Electronic Supplementary Information (ESI)

Ultrasound-triggered hydrogel formation through thiol-norbornene reaction

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Section 1. Experimental Section

1.1 Materials.

Dextran (Mw ca. 75,000) was purchased from Alfa Aesar. Dithiothreitol (DTT) was purchased from Fluorochem. Sodium chloride (NaCl) was purchased from SHOWA. Potassium persulfate (KPS) (99+%) and carbic anhydride (99+%) were purchased from Acros Organics. Phosphate-buffered saline (PBS) buffer was purchased by Bioman Scientific CO, LTD. Dialysis membranes with the molecular weight cut off (MWCO) of 12-14 kDa were purchased from Cellu·Sep®. Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), and alamarBlue assay were purchased from ThermoFisher.

1.2 Preparation of Ultrasound-triggered hydrogel.

Synthesis and characterization of norbornene-functionalized dextran (Nor-Dex).

The norbornene-functionalized dextran was synthesized according to the literature with some modification.[1] In general, dextran (1 g) was dissolved in deionized water (100 mL) to obtain dextran solution with 1 wt% under stirring. NaOH (10 M) was used to adjust the dextran solution to pH 12, carbic anhydride (6.00 g, 36.5 mmol) was added to the solution, and the reaction was carried at room temperature. During the reaction, the solution was maintained the pH between 9.5-10.5 by adding NaOH (10 M) dropwise. (**Scheme S1**) After all of the carbic anhydrides were dissolved, NaCl (3 g, 51.3 mmol) was added to the solution with vigorous stirring for 15 mins to enhance separation. The solution was added dropwise to ice-chilled acetone (1 L) for at least one hour to improve precipitation. The white precipitant was separated through filtration and re-dissolved in deionized water (100 mL). The norbornene-functionalized dextran solution was dialyzed using a dialysis membrane (Cellu. Sep T1 dialysis membrane, MWCO 3.5 kDa, MFPI, USA) for three days, and then lyophilized to obtain the final product. The final yield of the Nor-Dex was about 90%. The chemical structure of Nor-Dex was characterized using proton nuclear magnetic resonance (^{1}H NMR) spectroscopy (BRUKER AVIII 400 NMR) and Fourier-transform infrared spectroscopy (FTIR, PerkinElmer Spectrum Two).

Nor-Dex was characterized by ^{1}H NMR and FTIR spectroscopies. In the NMR spectrum, the board peaks at 3.4-4.1 ppm and 4.9 ppm were recognized as the polymeric backbone of dextran (**Fig. S1, ESI†**). The new peaks at 1.4 ppm and 3.0-3.3 ppm corresponded to the bridge protons and carboxyl-adjacent protons of carbic anhydride, and the signals at 6.1-6.3 ppm corresponded to the alkene protons of norbornene structure on carbic anhydride.[1] In the FTIR spectrum, the peaks of 1730 cm^{-1} and 1565 cm^{-1} were assigned to C=O stretching, and the peak at 1645 cm^{-1} was contributed from the C=C stretching of norbornene groups[2] (**Fig. S2, ESI†**).

Calculation of the modification percentage of norbornene in Nor-Dex. The alkene peaks from 6.1 ppm to 6.3 ppm ($I_{alkenes}$) and the main chain of dextran from about 3.4 ppm to 4.0 ppm ($I_{dextran}$) in the NMR spectrum of Nor-Dex were integrated and calculated based on the following equation. The norbornene functionalization of Nor-Dex was about 20%.

$$\frac{I_{alkenes}}{I_{dextran}} \times \frac{H_{dextran}}{H_{alkenes}} = \text{norbornene modification percentage of Nor-Dex}$$

Formation and characterization of ultrasound-triggered Nor-Dex hydrogels. The solutions of Nor-Dex (10 wt%), DTT (thiol/norbornene ratio *N*= 1), and KPS (1.5 wt%) were mixed homogeneously with pipetting and then transferred to a trimmed syringe. Upon the ultrasound treatment (120 Hz, 3 mins), the hydrogels were formed with a cylinder shape. The DTT-crosslinked Nor-Dex hydrogel was lyophilized for FTIR characterization. The characteristic peak of S-C stretching at 1385 cm^{-1} was observed in the FTIR spectra of Nor-Dex hydrogel,[3] indicating the Nor-Dex hydrogels were successfully formed by thiol-norbornene crosslinking under ultrasound irradiation (**Fig. S2**, **ESI†**).

Nor-Dex hydrogels were prepared with a fixed concentration of Nor-Dex (10 wt%, and the modification of norbornene was 20%), varied concentrations of DTT (thiol/norbornene ratios of 0.5, 0.75, 1, 1.25, and 1.5), and varied concentrations of KPS (0,0.5, 1.0 and 1.5 wt%) in phosphate-buffered saline (PBS) solution. The precursor solutions were placed in a syringe before treating with ultrasound irradiation. The ultrasound irradiation was carried out with different frequencies (40, 80, and 120 kHz), and the solutions were irradiated under ultrasound for different times (3, 5, 10, 30, and 60 mins) and temperatures (room temperature and 37 ^{o}C).

