

Synthetically tractable click hydrogels for 3D cell culture formed using tetrazine-norbornene chemistry

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Supporting Information

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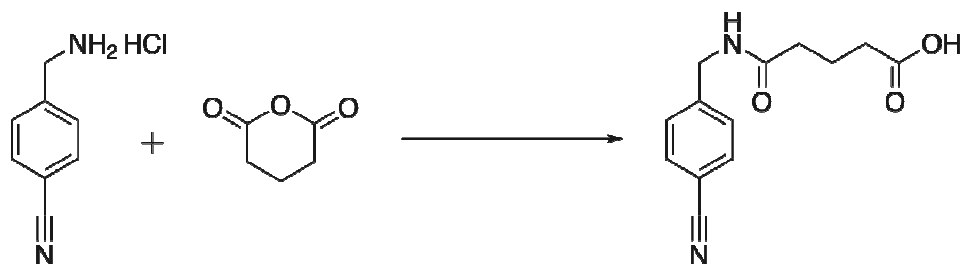
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1. Synthetic Procedures

1.1. General Considerations

Unless otherwise specified, all chemical reagents were purchased from Sigma-Aldrich and were used without purification. For small molecule synthesis, reactions were monitored by thin layer chromatography using silica gel 60 F254 coated aluminum plates from EMD Chemicals. Flash chromatography purification was done using 60-200 mesh silica gel with 60Å pore size from BDH Chemicals. ¹H NMR was performed on a 400 MHz Bruker Avance-III instrument and analyzed using MestReNova v6.1.1-6384. ESI-MS was performed by the central analytical mass spectrometry laboratory at the University of Colorado at Boulder on an Applied Biosystems QSTAR® instrument. Peptides were synthesized on a Protein Technologies Tribute automated peptide synthesizer using Rink Amide MBHA resin from Novabiochem. Fmoc protected amino acids and activators were purchased from Chem-impex International. All peptides were purified by reverse phase HPLC on a Waters Delta Prep 4000 instrument equipped with a Waters X-Select C-18 column and characterized by MALDI-TOF MS on an Applied Biosystems Voyager-DE™ STR instrument using α -cyano-4-hydroxycinnamic acid matrix.

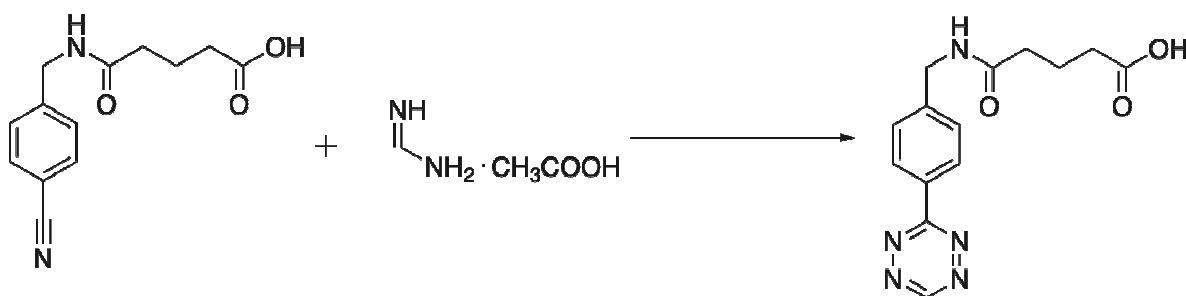
1.2. 5-(4-(cyano)benzylamino)-5-oxopentanoic acid



4-(aminomethyl)benzonitrile hydrochloride (11.32 g, 67.13 mmole) was added to a dry round bottom flask, purged with Ar, and dissolved in 500 ml of acetonitrile with triethylamine (10.29

ml, 73.85 mmole). Solid glutaric anhydride (7.66 g, 67.13 mmole) was then added, and the reaction was refluxed at 85°C for 15 h. After removal of the acetonitrile by rotary evaporation, the crude mixture was dissolved in 100 ml of dH₂O, acidified to pH 3, and extracted 3 x 500 ml with ethyl acetate. The combined organic layers were then washed with brine, dried over MgSO₄, and evaporated to dryness to yield 15.4 g of product as a white solid (93% yield). ¹H NMR (400 MHz, DMSO) δ 12.05 (s, 1H), 8.44 (t, *J* = 5.9 Hz, 1H), 7.78 (d, *J* = 8.5 Hz, 2H), 7.42 (d, *J* = 8.6 Hz, 2H), 4.33 (d, *J* = 6.0 Hz, 2H), 2.26 – 2.15 (m, 4H), 1.79 – 1.69 (m, 2H). ESI-MS [M-H] calculated for C₁₃H₁₃N₂O₃ 245.1; found 245.1.

