Supporting Information

Hydrogel synthesis and stabilization via tetrazine click-induced secondary interactions

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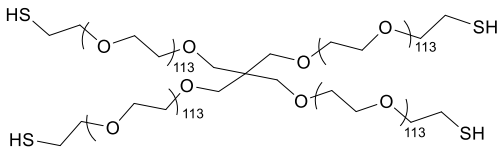
1. Materials and Methods

1.1 General procedures and methods

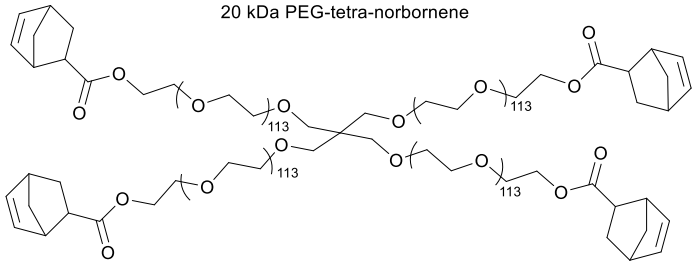
Unless otherwise reported, all chemicals and reagents were used as received from commercial sources. Lithium acylphosphinate (LAP) was synthesized according to established protocols[1]. 4-arm, 20 kDa PEG-tetra-thiol was purchased from Laysan Bio, Inc. and used without further modification.

20 kDa PEG-tetra-thiol

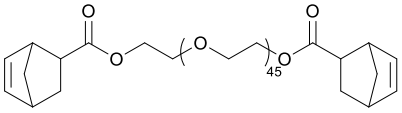


1.2 PEG-norbornene macromer functionalization

20 kDa PEG-tetra-norbornene



2 kDa PEG-di-norbornene



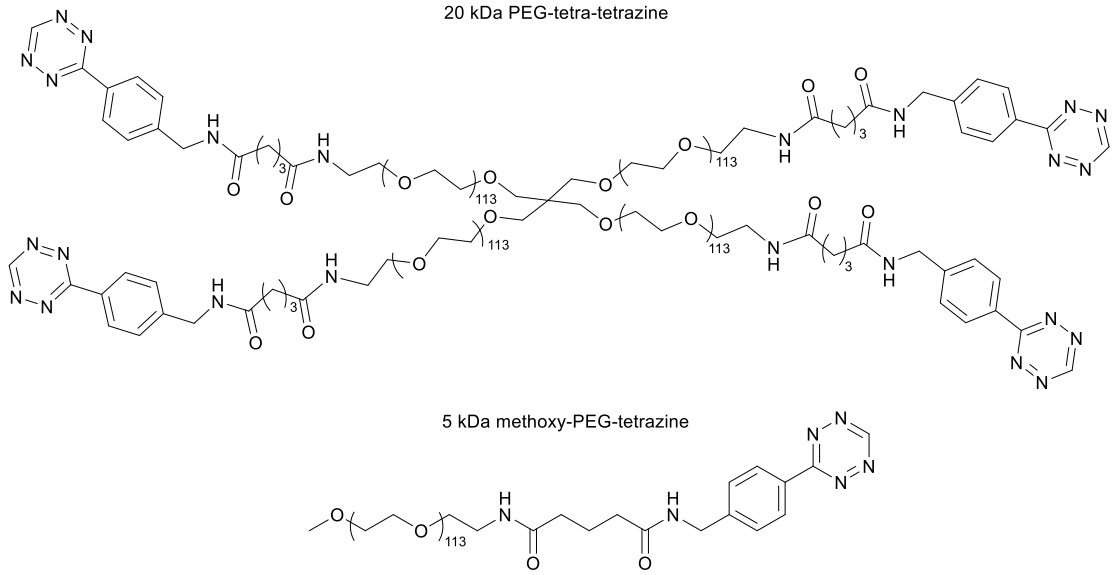
4-arm 20 kDa PEG-hydroxyl (JenKem Technologies USA) and 2 kDa linear PEG-hydroxyl (Laysan Bio, Inc.) were functionalized with norbornene acid as previously described[2] with slight modification to yield PEG-tetra-norbornene (PEG4NB) and PEG-di-norbornene (NB-PEG-NB).

PEG-tetra-norbornene synthesis. Briefly, 10 g 4-arm 20kDa PEG-hydroxyl (0.5 mmol), 0.122 g 4-(dimethylamino)pyridine (0.5X to PEG-OH, 1 mmol, Sigma-Aldrich), 0.81 mL pyridine (5X to PEG-OH, 10 mmol Sigma-Aldrich), and 60 mL anhydrous dichloromethane (Acros Organics) were dissolved in round-bottom flask under argon. Separately, 1.22 mL 5-Norbornene-2-carboxylic acid (10 COOH:1 PEG-OH, 10 mmol, Alfa Aesar), 0.77 mL diisopropylcarbodiimde (5 mmol, Alfa Aesar), and 30 mL anhydrous dichloromethane were mixed for 45 min at room temperature in a reaction vessel under argon to generate a dinorbornene anhydride, which was filtered to remove precipitated urea salts and then added to the round-bottom flask containing PEG. The solution was allowed to react overnight at room temperature, after which it was

precipitated on ice in 10-fold vol. excess of diethyl ether (Fisher Chemical) chilled to 4°C and vacuum filtered to yield a white precipitate of functionalized PEG. The product was then filtered twice and dried under vacuum for 24 h, dialyzed against deionized water for 48 h (MWCO = 10 kDa), and lyophilized to obtain purified PEG-norbornene.