1.3 Characterization

Compression tests. Compression tests were carried out by a material testing system (AGS 500N, Shimadzu). The hydrogel samples were formed in a cylinder shape with a height of ~4 mm and a diameter of ~4.7 mm. The operation speed was fixed at 0.5 mm/min. The compressive modulus of hydrogels was obtained by calculating the slope of the linear range (10%-20% strain) of the stress-strain curve. Three samples were measured for each condition to obtain the average compressive modulus of hydrogels. A summary of the hydrogel compositions used in the compression tests was shown in **Table S1**.

Table S1. Summary of the Nor-Dex hydrogel compositions used in the compression tests.

Tests [a]	KPS concentration (wt%)	thiol/norbornene ratios	Frequency (kHz)	Temperature (˚C)	Time (min)
Time dependence	1.5	1	120	R.T.	3, 5, 10, 30, 60, 120, 180
KPS concentration dependence	0, 0.5, 1, 1.5	1	120	R.T.	3
Frequency and temperature effects	1	1	40, 80, 120	R.T., 37 ˚C	3
thiol/norbornene ratios	1	0.5, 0.75,1, 1.25, 1.5	120	R.T.	3

[a] The Nor-Dex was fixed at 10 wt% in each test.

Swelling tests. The swelling ratio of the Nor-Dex hydrogels was measured through the gravimetric method. First, the hydrogels were frozen and lyophilized, and then recorded the dried weight (w_d). The freeze-dried hydrogels were immersed in the PBS at 37 ℃. At different times intervals, the weight of the wet samples (w_s) was obtained by removing the PBS solution on the surface. The swelling ratio and water content were determined by the following equations of **Eq. S1** and **Eq. S2**:

$$\text{Swelling ratio} = w_s/w_d \quad (\textbf{Eq. S1})$$

$$\text{Equilibrium water content} = [(w_s - w_d)/ w_s] \times 100\% \quad (\textbf{Eq. S2})$$

Degradation tests. The mass remaining ratio of the Nor-Dex hydrogels was also determined through the gravimetric method. In general, the lyophilized hydrogel samples (w_0) were immersed in PBS at 37℃. At specific time intervals, the PBS was removed from the hydrogels. The samples were frozen, lyophilized, and reweighted (w_2). The percentage of the degradation was calculated by the following equation (**Eq. S3**):

$$\text{Mass remaining (\%)} = w_2/w_0 \times 100\% \quad (\textbf{Eq. S3})$$

SEM images and pore size analysis. The Nor-Dex hydrogel samples were frozen and lyophilized overnight. The samples were sputter-coated with a layer of platinum before imaging. The microstructures of hydrogels were recorded using a scanning electron microscope (SEM, Hitachi TM-3000 tabletop SEM) under vacuum. Pore size analysis was performed with Image J with 30 different pores from each sample.

Radical Dosimetry. The radical amounts in the solution were quantified using terephthalic acid.[4] In general, terephthalic acid solution (0.2 mM, 0.5 mL) was mixed with KPS solution (1.75 mM, 0.5 mL) under continuous stirring. Samples were treated with ultrasound irradiation at different frequencies (40, 80, and 120 kHz) and

temperatures (R.T. and 37 °C). The fluorescent intensity of samples was measured by the microplate reader (BioTek Synergy H1), and the excitation wavelength was fixed at 310 nm to obtain the fluorescence range from 350 nm to 550 nm.

Free thiol detection through Ellman's assay. Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB) was used to determine the free thiols in the solution.[5] Herein, DTT (10 mM, 1000 µl) was mixed with KPS solution (0.617 M, 17.06 µl), and treated with ultrasound irradiation at different frequencies (40, 80, and 120 kHz) and temperatures (R.T. and 37 °C). Afterward, DNTB (2 mM, 100 µl) was added to the solution and mixed for 5 mins. The absorbance of the solution was measured by the microplate reader (BioTek Synergy H1) at 412 nm.

Effective crosslink density. The Nor-Dex hydrogels were swollen for 24 h at 37 °C, and the compressive modulus of hydrogels was measured using MTS. The effective crosslink density (ν_e) of hydrogels was calculated using **Eq. S4**[1] with ϕ_e was calculated using **Eq. S5**:

$$\nu_e = \frac{E}{3\phi_e^{\frac{1}{3}}RT} \quad \textbf{(Eq. S4)}$$

$$\phi_e = \frac{1}{1+\left(\frac{m_s-m_d}{m_d}\right)\frac{d_p}{d_{H_2O}}} \quad \textbf{(Eq. S5)}$$

where E is the compressive modulus, ϕ_e is the volume fraction of polymer calculated from the hydrogels after swelling at 37 °C for 24 h (m_s is the weight of the swollen hydrogel, m_d is the weight of dry hydrogel), the dextran density is denoted d_p (1.0373 g cm^{-3}), d_{H_2O} is the density of water (1 g cm^{-3}), R[a] and T[b] are the ideal gas constant and temperature in Kelvin, respectively. The numbers used for calculating the effective crosslink density of Nor-Dex hydrogels were summarized in **Table S2**.

Table S2. Summary of the numbers used for calculating the effective crosslink density of Nor-Dex hydrogels.

