Supplementary Information

Mechanochemical functionalization of disulfide linked hydrogels

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Experimental Section

Materials: All materials including laboratory chemicals and reagents were purchased from Sigma unless otherwise noted. Tissue culture plastic (12 well plates) and glass coverslips (18 mm circular) were purchased from Fisher Scientific. Thiol-terminated four-armed poly(ethylene glycol) (PEG4SH, MW 5000) was purchased from JenKem. Cell culture media and 0.25% trypsin were purchased from Gibco. Rabbit anti-Paxillin was purchased from Abcam (ab32084) and Mouse anti-Vinculin was purchased from Sigma (V9131). Maleimide and fibrinogen fluorescein were purchased from Fisher Scientific (62245) and (F13191), respectively. Fluoresceinamine was purchased from Sigma (Fluka-32615)) 4,6-diamidino-2-phenylindole (DAPI), Alexa488-phalloidin, Tetramethylrhodamine-conjugated anti-rabbit IgG antibody, and Alexa Fluor 647-conjugated anti-mouse IgG antibody and were purchased Invitrogen.

Preparation of hydrogels: Disulfide hydrogels were fabricated as previously described.²⁰ Briefly, 10 wt % solution of PEG-4SH (dissolved in D.I. water) was mixed with 30% hydrogen peroxide (1% final concentration) and sodium iodide (1mM concentration). To make bulk gels, the solution (100 µl was injected into a 1cc/1mL syringe of which front end was cut to make gels with a cylinder shape. After desired time, gels were gently taken out and put in D.I. water. To fabricate gels on a cover slip, the solution (15 µl) was injected between a hydrophobic treated glass slide (bottom) and a 3-(Trimethoxysilyl)propyl methacrylate (TMSPM) treated coverslip (top, 10 min with TMSPM and then baked at 95°C for 2 h). After polymerization, gels were gently detached from the hydrophobic-treated glass and put in D.I. water. Poly(ethylene glycol) diacrylate (PEGDA) of average molecular weight 10,000 was synthesized as described previously²⁶. Briefly, dried PEG (Sigma-Aldrich) in dissolved in dichloromethane was reacted with a 3x equivalence of acryloyl chloride and trimethylamine overnight at room temperature. Potassium carbonate was then added for 1.5 hours. The PEGDA was filtered, extracted with cold ether and lyophilized. PEGDA hydrogels were formed from 10% PEGDA solutions using 0.05% 2-Hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone as a UV activated initiator. The gels were formed as described above under ~ 5 mW/cm² UV crosslinker (Spectronics) for 10 minutes. For combinations of PEGDA and PEG-4SH, the 10%

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PEGDA & PEG-4SH solution were mixed at different ratios and initiators were added. The mixtures were gelled in the UV crosslinker for 10 minutes and then left for 2 hours to gel.

Gel density measurement: Gels were dried and then the dry gel densities were measured in weighting bottles with hexane (nonpolar solvent)²⁷. Briefly, the empty bottle weight (m_o) and the weight of the bottle filled with the solvent (m_1), dry gel (m_s), or both solvent and dry gel together (m_2) were measured. The density of dry gels was calculated with the following equation:

$$\rho_s = \frac{m_s}{m_1 - m_2 + m_s} \rho_L$$

where ρ_S and ρ_L are the dry gel density and the solvent density, respectively.

Gel swelling measurement: Gels were dried in a vacuum oven for 48 h and the weight of the dried gels (W_o) was measured. The dry gels were immersed in D.I. water at room temperature (~ 22°C) until swelling equilibrium was attained. The weight of swelling-saturated gels (W_t) was determined after the removal of the surface water. The swelling ratio was calculated with the following equation:

Swelling ratio =
$$\frac{W_t - W_0}{W_0}$$

The swelling ratios of all the prepared hydrogels were measured and compared.

Compression Testing: For 3D bulk gels, DMA tests were performed using RSA 3 (TA instrument) with parallel plates of 25 mm in diameter. Prepared gel specimens were cut to the size of 5.5 – 6.5 mm in diameter and 2.5 – 4.5 mm in length and placed on the bottom plate, and the top plate was closed until the preload of 2 g was applied. Quasi-static compression was applied to the specimen with the ramping rates of 1 – 50 µm /sec until failure. The buckling of specimen was carefully controlled during the compression to avoid any premature failure. For 2D gels fabricated on cover slips, compression was performed with patterned or non-patterned polydimethylsiloxane (PDMS, Polysciences, Inc.) stamps. To generate patterned surfaces, PDMS stamps were made by polymerization upon a patterned master of photoresist (SU-8, MicroChem) fabricated using UV photolithography through a laser printed mask. Maleimide and acrylate-