PEG-di-norbornene synthesis. 2 kDa PEG-hydroxyl (Alfa Aesar) was functionalized with norbornene acid to yield PEG-di-norbornene as described above with the following modifications. 5.0 g 2kDa PEG-hydroxyl (2.5 mmol, 5.0 mmol -OH) was used, along with 0.30 g 4-(dimethylamino)pyridine (0.5X to -OH, 2.5 mmol, Sigma-Aldrich), 2.0 mL pyridine (5X to -OH, 25 mmol Sigma-Aldrich), and 20 mL anhydrous dichloromethane (Acros Organics). Additionally, 6.1 mL 5-Norbornene-2-carboxylic acid (10 COOH:1 PEG-OH, 50 mmol, Alfa Aesar), 3.9 mL diisopropylcarbodiimde (25 mmol, Alfa Aesar), and 15 mL anhydrous dichloromethane were used in the dinorbornene anhydride reaction. After being precipitated, filtered, and dried, the product was dialyzed against deionized water for 48 h (MWCO=1kDa) and lyophilized to obtain purified PEG-norbornene.

1.3 PEG-tetrazine macromer functionalization



PEG-tetra-tetrazine synthesis. 4-arm, 20 kDa PEG-amine (JenKem USA) was functionalized with tetrazine carboxylic acid (Tz-COOH) to yield 4-arm PEG-tetrazine (PEG4Tz) as previously described with slight modification[3]. First, 5-(4-(1,2,4,5-Tetrazin-3-yl)benzylamino)-5-oxopentanoic acid (Tz-COOH) was synthesized by reacting 5-(4-(cyano)benzylamino)-5-oxopentanoic acid with hydrazine, formamidine acetate, and zinc triflate catalyst, as previously described[4]. 1.0 g 4 arm, 20 kDa PEG-NH_2 (0.05 mmol, 0.20 mmol -NH_2) was added to a dry, argon purged vessel, dissolved in 10 mL of 1-Methyl-2-Pyrrolidinone (NMP, Chem Impex) with 0.06 mL triethylamine (2X to -NH_2, 0.40 mmol, Alfa Aesar), and allowed to mix for approximately 15 min. In a separate dry, argon purged vessel 0.30 g of Tz-COOH (5X to -NH_2, 1.0 mmol) was dissolved in 5 mL NMP and activated with 0.38 g O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyl-uronium hexafluorophosphate (5X to -NH_2, 1.0 mmol, HBTU, Chem Impex) for 5 min. The activated Tz-COOH was mixed with the PEG amine and allowed to react at room temperature for 15 h. The reaction mixture was then precipitated in 40 mL cold diethyl

ether (4°C) and centrifuged to remove the salt byproducts, then dried under vacuum and dialyzed against ultrapure water for 48 h and lyophilized.

Methoxy-PEG-tetrazine synthesis. Linear 5 kDa methoxy-PEG-amine (mPEG-NH_2, Laysan Bio) was functionalized with Tz-COOH to yield methoxy-PEG-tetrazine (mPEG-Tz) as described above with the following modifications. 1.03 g of 5 kDa mPEG-NH_2 (0.20 mmol, 0.82 mmol -NH_2) was added to a dry, argon purged vessel and dissolved in 3 mL NMP with 0.23 mL triethylamine (2X to -NH_2,1.63 mmol). 0.37 g Tz-COOH (1.5X to -NH_2, 1.23 mmol) was dissolved in NMP and activated with 0.47 g HBTU (1.5X to -NH_2, 1.23 mmol) for 5 min.

1.4 Synthesis and in situ gelation of non-covalently crosslinked hydrogels

A solution of PEG-tetra-norbornene at 10% w/v in deionized water was combined with mPEG-Tz at a 1:1 ratio of norbornene to tetrazine. Gels were allowed to polymerize at room temperature for 30 min, and then immediately dried under vacuum for 48 h. The dry mass of the combined sol and gel fractions was recorded. Dried gels were then swelled in double deionized water for 24 h on the orbital shaker at room temperature to wash out the uncrosslinked sol fraction. The swelled gels were dried again for 24 h under vacuum, and the dry mass of the remaining gel fraction was recorded. Sol fraction was calculated as the ratio of the sol fraction dry mass to the combined dry mass of the sol and gel fractions. Swelling ratio was also calculated. Gel samples were swelled to equilibrium for 64 hours in deionized water, and the swollen mass was recorded. Gels were dried overnight at 60°C and dry mass was recorded. Swelling ratio, Q, was calculated using $Q = \frac{W_s - W_d}{W_d}$, where W_s is the mass of the sample at equilibrium swelling and W_d is the dry mass of the sample.

Gelation was monitored using time-sweep rheology for non-covalently crosslinked gels. A solution of 10% w/v 4-arm PEG-norbornene in deionized water with mPEG-Tz added at a 1:1 tetrazine-ene ratio was pipetted between the lower Peltier plate and the 8 mm parallel plate of an Anton Parr Physica MCR 301 rheometer. G' and G" were then monitored as a function of time at a constant frequency of 1 rad s^{-1} and constant strain of 1% over the course of 2 h at 21°C (room temperature).

1.5 Characterization of tetrazine-norbornene reaction kinetics

The kinetics of the tetrazine-norbornene IEDDA reaction were tracked by monitoring the characteristic absorbance of unreacted tetrazine at 520 nm[5]. Methoxy-PEG-tetrazine (mPEG-Tz) at a concentration of 12 mM in phosphate buffered saline (PBS) was combined in a 96-well plate with either 6 mM of NB-PEG-NB (2 kDa), 6 mM of NB-PEG-NB that had been reacted with L-cysteine at a 1:1 thiol-ene ratio (not reported), or no additional norbornene-containing macromer. Using an Infinite M 200 Pro plate reader (Tecan), absorbance at 520 nm was measured over the course of 1 h with one reading per minute. Absorbance over time was averaged over three samples.