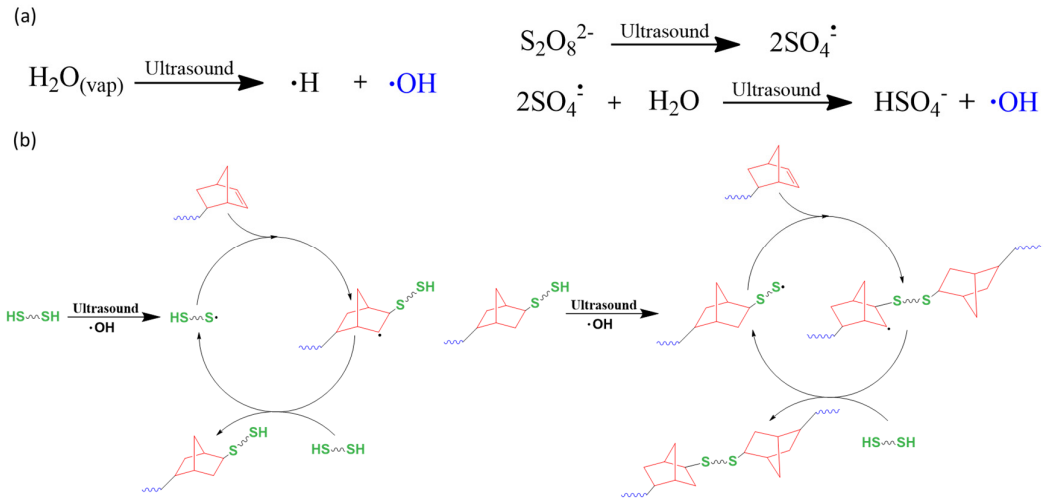
Frequency (kHz)	E (Pa)	Volume fraction (ϕ_e)
40	7.4 ± 0.42	0.15 ± 0.07
80	2301 ± 26.00	0.21 ± 0.04
120	1374 ± 235.99	0.25 ± 0.05
[a] Ideal gas constant (R) = 8.314 (J mol^{-1} K^{-1})		
[b] Temperature (T) = 310 (K)		

Cytocompatibility tests. Mouse embryo fibroblasts (MEFs) were harvested from C57BL/6N mice embryo and then immortalized. MEFs were incubated in the media (prepared by DMEM supplemented with 1% HEPES, 10% FBS, and 1% penicillin/streptomycin) at 37 ^{o}C under 5% CO_2 with the humidified condition.

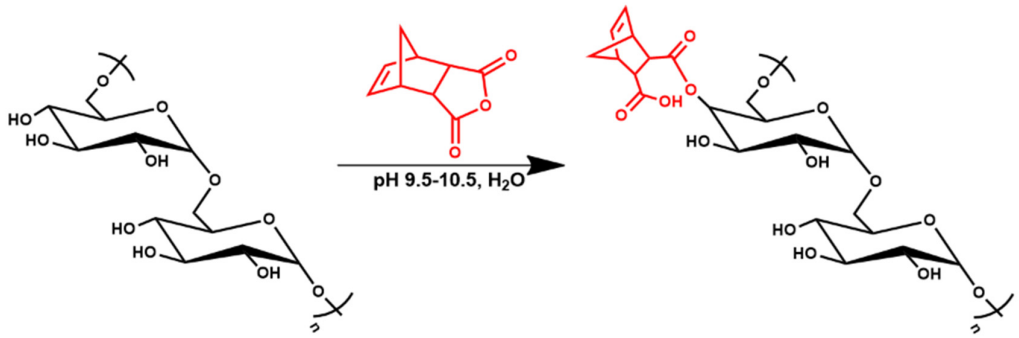
The Nor-Dex hydrogels were sterilized with UV irradiation for 1 h before immersing in the serum-containing DMEM for 5 days, where the volume ratio of hydrogel: media was 1: 10. The serum-containing DMEM included the degradation products of the Nor-Dex hydrogel was used for cell culture. The cytocompatibility of Nor-Dex/DTT hydrogel extracts was measured against MEFs by alamarBlue assay. MEFs were seeded in 96-well plates at a density of 3000 cells/well, and cultured with the hydrogel extracts. The metabolic activities of MEFs were evaluated by the alamarBlue assay. The fluorescence of samples was measured by the microplate reader (BioTek Synergy H1), and the excitation wavelength was fixed at 560 nm and emission of 590 nm. The values were normalized to readings at day 1, and the measurement was performed at least three replicates.

Statistical analysis. The standard deviation (SD) was the mean error of at least three independent experiments. Statistical analysis was computed with IBM® SPSS® Statistics version 25.0. The statistical significance between the group was analyzed with a one-way analysis of variance (ANOVA). Significance was taken to be $p< 0.05$ with *, **, or *** indicating $p< 0.05$, 0.01, or 0.001, respectively.

Section 2. Supporting Figures



Scheme S1. (a) Radicals generated from water vapor and KPS under ultrasound. (b) Nor-Dex hydrogel formation through the ultrasound-initiated thiol-norbornene reaction between Nor-Dex and DTT.



Scheme S2. Synthetic scheme of Nor-Dex.