fibrinogen fluorescein were employed to conjugate maleamide or acrylate groups to broken disulfide bonds in the gels (For cell culture, 25 µg/ml of acrylate fibronectin in PBS was used). Acrylation of fibrinogen was performed by conjugation of pendant amines with NHS-acrylate (10:1 ratio) in carbonate buffer of pH 9 with 1M NaCl for 4 hours²⁸. Diluted Maleimide or acrylate-fibrinogen fluorescein in D.I. water (or acrylate fibronectin in PBS) were pipetted onto patterned or non-patterned stamps for 30 min and dried with air. Fluorescein or Proteins were transferred from stamps to the surface of gels by micro-contact printing with compression (over 50 g for 30 min). Patterned gels were rinsed at least ten times and left for 1 day for fluorescence imaging or cell cultures.

Tensile strain testing: Disulfide hydrogels were fabricated on Au sputtered PDMS as described above. PDMS were fabricated with mixing Sylgard 184 silicone base and curing agent in a 10:1 ratio by weight. The mixture was degassed to remove any bubbles in vacuum for 2 hours and poured over a flat Petri dish. The PDMS was then baked at 75°C for 3 hours. After cooling, the PDMS was easily peeled and cut to 2 cm (length) x 1cm (width). Au was sputtered onto the exposed central region of 1 cm x 1 cm (Figure S4). The gels on the Au-sputtered PDMS were stretched in a uniaxial direction by 0-40% strain.

Fluorescence imaging and Image Analysis after compression test: Immunofluorescence microscopy was conducted using a Zeiss Axiovert 200 M inverted research-grade microscope (Carl Zeiss, Inc.) or an LSM 700 (Carl Zeiss, Inc.) which is a four laser point scanning confocal with a single pinhole. Immunofluorescent images from the immunofluorescence microscopy or the LSM 700 were analyzed using ImageJ; the fluorescence intensities of compressed (0-40%) gels were measured to compare different levels of fluorescence depending on the degree of compression. All results were confirmed at least three times. The relative intensity of the fluorescence was determined by comparing each intensity value to the average intensity of zero compression.

Modeling of disulfide gel compression: The disulfide gel model was implemented as a vumat (user defined material) within a finite element framework (Abaqus). The material was modeled based on our previous work on elastomers²⁹ with the simplification of assuming entirely chemical crosslinks (no network slip

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allowed). The disulfide disassociation was modeled according to transition state theory assuming a linear decrease in the energy barrier with force. The percent of intact disulfide bonds along a particular orientation was taken to directly determine the percent of intact polymer chains along that orientation. The initial crosslink density was fit to the mechanical response of the gel under monotonic compression. As is standard in the literature the gel is assumed to be nearly incompressible. The disulfide bond energetic parameters were then fit to the monotonic compression data at three strain rates. The percent of broken disulfides for the experiments with the 30s hold at constant strain (stress relaxation) were then predicted from these monotonic parameters.

Cell source and culture: Human MSCs isolated from bone marrow were purchased from Lonza. Cells were tested for positive expression of CD₂₉, CD₄₄, CD₁₀₅, and CD₁₆₆ and negative expression of CD₁₄, CD₃₄, and CD₄₅ by flow cytometry (<u>http://www.lonza.com</u>). Mouse embryonic fibroblasts (MEFs) and murine B₁₆ melanoma cancer cell lines (B₁₆Fo and B₁₆F₁₀) were purchased from ATCC. Purchased cells were cultured and then expanded. Cells were frozen in cryopreservation media (10% DMSO in Dulbecco's Modified Eagle's Medium (DMEM)) with passage 2 and thawed and cultured in low glucose DMEM (1 g/mL) for MSCs or high glucose for MEFs and B₁₆S media supplemented with 10% fetal bovine serum (Invitrogen), and 1% penicillin/streptomycin. Cells were passaged around 80-90% confluency. Media was changed every 3 or 4 days and cells were fixed after cultured for 5 days.