1.6 Dynamic Light Scattering

Dynamic light scattering (DLS) was performed using a Malvern Zetasizer Nano ZS. Samples were prepared at a concentration of 1 mg/mL in 0.2 μm syringe-filtered PBS. Those samples were then filtered through a 0.2 μm syringe filter with a PVDF membrane. Samples were added

into 70 μL cuvettes which had been rinsed twice with 0.2 μm syringe-filtered PBS. Measurements were performed in triplicate in sets of 11 acquisitions.

1.7 Covalently crosslinked hydrogel preparation

Two types of gel samples were prepared for characterization: IEDDA tetrazine-norbornene polymerized gels, referred to as tetrazine gels, and radical-mediated thiol-norbornene polymerized gels, referred to as thiol-ene gels.

Tetrazine gels were prepared by combining 7.5% w/v (3.54 mM) 20 kDa PEG4Tz with 1 mM norbornene-functionalized peptide GRGDS (synthesized as previously described) and 7.08 mM NB-PEG-NB in PBS. The total ratio of tetrazine to norbornene in the pre-gel solution was 1:1. Pre-gel solution was added to 8 mm diameter and 1 mm thick silicone gaskets on top of glass slides treated with Sigmacote (Sigma Aldrich). Tetrazine gels were allowed to polymerize at room temperature for 30 min.

Thiol-ene gels were prepared by combining 7.5% w/v (3.73 mM) 20 kDa PEG4SH with 2 mM photoinitiator LAP, 1 mM norbornene-functionalized peptide GRGDS, and 7.45 mM NB-PEG-NB in PBS. To prevent disulfide bond formation, PEG4SH was kept at -20°C in small aliquots used within two freeze-thaw cycles, kept at room temperature for less than 1 hr, and mixed thoroughly via vortex before use. The total ratio of thiol to norbornene in the pre-gel solution was 1:1. Pre-gel solution was added to 8 mm diameter and 1 mm thick silicone gaskets on top of glass slides treated with Sigmacote (Sigma Aldrich). Thiol-ene gels were then crosslinked via 365 nm UV light for 5 min at 10 mW/cm^2. For a 1 mm thick gel sample with a LAP concentration of 2 mM and light exposure for 5 min, light attenuation through the sample is expected to be less than 10%.[1] Post-polymerization, both tetrazine and thiol-ene gels were swelled in excess PBS.

1.8 Gel hydrolysis for NMR

Thiol-ene crosslinked gel samples were prepared as described above with the slight modification of the use of an 8 mm diameter, 2 mm height round silicone mold to create a sample with a pre-swollen volume of 105 μL. Gel samples were individually incubated in 1 mL 0.1 N NaOH at 37°C for 1 h, then frozen at -80°C for 4 h. Frozen samples were lyophilized overnight. The mass of lyophilized samples was measured and samples were dissolved in 800 μL $CDCl_3$ for 48 h to ensure complete solvation. After 48 h, 400 μL of $CDCl_3$ was added to bring the sample volume back to 800 μL, and samples were centrifuged at 21,380 RCF for 5 min to separate residual salts. 1H NMR was analyzed using a Bruker Avance Neo 400 Hz console.

1.9 Characterization of swelling ratio and gel fraction

Swelling ratio was characterized for thiol-ene and tetrazine click-crosslinked gels. Post-polymerization, gel samples were swelled to equilibrium overnight in PBS, and the swollen mass was recorded. Gels were dried overnight at 60°C and dry mass was recorded. Swelling ratio, Q, was calculated using $Q = \frac{W_s - W_d}{W_d}$, where W_s is the mass of the sample at equilibrium swelling and W_d is the dry mass of the sample.

Gel fraction was also characterized. Immediately post photopolymerization, thiol-ene and tetrazine click gels were dried under vacuum for 24 h and the dry mass of the combined sol and

gel fractions was recorded. Dried gels were then swelled in double deionized water for 24 h on the orbital shaker at room temperature to wash out the uncrosslinked sol fraction. The swelled gels were dried again for 24 h under vacuum, and the dry mass of the remaining gel fraction was recorded. Sol fraction was calculated as the ratio of the sol fraction dry mass to the combined dry mass of the sol and gel fractions.

1.10 Rheological characterization

Gel samples were swelled overnight (~18 hours) to equilibrium. Storage modulus (G') was assessed by taking the average over the linear viscoelastic region of a strain sweep from 0.01% to 20% strain at a frequency of 1 rad/s. Tests were performed on a TA Discovery HR-2 rheometer with parallel-plate geometry (8 mm diameter).

In-situ gelation experiments were performed using an Anton Paar Physica MCR 301 rheometer. For thiol-ene crosslinked gels, 40 μL of pre-gel solution prepared as described previously was added to the stage and a time sweep was performed at 1% strain and 1 rad/s at 37°C using an 8 mm diameter parallel-plate geometry. After 1 min the sample was exposed to 365 nm UV light at an intensity of 10 mW/cm^2 for 5 min, at which point the time sweep was terminated. Tetrazine click-crosslinked samples were prepared by adding 4-arm 20 kDa PEG-tetrazine to the remaining components of the pre-gel solution immediately before adding a 40 μL sample to the stage. Time sweeps were performed at 1% strain and 1 rad/s at 37°C using an 8 mm diameter parallel-plate geometry over the course of 30 min.