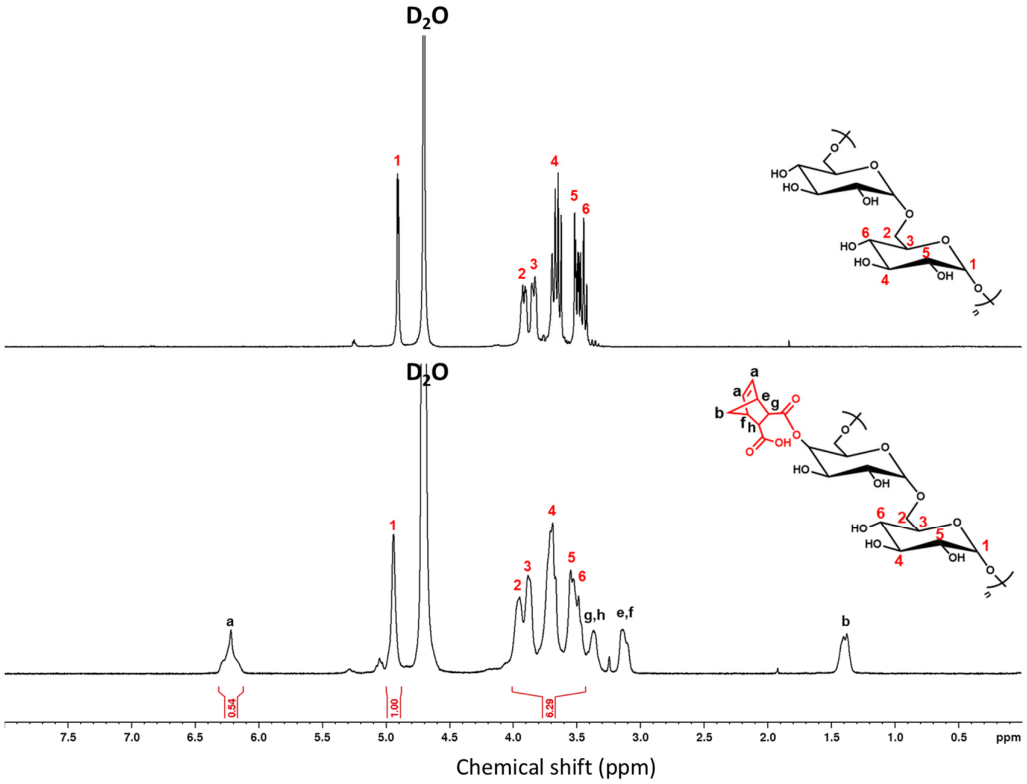


Fig. S1 ^{1}H NMR spectra of dextran and Nor-Dex.

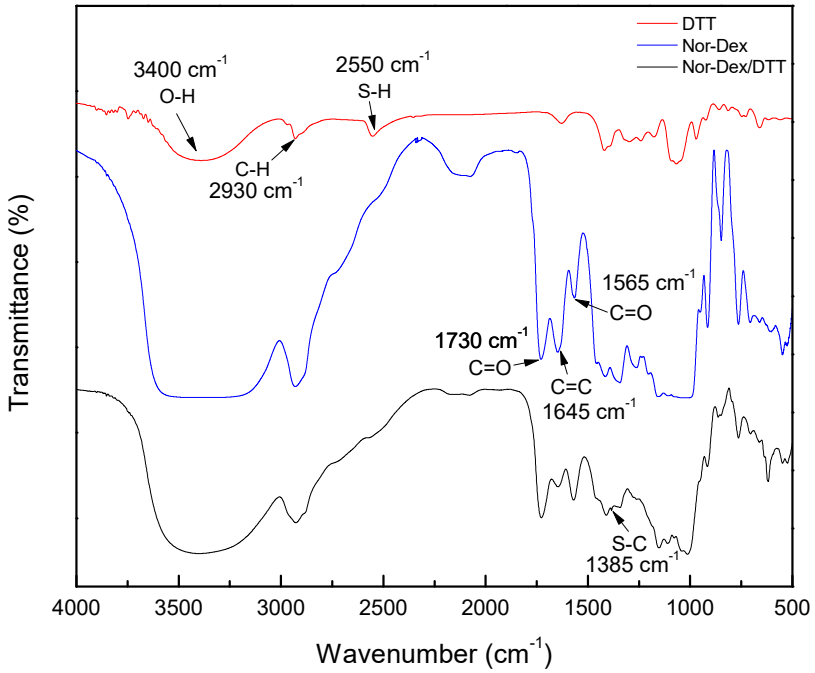


Fig. S2 FTIR spectra of DTT, Nor-Dex polymer, and Nor-Dex hydrogel crosslinked with DTT (Nor-Dex/DTT). Nor-Dex hydrogel was prepared using Nor-Dex (10 wt%), DTT (thiol/norbornene ratio *N*= 1), KPS (1.5 wt%), and 120 kHz for 3 mins at R.T.