Immunocytochemistry: Cells cultured on the patterned or non-patterned surfaces for 5 days were fixed with 4% paraformaldehyde (Alfa Aesar) for 20 min. 0.1% Triton X-100 in PBS was used for 30 min to permeabilize cells and these cells were blocked with 1% bovine serum albumin (BSA) for 15 min and then labeled with primary antibodies in 1% BSA in PBS (1:500 dilution) for 2h at room temperature (~20 °C). Surfaces were rinsed three times and then secondary antibody labeling was conducted in 2% goat serum contained 1% BSA in PBS for 20 min at a humid incubator (5% CO₂ and 37 °C) with 4,6-diamidino-2-phenylindole (DAPI, 1:2500 dilution), Alexa488-phalloidin (1:200 dilution), Tetramethylrhodamine-conjugated anti-rabbit IgG antibody (1:200 dilution), and Alexa Fluor 647-conjugated anti-mouse IgG antibody (1:200 dilution). Surfaces were rinsed three times and then mounted on a glass slide to conduct immunofluorescence

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microscopy by using a Zeiss Axiovert 200M inverted research-grade microscope (Carl Zeiss, Inc.). ImageJ

was employed to count cell numbers and measure cell area.

Statistical analysis: Error bars represent standard deviation (SD) and data was obtained from 3 independent

experiments unless otherwise specified. For statistical analysis, two-tailed p-values from unpaired t-test for

comparing two groups were employed and values of P < 0.05 were considered statistically significant.

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Gelation Time	5% S-S Density (g/ml)	10% S-S Density (g/ml)	15% S-S Density (g/ml)	20% S-S Density (g/ml)
1h	1.26 ±0.2	1.35 ±0.2	-	-
2h	1.36 ±0.1	1.53 ±0.4	1.53 ±0.4	1.57 ±0.1
3h	1.39 ±0.2	1.57 ±0.2	1.57 ±0.2	1.62 ±0.3
4h	1.42 ±0.2	1.58 ±0.2	1.58 ±0.2	1.62 ±0.1

Table S1. Density of different concentrations (5-20%) of disulfide hydrogels with different gelation time.

Gelation Time	5% S-S Swelling (m/m₀)	10% S-S Swelling (m/m₀)	15% S-S Swelling (m/m₀)	20% S-S Swelling (m/m₀)
1h	17.73 ±0.7	36.18 ±0.5	-	-
2h	17.32 ±0.3	10.28 ±1.2	12.36 ±0.2	12.36 ±0.2
3h	18.25 ±0.6	10.44 ±0.6	10.43 ±0.5	10.43 ±0.5
4h	19.03 ±0.3	9.01 ±0.2	10.99 ±0.4	10.99 ±0.4
				- gelation

Table S2. Swelling of (5-20%) disulfide hydrogels with different gelation time.



Figure S1. Swelling properties of polymer blend hydrogels. (a) Swelling ratio of the gels with different concentrations of 4arm-PEG-thiols (gelation for 3 h) and different portion of PEGDA and 4arm-PEG-thiols. (b) Representative laser scanning confocal microscope images and fluorescence intensity of fluoresceine-5-maleimide across different swelling ratios.



Figure S2. Simulation of polymer chain force for disulfide hydrogels to predict the force-strain required for disulfide bond rupture.



Figure S3. Nuclear magnetic resonance spectroscopy (NMR) confirmation of the kinetics of crosslinking with the pure and mixed gels to assess the formation of thioethers via Michael addition at different time points. NMR spectra were obtained with the polymers in D20. Spectra were referenced with the D2O peak as 4.67ppm. Spectra was obtained for mixing of the polymers at various timepoints from the same tube and normalized to total area.



Figure S4. Plot of the stress-strain curve for the gels with different portion of PEGDA and 4arm-PEG-thiols and different molecular weight of 4arm-PEG-thiols.



Figure S5. (a) Simulation of the stress-strain curve for the disulfide gels with different degree of cross-linking in the dry polymer without changing the volume fraction of water in the hydrated. (b) Average failure strain (N=5) for the gels with different molecular weight (5,000 & 10,000 Da) of 4arm-PEG-thiols.



50 ul

Figure S6. Tension initiates disulfide bond rupture in hydrogels. (a) Schematic illustrating the process of fabricating disulfide gels on PDMS sputtered with Au and application of tensile stress. Representative optical images of gels on the device. (b) Fluorescence intensity of fluoresceine-5-maleimide during tensile testing with accompanying simulation (c) representative laser scanning confocal microscope images of disulfide hydrogels across degrees of tensile strain.



Figure S7. Ruptured disulfide bonds via compression lead to chemical reaction. (a) Representative immunofluorescence microscopy images of patterns through compression with fluorescein-5-maleimide and fluoresceinamine for 30 min. (b) Immunofluorescence images of gels with or without treatment of Triton X-100 after compression with acrylate fibrinogen fluorescein.