1.3. 5-(4-(1,2,4,5-tetrazin-3-yl)benzylamino)-5-oxopentanoic acid



The desired tetrazine carboxylic acid (Tz-COOH) was synthesized using the method of Devaraj *et al.*¹, with slight modification. 5-(4-(cyano)benzylamino)-5-oxopentanoic acid (0.47 g, 1.91 mmole) was combined with formamidine acetate salt (0.79 g, 7.64 mmole), and elemental sulfur (0.06 g, 1.91 mmole) in a round bottom flask and thoroughly mixed. Anhydrous hydrazine (1.1 ml, 35.0 mmole) was then added to the flask and, after vigorous gas evolution, the yellow slurry was stirred at room temperature for 20 h. Glacial acetic acid (30 ml) was slowly added to the crude mixture and allowed to stir for approximately 10 min, after which the suspension was filtered through a glass frit. The resulting orange solution was then cooled to 0°C and sodium nitrite (0.66 g, 9.55 mmole) dissolved in 1.5 ml of dH₂O was added dropwise over 15 min,

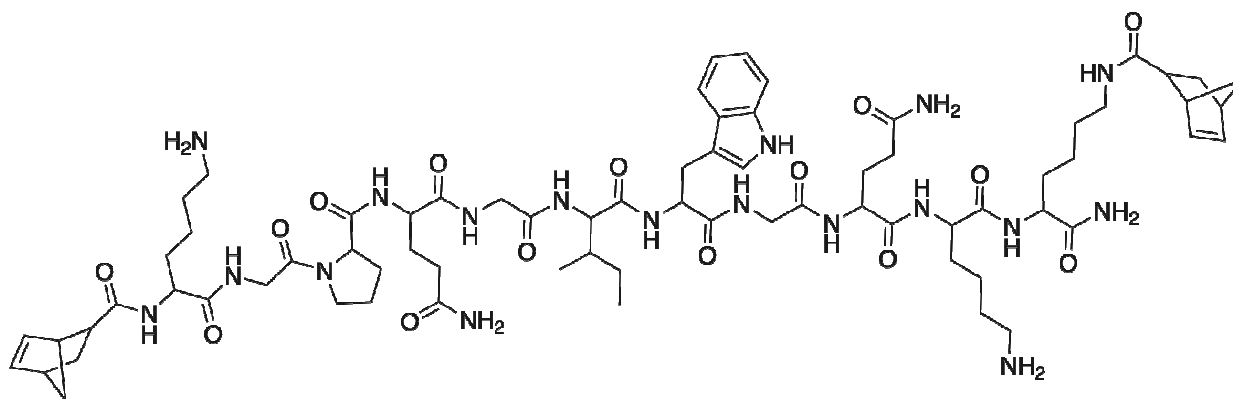
during which the solution turned bright pink and gas evolution was vigorous. Once the gas evolution was complete, the solution was evaporated to dryness, being careful to avoid excessive heating. The pink solid was then redissolved in 100 ml of 2% HCl, extracted 3 x 200 ml with dichloromethane, washed with brine, dried over MgSO₄, and purified by flash chromatography (75-100% ethyl acetate in hexanes + 1% acetic acid) to yield 100 mg of the tetrazine carboxylic acid as a pink solid (17% yield). Similar yield was obtained for a larger scale reaction starting with 3 g of 5-(4-(cyano)benzylamino)-5-oxopentanoic acid. ¹H NMR (400 MHz, DMSO) δ 12.06 (s, 1H), 10.58 (s, 1H), 8.52 – 8.40 (m, 3H), 7.53 (d, *J* = 8.6 Hz, 2H), 4.40 (d, *J* = 5.9 Hz, 2H), 2.28 – 2.17 (m, 4H), 1.77 (p, *J* = 7.3 Hz, 2H). ESI-MS [M-H] calculated for C₁₄H₁₄N₅O₃ 300.1; found 300.1.