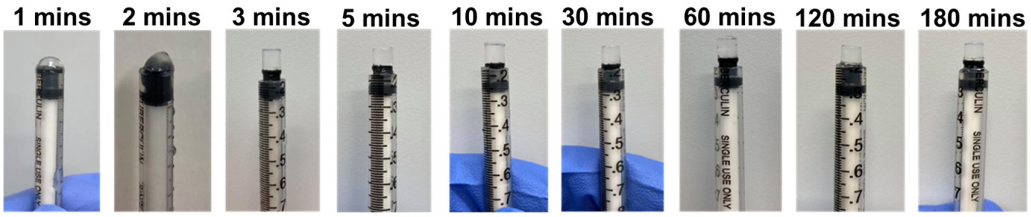


Fig. S3 Representative images of Nor-Dex hydrogels prepared with varied sonication times (Nor-Dex (10 wt%), DTT (thiol/norbornene ratio *N*= 1), KPS (1.5 wt%), and 120 kHz at R.T.).

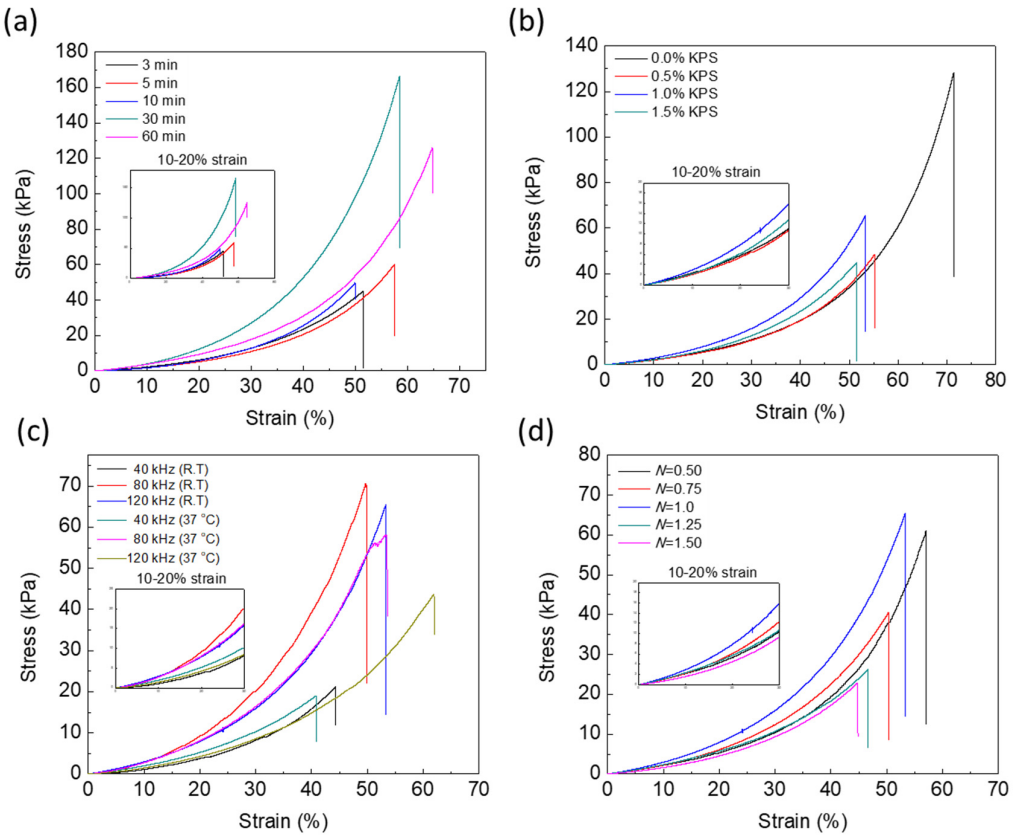


Fig. S4 Representative stress-strain curves of Nor-Dex hydrogels prepared with (a) varied sonication time (Nor-Dex (10 wt%), DTT (thiol/norbornene ratio *N*= 1), KPS (1.5 wt%), and 120 kHz at R.T.), (b) varied concentration of KPS (Nor-Dex (10 wt%), DTT (*N* =1), KPS (0, 0.5, 1, and 1.5 wt%), sonication time of 3 mins, and 120 kHz at R.T.), (c) varied frequency and temperature (Nor-Dex (10 wt%), DTT (*N* =1), KPS (1 wt%), and sonication time of 3 mins), and (d) varied thiol/norbornene ratio (Nor-Dex (10 wt%), DTT (*N*= 0.5, 0.75, 1, 1.25, and 1.5), KPS (1 wt%), sonication time of 3 mins, and 120 kHz at R.T.).