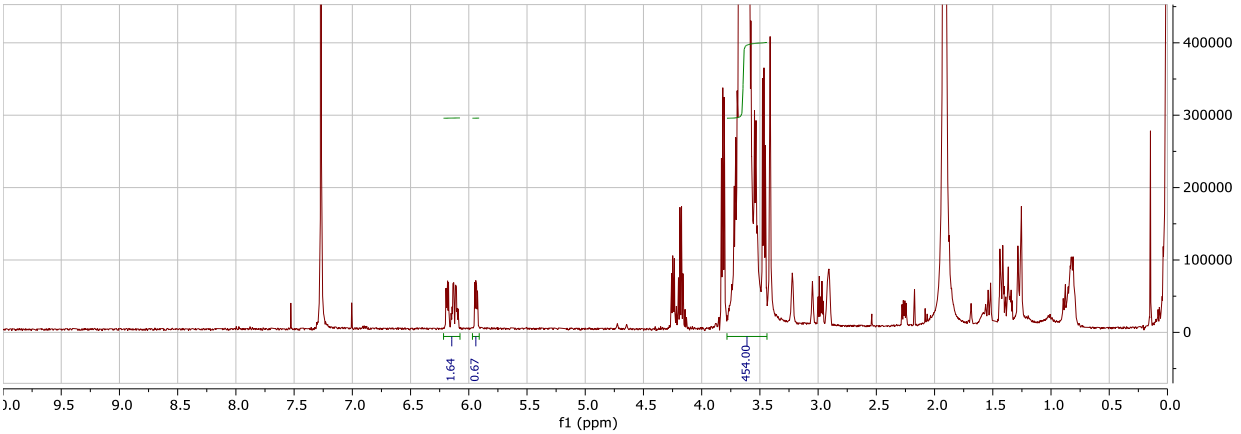
1.11 Degradation studies

Degradability via base-catalyzed hydrolysis was assessed. Gel samples were synthesized and swelled to equilibrium overnight. The starting mass of each sample was then recorded, and then gels were submerged in 0.1 N sodium hydroxide and kept at 37°C. The remaining wet mass was recorded every 15 minutes for the first 2 h, then every 2 h up to 8 h total, and then again at 24 h. After 24 h, remaining gel samples were rinsed in PBS and subjected to rheological analysis.

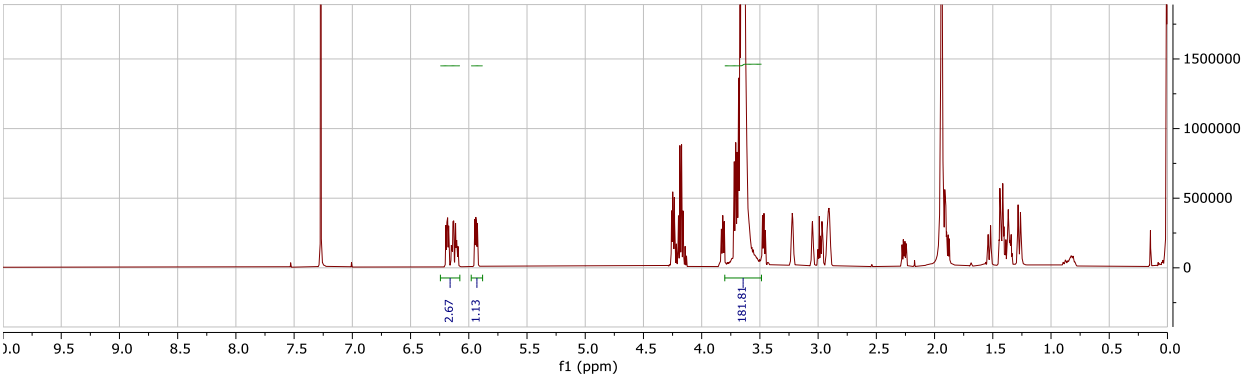
1.12 Computational Details

Model systems for thiol-norbornene and tetrazine norbornene were subjected to solvent-explicit, all-atom molecular dynamics simulations using the GPU-accelerated DESMOND[6] software and the OPLS3 force field.[7] A periodic TIP4P water orthorhombic box with a 20Å buffer was used for the solvation box. The NPT ensemble class with a temperature of 300 K and a pressure of 1.01325 bar was used. Each molecular dynamic production run was carried out for 60.0 ns which included the multisim relaxation procedure. The recording interval was set to 20.0 ps for the trajectory and energy.

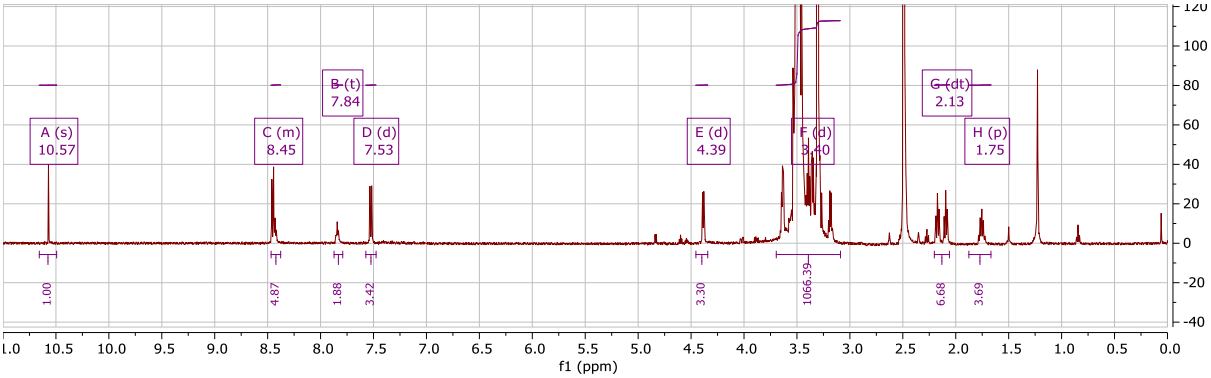
2. NMR Spectra



2.1 20 kDa PEG-tetra-norbornene. Analysis by ^{1}H NMR indicated 96% end group functionalization. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.22 – 6.07 (m, 1H), 5.94 (dd, *J* = 5.7, 2.8 Hz, 1H), 4.28 – 4.11 (m, 2H), 3.64 (s, ~454H per arm).