1.4. PEG-Tz Macromer

The key PEG-Tz macromer was synthesized by coupling the Tz-COOH product from the previous step to a multi-functional PEG amine (JenKem Technologies USA). Briefly, 4 arm, 20 kDa PEG-NH₂ hydrochloride salt (0.514 g, M_n = 20 kDa, 0.103 mmole NH₂) was added to a dry, Ar purged vessel, dissolved in 3 ml of anhydrous N,N-dimethylformamide (DMF) with triethylamine (0.022 ml, 0.15 mmole), and allowed to mix for approximately 15 min. In a separate dry, Ar purged vessel Tz-COOH (0.124 g, 0.41 mmole) was dissolved in 2 ml of anhydrous DMF and activated with 2-(1H-7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate (HATU) (0.156 g, 0.41 mmole, from Chem-Impex International) 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 cold diethyl ether, dissolved in 2 ml of dichloromethane, centrifuged to remove the salt byproducts, washed 2 x 1 ml with

saturated sodium bicarbonate, 1 x 1 ml with dH₂O, and finally precipitated in cold diethyl ether to obtain 350 mg of product (68% yield, ~ 75% functionalized based on NMR). ¹H NMR (400 MHz, DMSO) δ 10.58 (s, 1H), 8.46 (m, 3H), 7.85 (t, *J* = 5.6 Hz, 1H), 7.53 (d, *J* = 8.6 Hz, 2H), 4.39 (d, *J* = 5.9 Hz, 2H), 3.70 – 3.37 (m, [CH₂CH₂O]_n), 2.18 (t, *J* = 7.5 Hz, 2H), 2.10 (t, *J* = 6.3 Hz, 2H), 1.77 (m, 2H).

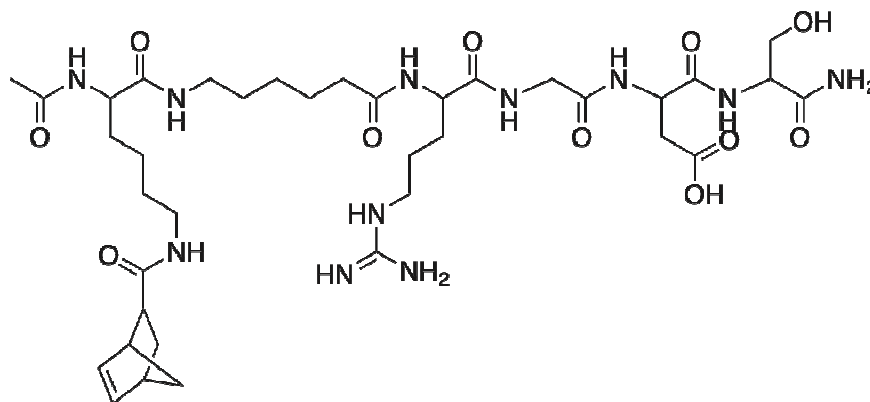
1.5. Di-norbornene peptide crosslinker norb-KGPQGIWGQKK-norb



To make the hydrogels degradable via matrix metalloproteinase enzymes produced by the encapsulated cells, a peptide with the sequence KGPQGIWGQKK(Dde) was synthesized at the 0.25 mmole scale. Fmoc deprotection was performed by treatment with 20% piperidine and 2% 2,8-diazobicyclo[5.4.0]undec-7-ene in N-methylpyrrolidone. Amino acid coupling was performed in peptide synthesis grade DMF (from Applied Biosystems) using 4 equivalents of the amino acid, 4 equivalents of O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluorophosphate (HBTU) activator, and 8 eq. of 4-methylmorpholine. After the synthesis was complete, the resin was transferred to a manual peptide synthesis vessel and the Dde protecting group was removed from C-terminal Lys residue by treatment with 2% hydrazine monohydrate in DMF for 3 x 10 min. 5-norbornene-2-carboxylic acid (mixture of endo- and exo-) was then simultaneously coupled to the N-terminus of the peptide and the deprotected ε amine of the C-

