

Electronic Supplementary Information (ESI)

Design of functionalized cyclic peptides through orthogonal click reactions for cell culture and targeting applications

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Experimental

Materials

General organic reagents were purchased from commercial sources and used as received unless otherwise stated. 8-arm PEG amine (20 kDa) was purchased from JenKem Technology USA Inc. (Allen, TX). PEG-*bis*-azide (3.4 kDa) was purchased from Creative PEGworks (Chapel Hill, NC). MBHA Rink-Amide ChemMatrix resin and all amino acids were purchased from ChemPep (Wellington, FL). Deionized water (18 M Ω -cm) was purified onsite using a Milli-DI water purification system (Millipore Sigma, Burlington, MA). All other specialty reagents used are specified within methods below.

Linear Peptide Synthesis

The peptide sequences **cGRGDdvK(alloc)DRK(azide)** (lower case letters indicate D-isomers) and **K(azide)RDVPMS↓MRGGDRK(azide)** were synthesized using standard Fmoc chemistry for solid phase peptide synthesis (SPPS) on a PS3™ Benchtop Peptide Synthesizer (PS3). Briefly, 0.25 mmol equivalents of MBHA Rink-Amide ChemMatrix (0.32 g mmol⁻¹) resin was placed into a standard PS3 reaction vessel. The resin was swollen in dimethylformamide (DMF) for 10 min before the addition of amino acids. Vials containing 4x excess equivalents of amino acid and 3.5x excess equivalents of N,N,N',N'-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), which was used as the activating agent, were loaded onto the machine. All amino acid additions were done using a deprotection, activation, and addition sequence programmed into the PS3. Fmoc deprotection was performed twice for 5 min with 20% piperidine in DMF. Amino acid activation consisted of 30 s mixing with 0.4 M N-methylmorpholine (4-MMP) in DMF followed by addition to the resin for 1 h with mixing. All amino acids were double coupled and additional DMF wash steps were added before and after deprotection and amino acid addition. Capping of the cRGDdvK(alloc)DRK(azide) peptide with

acetic anhydride was done after the addition of K(azide), K(alloc), and each amino acid of the RGD sequence to ensure all the functional components of the peptide for immobilization, cyclization, and integrin binding, respectively, were present in the final purified sequence.

After synthesis, the resin was transferred to a glass reaction vessel for manual SPPS, and the peptide cleaved from the resin using a cleavage solution of 95 % trifluoroacetic acid (TFA) (v/v), 2.5 % (v/v) DI H₂O, 2.5 % (v/v) triisopropylsilane (TIPS), and 5 % (w/v) tris(2-carboxyethyl)phosphine (TCEP). After 2 h of mixing, the cleavage solution was collected from the vessel, and the peptide precipitated into ice-cold ethyl ether. The peptide was centrifuged, filtered, and washed three times with fresh cold ethyl ether. The peptide was allowed to dry at room temperature overnight. The dried peptide was dissolved in a solution of deionized (DI) water:acetonitrile (95 % (v/v): 5 % (v/v)) to a concentration of approximately 20 mg/mL. The peptide was purified using reverse phase high performance liquid chromatography (HPLC) (XBridge BEH C18 OBD 5 µm column, linear gradient from 95:5 to 5:95 water:acetonitrile over 25 min) (Waters Corporation, Milford, MA). The collected fraction was frozen and lyophilized. The peptide sequence was confirmed using electrospray ionization (ESI+) mass spectrometry (Acquity UPLC H-class/SQD2) (Waters Corporation, Milford, MA).

Synthesis of the Photoinitiator Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP)

Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) was synthesized following a previously published protocol.^{1,2} Briefly, 2,4,8-trimethylbenzoyl chloride (9 mmol) was added dropwise to dimethyl phenylphosphonite (9 mmol). The solution was reacted overnight under argon at room temperature. Lithium bromide (36 mmol) in 2-butanone was added to the reaction solution and heated to 50 °C for 10 min when a solid white precipitate formed. Once the solution returned to room temperature the solution was filtered and rinsed three times with 2-butanone to obtain the precipitate. The final product was dried under vacuum and analyzed by ¹H NMR

(Bruker AVIII 600) (Bruker Daltonics, Billerica, MA). ^1H NMR (D_2O , 600 MHz): $\delta=7.59$ (m, 2H), $\delta=7.49$ (m, 1H), $\delta=7.34$ (m, 2H), $\delta=6.77$ (s, 2H), $\delta=2.11$ (s, 3H), $\delta=1.90$ (s, 6H).

Synthesis of Cyclic RGD

The linear RGD peptide was cyclized in solution utilizing a photoinitiated thiol-ene reaction between the thiol of the Cys on the N-terminus and the alloc protecting group installed on the Lys in the eighth position. The linear peptide was added to a scintillation vial and dissolved to 5 mM in DI water containing 5 mM LAP. The peptide/LAP solution was irradiated with 365 nm light at 20 mW cm^{-2} for 10 min while stirring. After 10 min of irradiation, an Ellman's test was performed to qualitatively check for any free thiols. Briefly, 20 μL of the peptide solution was added to 180 μL of Ellman's reagent in Ellman's reaction buffer. After mixing the solution with a syringe, the color of the resulting solution was assessed qualitatively to determine if free thiols remained; in all batches to date, no free thiols were observed after 10 min of irradiation. The cyclized peptide was purified by HPLC using the protocol above.