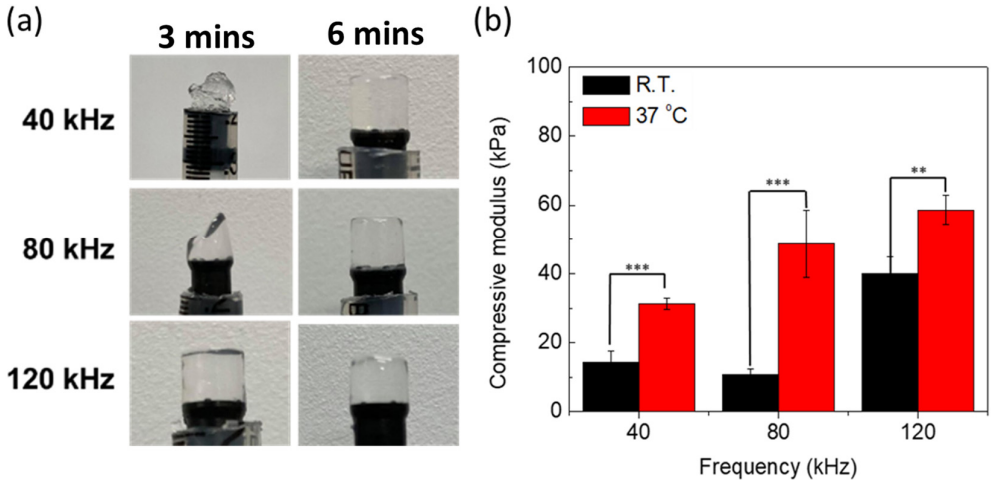


Fig. S5 (a) Representative images of Nor-Dex hydrogels prepared in the absence of KPS at different frequencies with varied sonication times (Nor-Dex (10 wt%), DTT (*N*= 1)). (b) Compressive moduli of Nor-Dex hydrogels prepared in the absence of KPS with varied frequencies and temperatures (Nor-Dex (10 wt%), DTT (*N*= 1), and sonication time of 6 mins). Significance was set at $p< 0.05$ with ** or *** indicating $p< 0.01$ or 0.001, respectively.

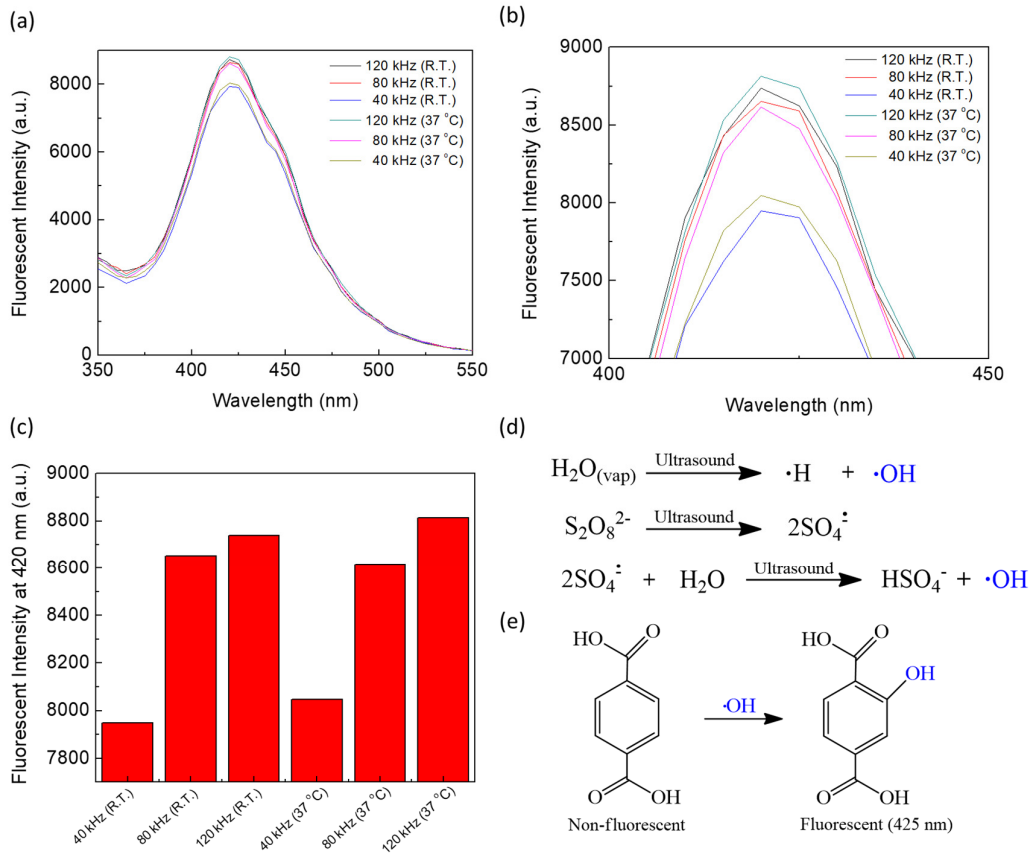


Fig. S6 Measurements of radical amounts in the solution. (a, b) Fluorescence spectra and (c) fluorescent intensity at 420 nm of the solutions of terephthalic acid and KPS mixtures under ultrasound irradiation at different frequencies and temperatures. Schematic illustrations of (d) radical formation under ultrasound irradiation and (e) reaction of hydroxyl radical (·OH) with terephthalic acid.