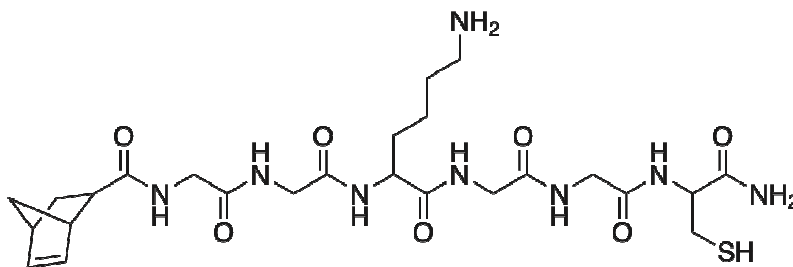
terminal Lys residue using the same protocol as above, except that HATU was used as the activator. Norbornene coupling was verified using the ninhydrin test. Peptide cleavage and deprotection was achieved by treatment with trifluoroacetic acid/phenol/triisopropylsilane/dH₂O (90/5/2.5/2.5). The peptide was recovered by precipitation in cold diethyl ether, washed twice with cold diethyl ether, and then dried in a vacuum desiccator. The crude peptide was subsequently purified by reverse phase HPLC (5% acetonitrile in water for 3 min, 5-20% acetonitrile over 7 min, 20-40 acetonitrile over 20 min; 20 ml/min flow rate) and lyophilized. The purified peptide was reconstituted in PBS and sterile filtered prior to cell encapsulation. MALDI-TOF MS [M+H] calculated for C₇₂H₁₀₈N₁₈O₁₅ 1465.83; found 1466.61.

1.6. Cell adhesive norb-AhxRGDS



To make the hydrogels cell adhesive, a peptide with the sequence Ac-K(Dde)AhxRGDS was synthesized on the 0.25 mmole scale. 5-norbornene-2-carboxylic acid was coupled to the ϵ amine of the Lys residue using the same protocol as for the di-norbornene crosslinker. The peptide was cleaved from the resin and purified, as described above. MALDI-TOF MS [M+H] calculated for C₃₇H₆₁N₁₁O₁₁ 836.46; found 836.80.

1.7. Heterobifunctional norb-GGKGGC



To leave pendant thiols in the hydrogel that could later be exploited for protein patterning via thiol-ene photochemistry, a peptide with the sequence GGKGGC was synthesized on the 0.25 mmole scale. 5-norbornene-2-carboxylic acid was coupled to the N-terminus of the peptide to result in a heterobifunctional norbornene-thiol peptide and the peptide was cleaved from the resin and purified, as described above. ESI-MS [M+H] calculated for C₂₅H₄₁N₈O₇S 597.3; found 597.3

1.8. Norbornene functionalized fluorescent BSA

For protein patterning experiments, commercially available fluorescent BSA proteins were modified with a norbornene succinimidyl ester. Briefly, 5-norbornene-2-carboxylic acid (0.6 g, 4.35 mmole), N-hydroxysuccinimide (0.5 g, 4.35 mmole), and 4-(dimethylamino)pyridine (0.026 g, 0.22 mmole) were added to dry round bottom flask, purged with Ar, and then dissolved in 5 ml of dry dichloromethane plus a minimal amount of anhydrous DMF. The solution was then cooled on ice and N,N'-dicyclohexylcarbodiimide (0.897 g, 4.35 mmole) dissolved in 1 ml of dry dichloromethane was added dropwise over approximately 5 min. The solution was allowed to warm to room temperature and react overnight, after which the urea salts were removed by filtration and the solution concentrated *in vacuo* to yield a viscous clear liquid. The crude product was verified by mass spectroscopy and was used without further purification. ESI MS

[M+Li] calculated for $C_{12}H_{13}NO_4$ 242.1; found 242.1. For protein functionalization, fluorescein and tetramethylrhodamine labeled BSA (1 mg, Invitrogen) were each reacted with the norbornene-NHS ester (40 μ l, \sim 10 eq.) in 460 μ l of cell culture grade phosphate buffered saline at pH 7.4 and room temperature for two hours. The resulting norbornene functionalized fluorescent BSA proteins were used without purification in the photopatterning experiments.

