu, P., *Mol. Pharm.* **2012,** *9* (9), 2719-2729.
4. Jackson, A. W.; Fulton, D. A., *Macromolecules* **2012,** *45* (6), 2699-2708.