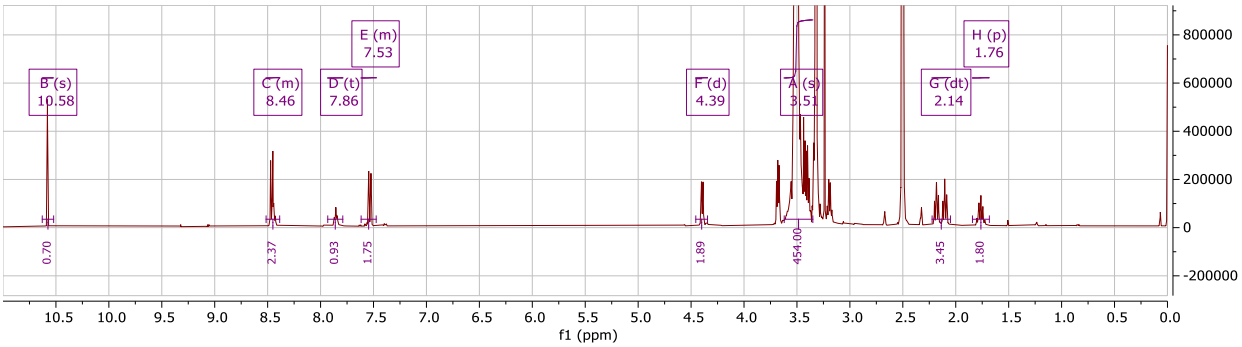


2.2 2 kDa PEG-di-norbornene. Analysis by ^{1}H NMR indicated 90% end group functionalization. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.21 – 6.08 (m, 3H), 5.94 (dd, *J* = 5.7, 2.8 Hz, 1H), 4.30 – 4.09 (m, 4H), 3.64 (s, ~182H).



2.3 20 kDa PEG-tetra-tetrazine. Analysis with ^{1}H NMR indicated 80% end group functionalization. ^{1}H NMR (500 MHz, DMSO-d6) δ 10.57 (s, 1H), 8.48 – 8.40 (m, 3H), 7.84 (t,

J = 5.7 Hz, 1H), 7.53 (d, *J* = 8.1 Hz, 2H), 4.39 (d, *J* = 6.0 Hz, 3H), 3.50 (s, ~454H), 2.17 (t, *J* = 7.5 Hz, 2H), 2.09 (t, *J* = 7.5 Hz, 2H), 1.75 (p, *J* = 7.5 Hz, 2H).



2.4 5 kDa Methoxy-PEG-tetrazine. ^{1}H NMR indicated 90% end group functionalization with tetrazine. ^{1}H NMR (400 MHz, DMSO) δ 10.58 (s, 1H), 8.50 – 8.40 (m, 3H), 7.86 (t, *J* = 5.6 Hz, 1H), 7.57 – 7.50 (m, 2H), 4.39 (d, *J* = 6.0 Hz, 2H), 3.51 (s, ~454H), 2.14 (dt, *J* = 31.1, 7.5 Hz, 4H), 1.82 – 1.70 (m, 2H).

3. Supplemental Table

Table S1. Characterization of non-covalently crosslinked hydrogels. Storage modulus G‘ is the final modulus post-crosslinking measured via in situ oscillitory rheology. (n=3)

Storage Modulus, G‘ [kPa] Pre-Swelling	Swelling Ratio, Q	Gel Fraction [%]
8.3 ± 0.5	163 ± 25	82 ± 2.1

4. Supplemental Figures

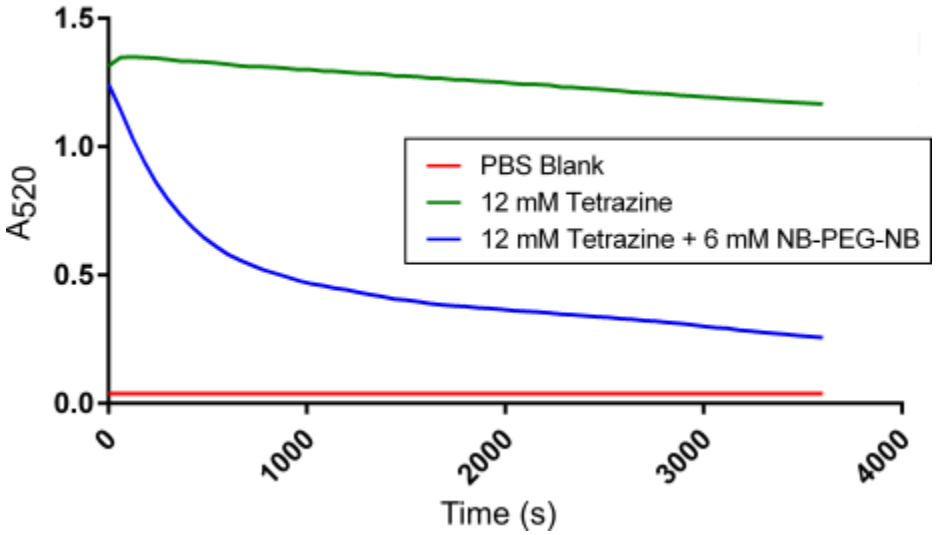


Figure S1. Monitoring tetrazine consumption over time via 520 nm absorbance. 12 mM mPEG-Tz was reacted with 6 mM PEG-di-norbornene over the course of 1 hour and absorbance was

measured once per minute. The rate of tetrazine consumption mirrors the *in situ* evolution of the storage modulus of non-covalently crosslinked tetrazine gels.