2. Experimental Methods

2.2. Cell encapsulation and viability assay

To verify the cytocompatibility of the tetrazine-norbornene crosslinking reaction, human mesenchymal stem cells (hMSC) were encapsulated in PEG-peptide hydrogels and their viability was assessed 24 h post-encapsulation. Briefly, hMSC were isolated from human bone marrow (purchased from Lonza) based on their adherence to tissue culture plastic, as we have previously described². The cells were expanded up to passage 3 and cultured at 37°C and 5% CO₂ in growth medium (i.e., Dulbecco's modified eagles medium (DMEM, low glucose) supplemented with 10% fetal bovine serum, 50 U/ml each penicillin and streptomycin (all from Invitrogen), and 1 ng/ml fibroblast growth factor-2 (Peprotech)). For the encapsulation, the cells were detached from the cell culture substrate using trypsin/EDTA and resuspended at a final cell density of 5×10^6 cells/ml in a solution of 7.5 wt. % PEG-Tz, 4.75 mM norb-KGPQGIWGQKK-norb, and 1 mM norb-RGDS. The cell suspension was pipette mixed and then quickly transferred to sterile syringe tip molds (i.e., 1 ml syringes that had been cut to remove the tips and then inverted) in 30 μ l aliquots. After allowing 15 min for gelation, the cell-laden hydrogels were transferred to a 24 well plate and cultured individually in 1 ml of growth medium without FGF-2.

hMSC viability was assessed at 24 h post-encapsulation using the commercially available live/dead staining kit. Briefly, the cell-laden hydrogels were transferred from the cell culture medium to sterile cell culture grade PBS and incubated at room temperature on an orbital shaker for approximately 15 min. The gels were subsequently transferred to PBS with 20 μ M ethidium homodimer and 5 μ M calcein-AM and incubated at room temperature on an orbital shaker for 45 min while protected from light. The gels were then imaged at 10X magnification through a water immersion lens on a Zeiss 710 LSM NLO laser scanning confocal microscope using 488 nm and 546 nm lasers to excite the calcein and ethidium fluorophores, respectively. Finally, to calculate percent viability, maximum intensity projections of 105 μ m z-stacks collected with a 15 μ m step size were thresholded and then analyzed in ImageJ using the particle analyzer plugin to count discrete cells in the live and dead channels.

2.3. Protein photopatterning

Hydrogels were prepared using 7.5 wt. % PEG-Tz, 4.75 mM norb-KGPQGIWGQKK-norb, and 1 mM norb-GGKGGC. The gels were formed between 22 mm square glass coverslips, one of which was treated with Sigmacote in order to facilitate removal from the gel. After gelation, the gels were then immersed in a solution of 0.1 mg/ml norbornene functionalized fluorescein-BSA and 2.2 mM I2959 photoinitiator in PBS, incubated at room temperature on an orbital shaker for 2 h, and then irradiated with collimated UV light (365 nm, 10 mW/cm², Omnicure lamp) through a chrome on quartz photomask (100 μ m lines with 100 μ m spacing) for 10 min. The patterned gel was transferred to fresh PBS, incubated for 1 hr at room temperature on an orbital shaker, and the patterning process was repeated with norbornene functionalized tetramethylrhodamine-BSA. In the second patterning step, the chrome photomask was rotated approximately 90° to

generate a grid pattern. Single and dual protein patterned hydrogels were imaged at 10X magnification through a water immersion lense on a Ziess widefield fluorescence microscope. Quantitative measurements of the patterned lines confirmed excellent pattern fidelity, as the average line width was $101.02 \pm 1.46 \mu\text{m}$ ($n = 12$).