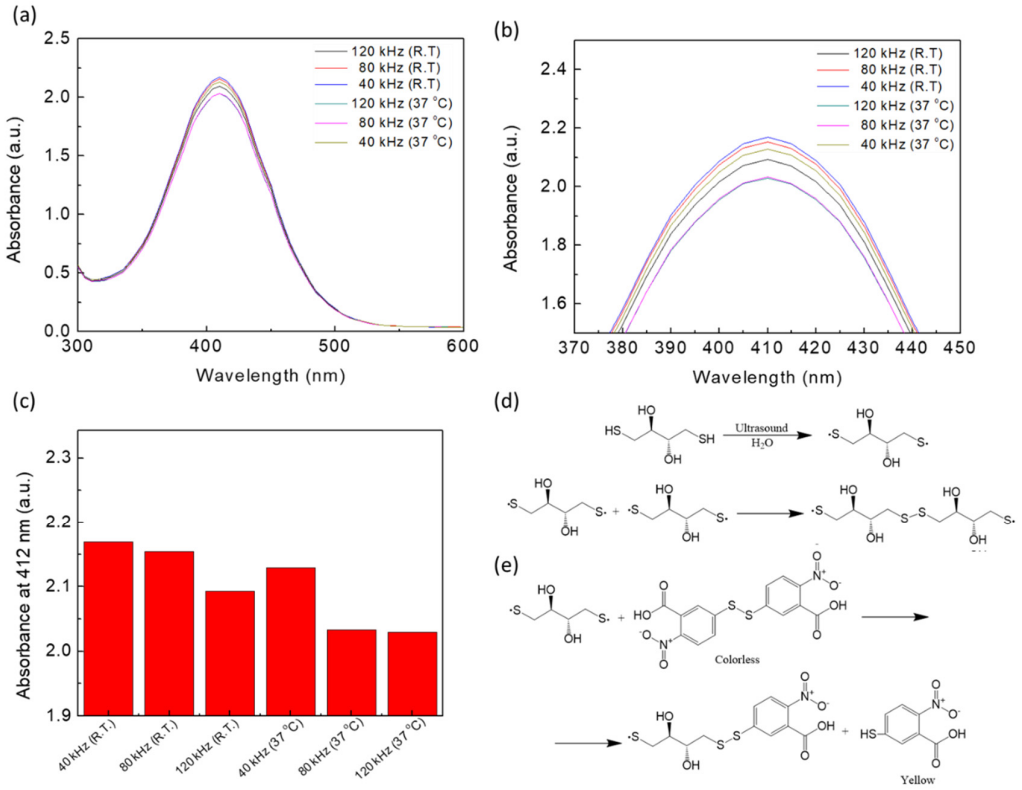


Fig. S7 Measurements of free thiols in the solution using Ellman's assay. (a, b) Absorption spectra and (c) absorbance at 412 nm of the solutions of DTT (10 mM), KPS (0.617 M), and DTNB (2 mM) mixtures under ultrasound irradiation at different frequencies and temperatures. Schematic illustrations of (d) disulfide bond formation between DTT molecules under ultrasound irradiation and (e) reaction of thiolate radicals with DTNB.

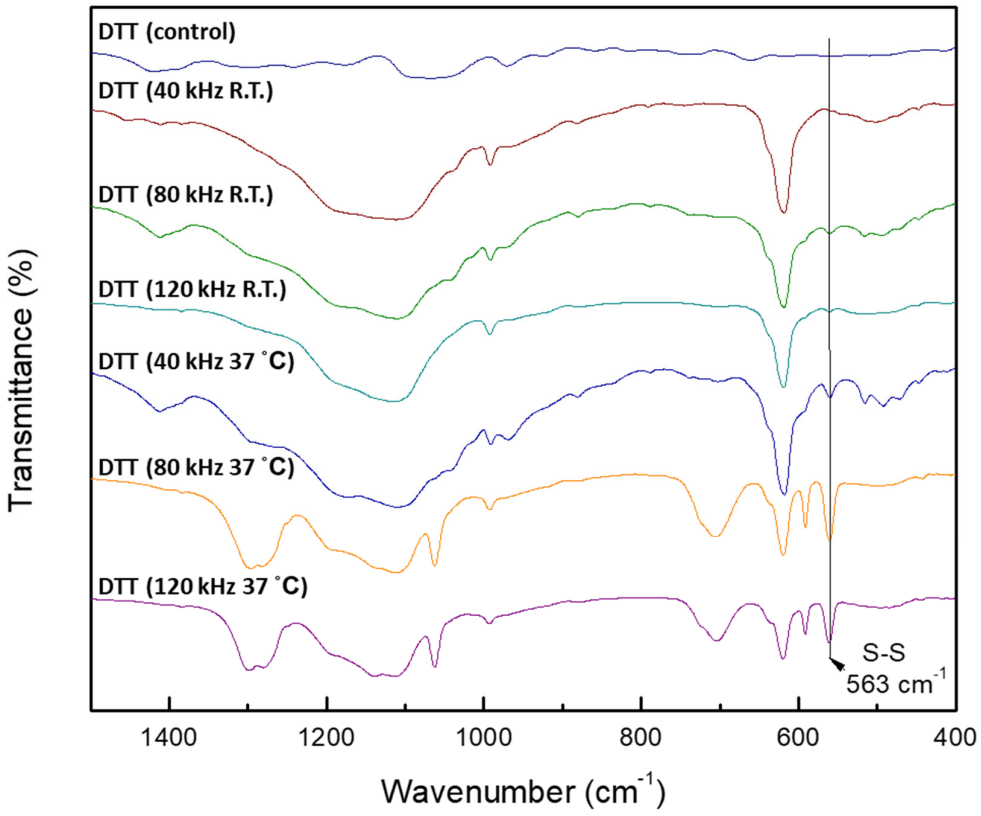


Fig. S8 FTIR spectra of DTT molecules after the ultrasound treatments in the presence of KPS (1 wt%) under different frequencies at room temperature or 37 ºC.