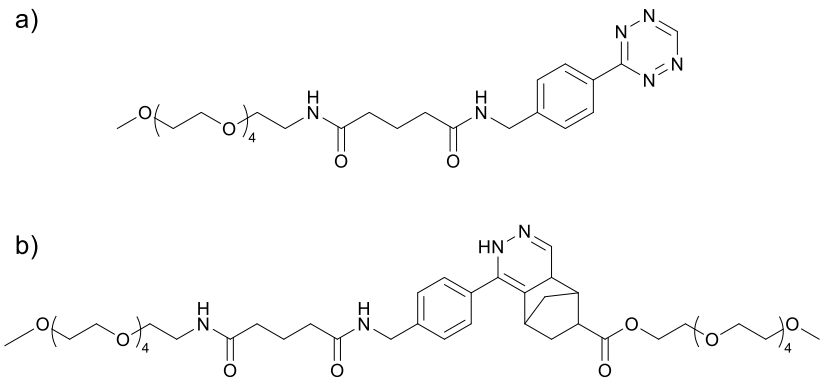


Figure S2. Structures of molecules used for molecular dynamic simulations comparing tetrazine and its norbornene cycloaddition product

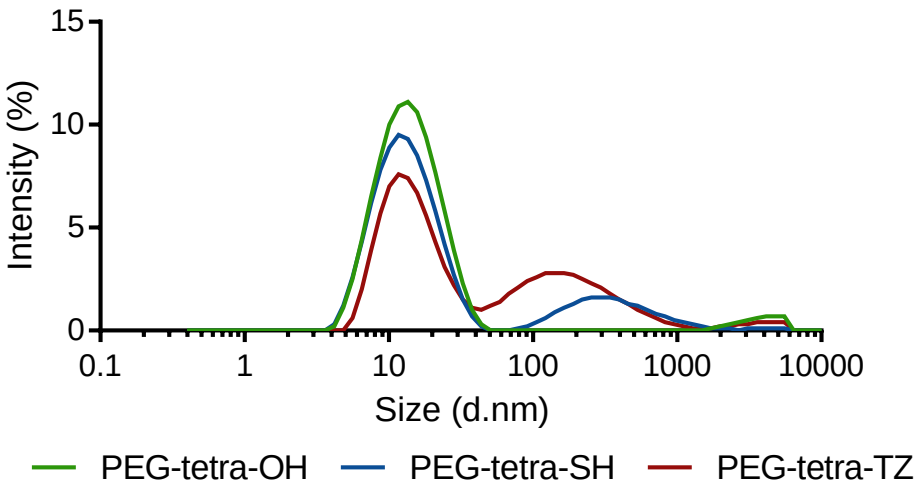


Figure S3. Hydrodynamic diameter of tetrafunctional PEGs in nm according to DLS. Samples were prepared at a concentration of 1 mg/mL in 0.2 µm syringe-filtered PBS, then 0.2 µm-filtered. Dynamic light scattering (DLS) was performed using a Malvern Zetasizer Nano ZS. Measurements were performed in triplicate in sets of 11 acquisitions.

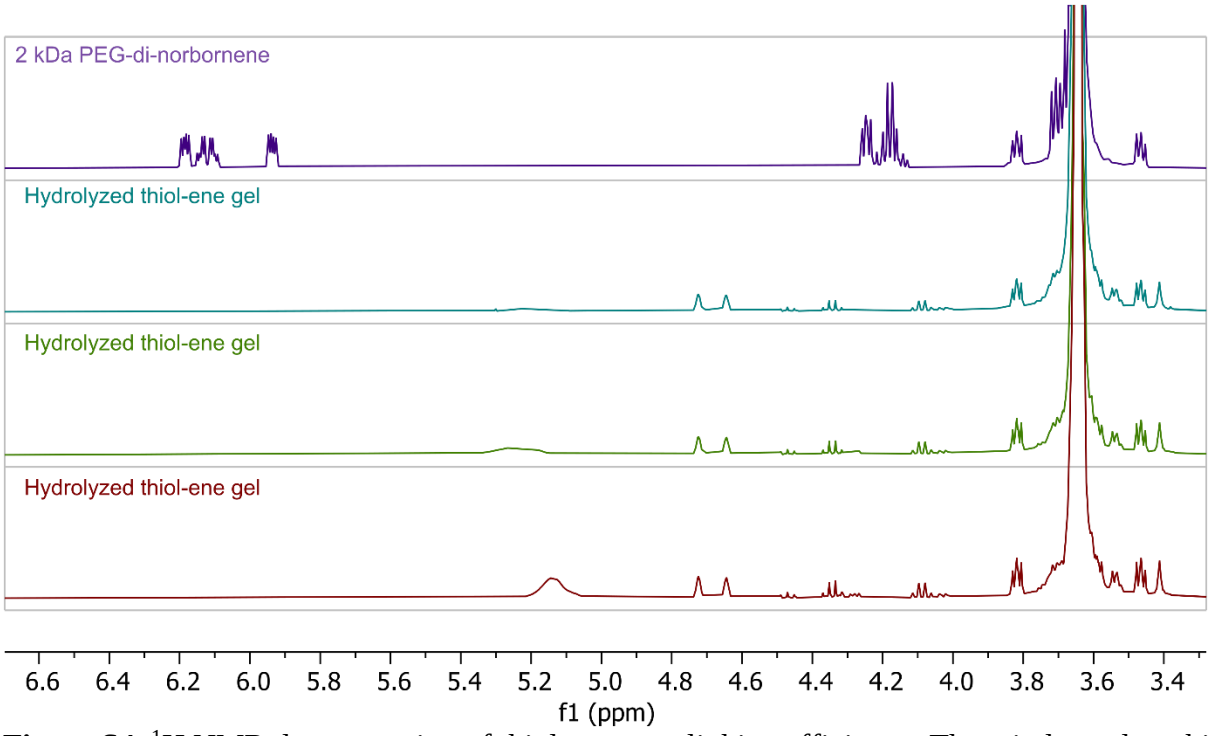


Figure S4. ^{1}H NMR demonstration of thiol-ene crosslinking efficiency. Three independent thiol-ene crosslinked gel samples were individually incubated in 1 mL 0.1 N NaOH at 37°C for 1 h, then the degraded samples in base were lyophilized. The mass of lyophilized samples was measured and samples were dissolved in $CDCl_3$ for 48 h to ensure complete solvation. Peaks indicating the presence of unreacted norbornene from δ5.93-5.95 and 6.09-6.20 are not seen in hydrolyzed thiol-ene crosslinked hydrogels.