3. Calculations of Critical Crosslinking Density

Using Flory-Stockmayer theory for statistical gelation of step growth networks, the critical crosslinking density to achieve gelation was calculated for each hydrogel formulation using the following equation

$$\rho_c = \frac{1}{\sqrt{\frac{[\text{norb}]}{[\text{Tz}]}} (f_{\text{PEG-Tz}} - 1)(f_{\text{crosslinker}} - 1)} = \frac{1}{\sqrt{\frac{[\text{norb}]}{[\text{Tz}]}} (2)}$$

where ρ_c is the critical crosslinking density, $[\text{norb}]$ is the concentration of norbornene from the crosslinker, $[\text{Tz}]$ is the concentration of tetrazine, $f_{\text{PEG-Tz}}$ is the functionality of the PEG-Tz macromer ($f_{\text{PEG-Tz}} = 3$), and $f_{\text{crosslinker}}$ is the functionality of the crosslinker ($f_{\text{crosslinker}} = 2$). The results from these calculations are below in Table S1.

Table S1. Hydrogel formulations and calculated ρ_c values.

	PEG-Tz wt. %	[Tz] (mM)	[pendant peptide] (mM) ^a	[norb] (mM) from crosslinker	ρ_c
1	7.5	10.5	0	10.5	0.71
2	7.5	10.5	1	9.5	0.74
3	5	7	1	6	0.76
4	10	14	1	13	0.73