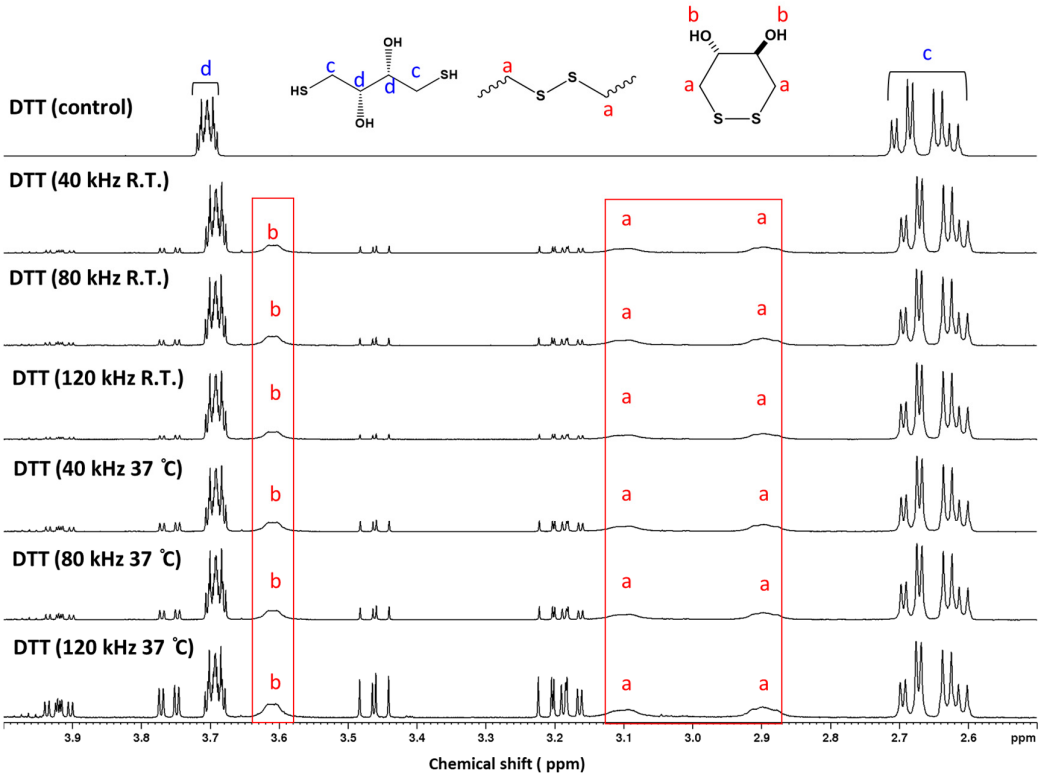


Fig. S9 NMR spectra of DTT molecules after the ultrasound treatments in the presence of KPS (1 wt%) under different frequencies at room temperature or 37 °C.

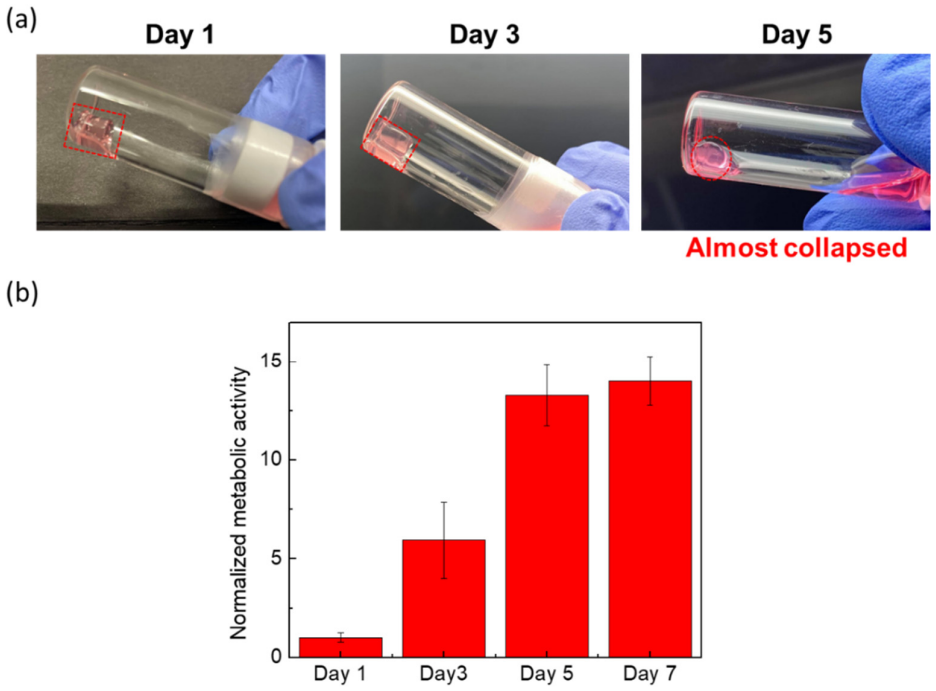


Fig. S10 (a) Stability test of Nor-Dex hydrogel in cell culture media. (b) Normalized metabolic activity of MEFs incubated with the cell culture media including the degradation products of the Nor-Dex hydrogels.

Section 3. Supporting References

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