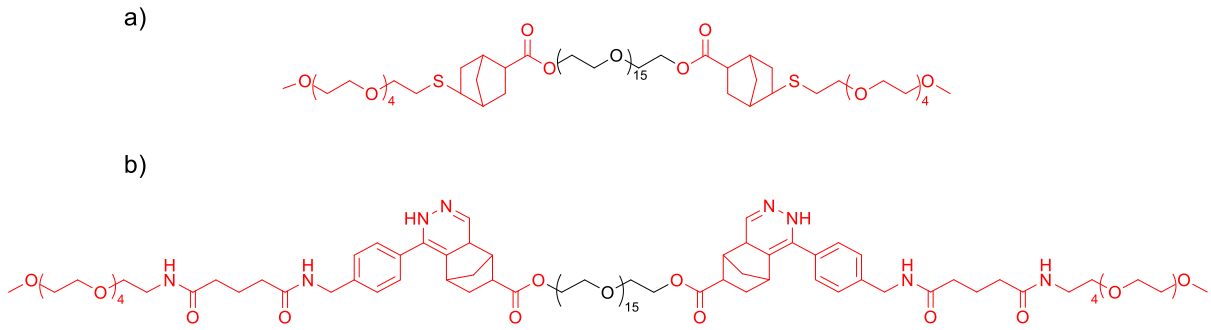


Figure S5. Structures of PEG-tethered click product molecular dynamic simulations. Regions of interest that were analyzed for intramolecular interactions are highlighted in red. a) Two thiol-ene products flanking a segment of PEG and capped at either end with short segments of PEG. b) Two tetrazine-norbornene products flanking a segment of PEG and capped at either end with short segments of PEG.

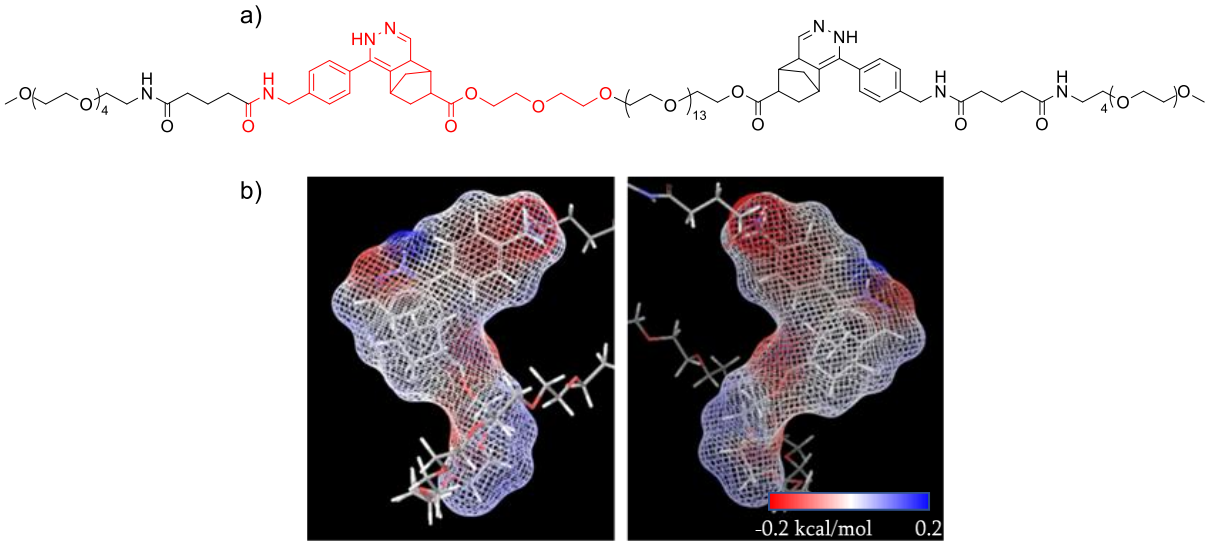


Figure S6. Electrostatic potential map of tetrazine-norbornene click product. A surface map of the electrostatic potential of the tetrazine-norbornene cycloaddition product and its amide linkages to the PEG backbone show a relatively large, electrostatically neutral region around the bridged cyclohexane. a) The structure of the model tetrazine-norbornene product, with the region reflected in the electrostatic potential maps highlighted in red. b) 3D surface map of the front and back of the region of interest.