Characterization of Cyclic RGD

Peptide cyclization was confirmed using ESI+ mass spectrometry, ^1H NMR, and 2D COSY, TOCSY and NOESY (Bruker Avance 600 MHz spectrometer). ESI+ mass spectrometry was performed to confirm the molecular weight of the purified peptide and confirm that no peptide oligomers were present. ^1H NMR was performed on both the linear and cyclic peptides with 128 scans at 15 mg/mL in deuterated DMSO. ^1H NMR spectra confirmed the disappearance or shift of peaks associated with the alloc protecting group of Lys ($\delta=5.90$ (m, 1H), $\delta=5.26$ (d, 1H), $\delta=5.17$ (d, 1H), $\delta=4.45$ (d, 2H)) and the Cys ($\delta=4.06$ (m, 1H), $\delta=2.97$ (d, 1H), $\delta=2.91$ (d, 1H), and $\delta=1.36$ (s, 1H)).

2D NOSEY, TOCSY, and COSY spectrum were obtained for both the linear and cyclic peptides using the same samples prepared for ^1H NMR (Bruker Avance 600 MHz spectrometer). Bruker standard pulse programs (noesygp, dipsi2etgp, and cosygpmfph) were used to acquire these 2D data. Typically, 2K data points were acquired in the acquisition dimension, and at least 400 points were acquired along the evolution dimension. ^1H chemical shift assignment was performed by first identifying the protons in the linear spectrum then using these assignments to find proton shifts and correlations in the cyclic spectrum. To identify protons in the linear spectrum, the amide proton NOSEY correlations were first used to trace the shifts associated with the amide peaks of the peptide backbone. After the amide protons had been identified the TOCSY and COSY, spectrums were used to identify the protons of the amino acid side chains. Once the linear peptide was characterized, a combination of the ^1H NMR and TOCSY spectrums were used to identify new peaks that appeared after peptide cyclization and the proton associations of those peaks. This analysis provided a final confirmation of peptide cyclization through the identification of protons associated with the alkyl-sulfide bond formed by the thiol-ene reaction.

Synthesis of DBCO end-functionalized eight-arm PEG

Eight-arm dibenzocyclooctyne end-functionalized poly(ethylene glycol) (*PEG-8-DBCO*) (40 kDa) was synthesized using carbodiimide coupling chemistry.³ Briefly, dibenzocyclooctyne-acid (DBCO-C6-Acid, 0.088 mmol) (Click Chemistry Tools, Scottsdale, AZ) was dissolved in anhydrous DMF (0.25 mL). 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate (HATU) (0.08 mmol), diisopropylethylamine (DIPEA) (0.18 mmol), and amine end-functionalized 8-arm PEG (0.005 mmol) were added to the reaction vessel, and the reaction was allowed to proceed while mixing overnight at room temperature. The reaction solution was subsequently precipitated in cold ethyl ether. The polymer was recovered by

filtration and washed three more times with cold ethyl ether. The polymer was recovered by centrifugation and dried overnight at room temperature under reduced pressure. The functionality of the resulting polymer was confirmed using ^1H NMR (600 MHz, DMSO). ^1H NMR (DMSO- d_6 , 600 MHz): $\delta=7.65$ (t, 1H), $\delta=7.46$ (m, 8H), $\delta=5.03$ (d, 1H), $\delta=3.51$ (m, PEG backbone), $\delta=3.11$ (m, 2H), $\delta=2.14$ (m, 1H), $\delta=1.84$ (m, 1H), $\delta=1.74$ (m, 1H), $\delta=1.17$ (m, 3H).

Synthesis of Fluorescent cyclic RGD

Cyclic RGD-Az (2 mM in PBS, 1 eq) was reacted with Alexa FluorTM 488 DIBO Alkyne (2 mM in DMSO, 1.1 eq, AF488-DIBO) (ThermoFisher, Waltham, MA) overnight at room temperature. The reaction mixture was subsequently diluted to 100 μM in PBS before further use. Conjugation of AF488-DIBO to cyclic RGD-Az was confirmed using ESI+ mass spectrometry as described above.

Formation of SPAAC hydrogels

Hydrogels were formed using a strain promoted azide alkyne cycloaddition (SPAAC) by mixing PEG-8-DBCO with an azide functionalized crosslink. For 2D cell experiments, PEG-8-DBCO (14 mM final DBCO concentration, 2.0 mM final PEG-8-DBCO concentration) was mixed with PEG-2-Az (12 mM final azide concentration, 6 mM final PEG-2-Az concentration) in PBS. For cyclic RGD incorporation into the hydrogel, cyclic RGD (2 mM final azide concentration, 2 mM final cyclic RGD concentration) was mixed with PEG-8-DBCO and allowed to react for 20 min at room temperature prior to hydrogel formation. The gel precursor solutions were aliquoted into 1 mL syringe molds and mixed by pipette to form 20 μL cylindrical hydrogels. Gels were polymerized in the syringe mold for 1 h, which was determined to be adequate time for complete gelation based on rheometric measurements (see below), before transferring to a 48-well plate.