^apendant peptide was either norb-AhxRGDS or norb-GGKGGC

4. NMR Spectra

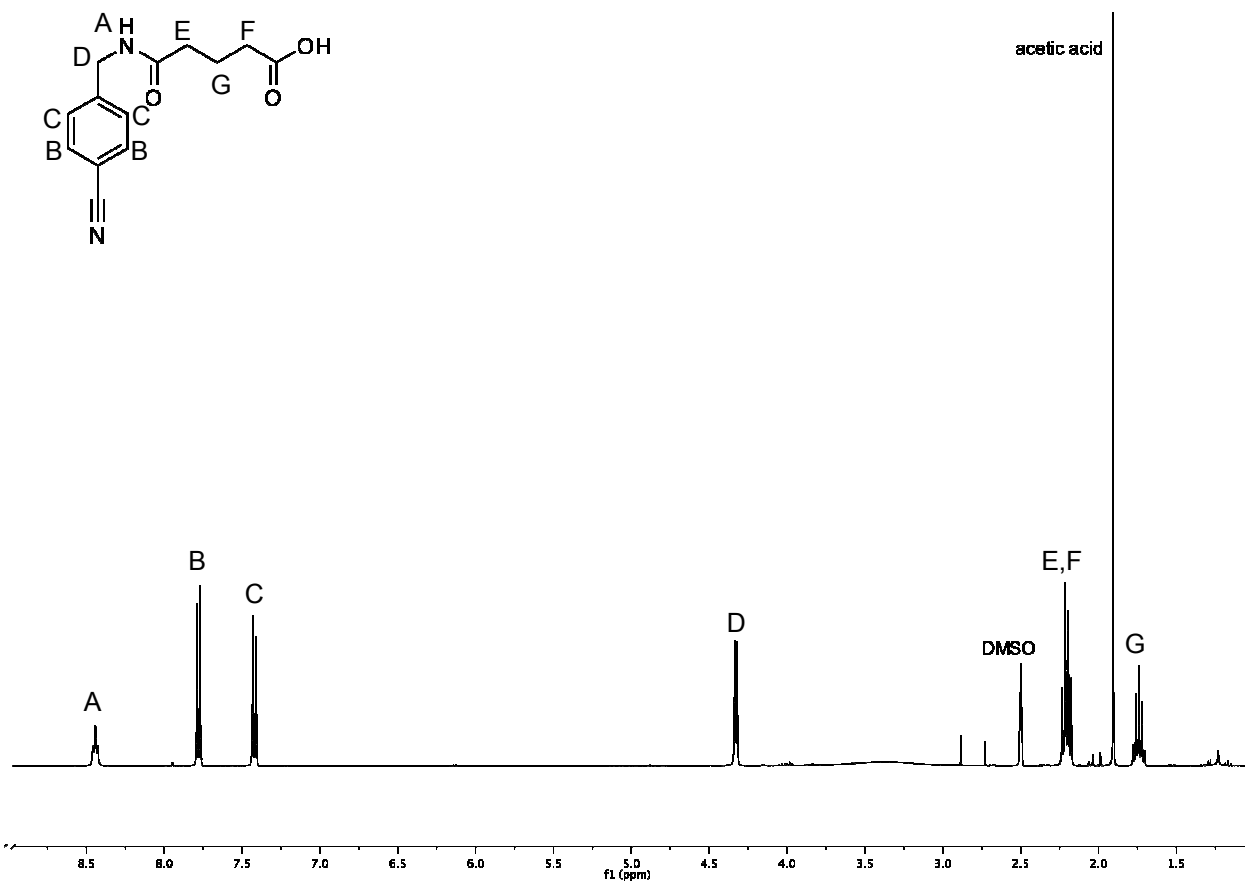


Figure S1. ¹H NMR of 5-(4-(cyano)benzylamino)-5-oxopentanoic acid.

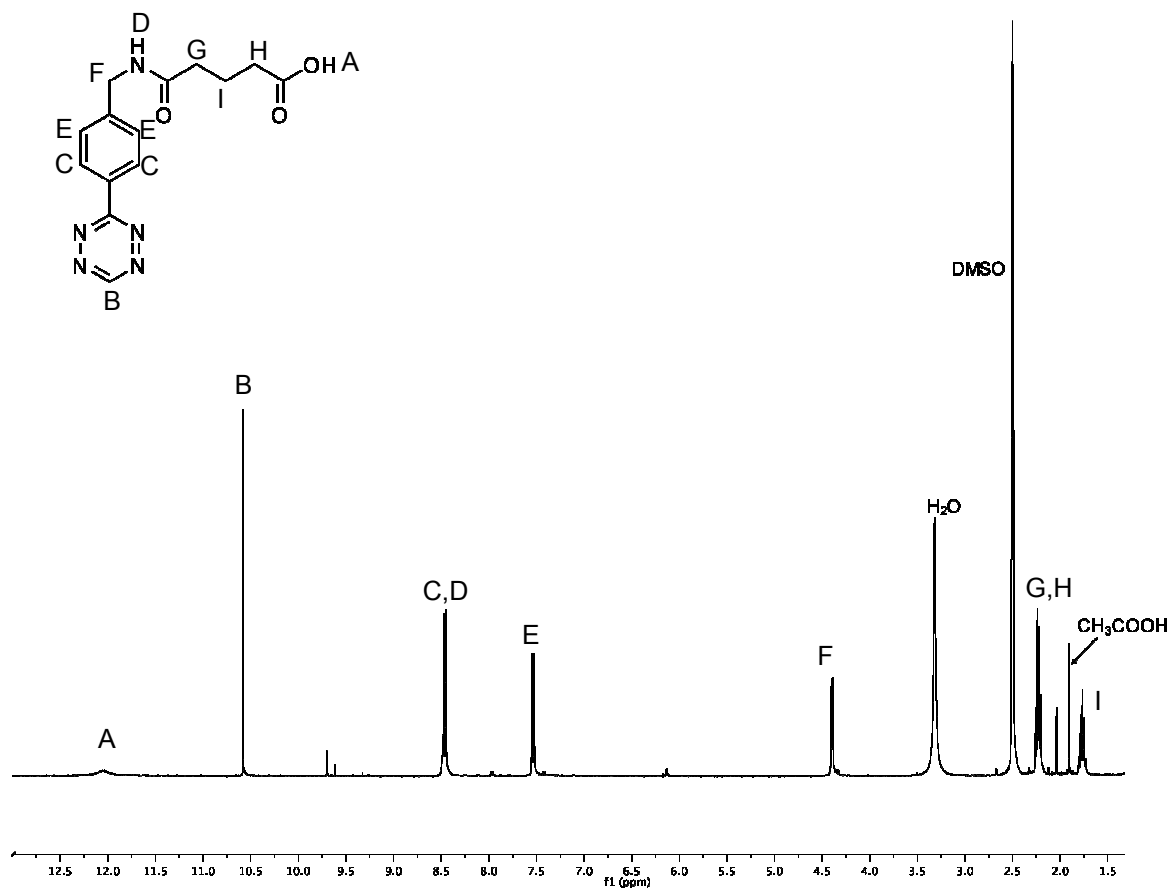


Figure S2. ¹H NMR of 5-(4-(1,2,4,5-tetrazin-3-yl)benzylamino)-5-oxopentanoic acid.