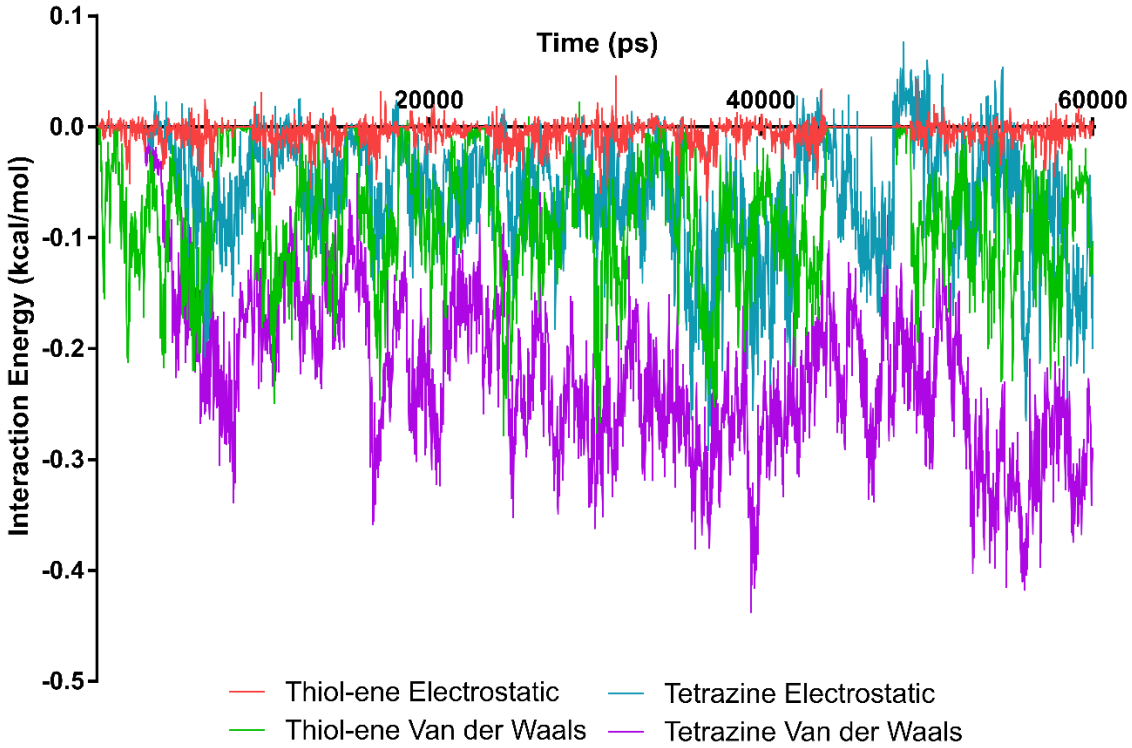


Figure S7. Simulated electrostatic and van der Waals interaction energies per atom between tetrazine-norbornene click product and thiol-ene click product regions of interest (highlighted in red in Figure S5) over time. A more negative value indicates a stronger interaction.

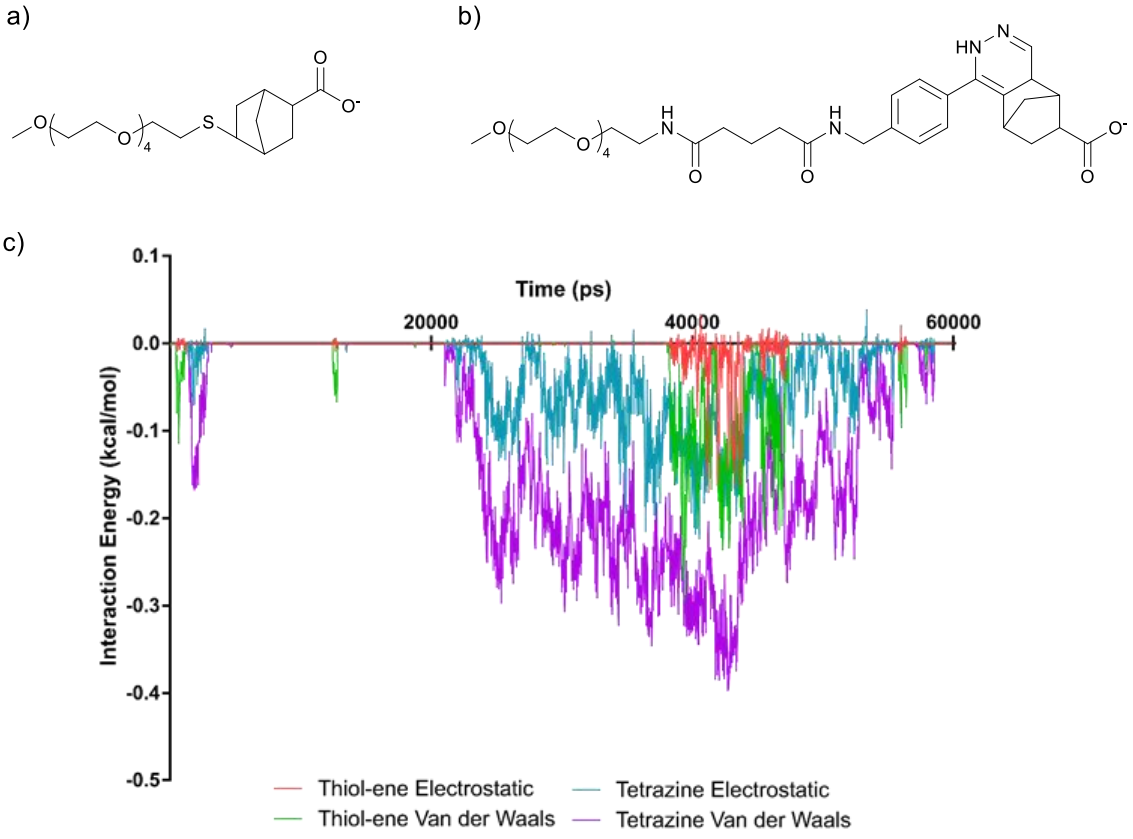


Figure S8. Molecular dynamic simulations of hydrolyzed gels crosslinked by click products. a) Model thiol-ene product after base-catalyzed ester hydrolysis. b) Model tetrazine-norbornene product after base-catalyzed ester hydrolysis. c) Simulated interaction energies per atom between two thiol-ene products and two tetrazine-norbornene products over time. A more negative value indicates a stronger interaction.

4. References

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