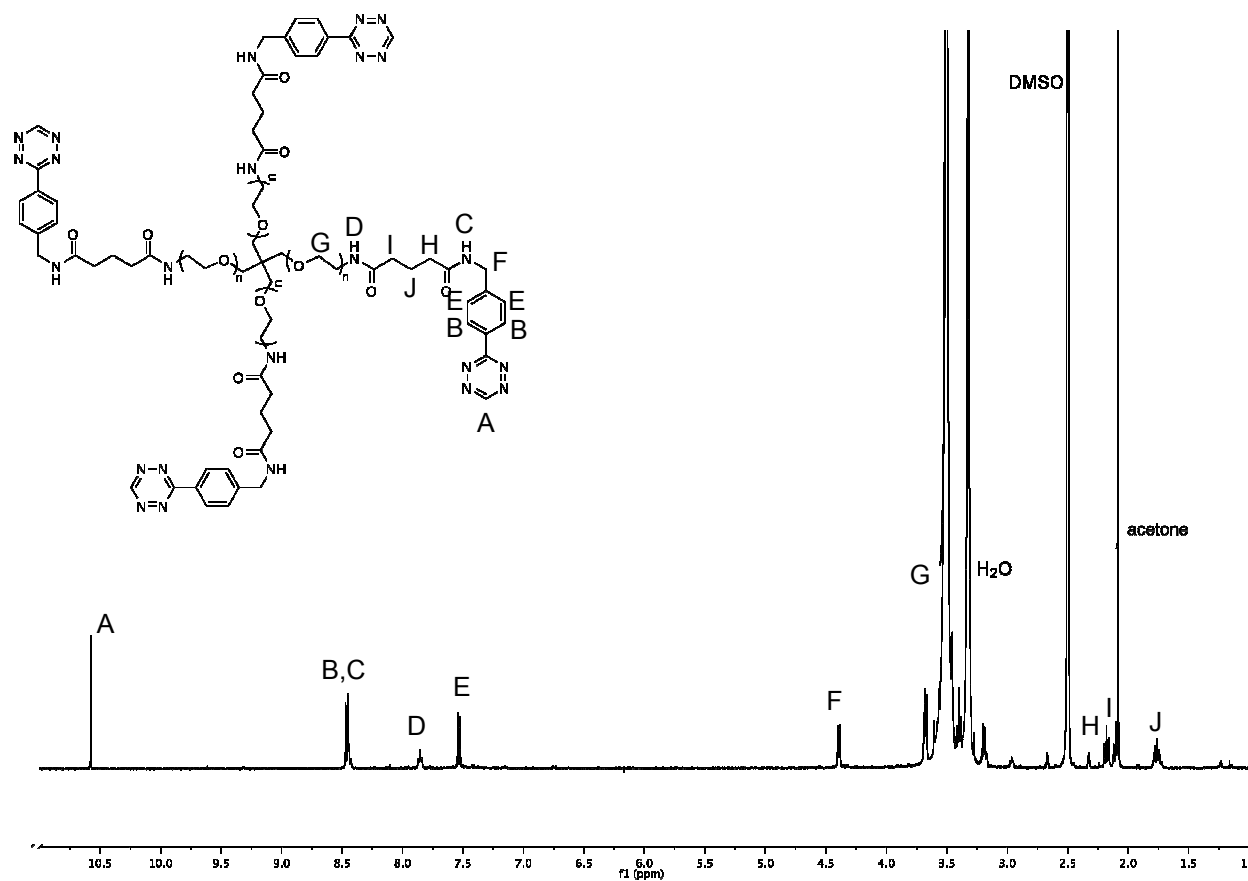


Figure S3. ¹H NMR of PEG-Tz.

5. References

- (1) Devaraj, N.K.; Weissleder, R.; Hilderbrand, S.A. *Bioconjug. Chem.* **2008**, *19*, 2297.
- (2) Anderson, S.B.; Lin, C.C.; Kuntzler, D.V.; Anseth, K.S. *Biomaterials.* **2011**, *32*, 3564.