For 3D cell experiments, PEG-8-DBCO (10 mM final DBCO concentration, 1.5 mM final PEG-8-DBCO concentration) was mixed with K(Az)RDVPMS↓MRGGDRK(Az) (8 mM final azide concentration, 4 mM final peptide concentration) in phosphate buffered saline (PBS). For cyclic RGD (2 mM final azide concentration, 2 mM final cyclic RGD concentration) was mixed with PEG-8-DBCO and allowed to react overnight at room temperature prior to hydrogel formation. Hydrogels (20 μ L) were polymerized in 1 mL syringes for 20 min at 37 $^{\circ}$ C before being transferred to a 48-well plate (see 3D hydrogel cell culture procedure below).

The mechanical properties of the hydrogels were determined by rheological measurements. Hydrogel precursor solution was prepared at a total volume of 15 μ L, 7 μ L was pipetted onto a Peltier plate on a rheometer fitted with an 8 mm flat plate geometry (AR-G2) (TA Instruments, New Castle, DE). Measurements were collected at room temperature at a gap height of 120 μ m. Time sweep measurements of the storage (G') and loss (G'') moduli were recorded at 1 % applied strain and 6 rad s^{-1} frequency, which is in the linear-viscoelastic regime for these hydrogels. Mineral oil was added to the outside of the geometry to ensure the hydrogel stayed hydrated throughout the time course. The time to complete gelation, defined when the percent change in modulus between consecutive data points was less than 10 %, and the final storage moduli were 4450 ± 1060 Pa and 2770 ± 190 Pa for the 2D and 3D hydrogel formulations, respectively. Respectively, these moduli are typically used for 2D and 3D cell culture applications.³

Cell Culture

MDA-MB-231 breast cancer cells were cultured in complete growth medium, which included Dulbecco's Modification of Eagle's Medium (DMEM) (Corning; Tewksbury, MA) with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (ThermoFisher). Cells were expanded in 75 cm^2 tissue culture flasks (T-75) (Celltreat; Pepperell, MA) and passaged every 3

to 5 days. To passage cells, the T-75 was rinsed with 2.5 mL of PBS, incubated with 2.5 mL of 0.25% Trypsin/0.1% EDTA (Corning) at 37 °C for 5 minutes for cell dissociation, spun down at 1000 RPM for 5 minutes, and re-suspended in complete medium. Cells were passaged every 3 to 5 days (~80% confluency).

2D Hydrogel Cell Attachment Assay

MDA-MB-231 cells were seeded onto hydrogels presenting cyclic RGD, linear RGD, or no peptide (n = 3) to study cell attachment. The day prior to cell seeding, SPAAC hydrogels were formed as discussed above. The hydrogels were placed in 48-well non-tissue culture treated plates (Celltreat). Before seeding, hydrogels were incubated in 0.5 mL of medium per well for 1 h at 37 °C. Cells were dissociated and re-suspended in complete medium as described above. Cells were counted and seeded at 15,000 cells per cm² in 0.5 mL of media per well. 8 hours after seeding, the culture medium was replaced in each well, and bright-field images were taken to examine preliminary cell attachment. At 24 h, cells were fixed and stained for cell cytoskeleton (F-actin, phalloidin-TRITC) and nuclei (DAPI) as detailed below.

3D Hydrogel Cell Culture Platform Investigation

MDA-MB-231 cells were encapsulated at 5x10⁶/mL within 20 µL hydrogels. For preparation of the hydrogel precursor solution, azido-cyclic RGD was pre-reacted overnight with PEG-8-DBCO. The next day, PEG precursor solutions with cyclic RGD or without cyclic RGD (negative control) were aliquoted into 1 mL syringes for formation of individual hydrogels. Cells subsequently were counted and resuspended in di-azide MMP-degradable crosslinker (VPMS↓MRGG) and PBS, and this cell suspension was mixed with precursor solution aliquoted in syringes to encapsulate cells in hydrogels formed with SPAAC chemistry. Hydrogels were

incubated at 37 °C for 20 minutes, allowing sufficient time for gel formation. Hydrogels subsequently were placed in 48-well non tissue culture treated plates and cultured in 0.5 mL of complete medium. Culture medium was replaced with fresh medium every 2 to 3 days.

Live/Dead Staining

Viability of MDA-MB-231 cells encapsulated in hydrogels was determined with a LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen; Eugene, OR) on day 1 and day 7 after encapsulation. Live cells were stained by Calcein AM (ex/em ~ 495 nm/~515 nm), and dead cells were stained by Ethidium homodimer-1 (ex/em~495 nm/635 nm). Solutions were added to PBS at final concentrations of 2 mM and 4 mM, respectively. Hydrogels were washed 2 times with 0.5 mL of PBS per hydrogel for 5 min at 37°C before and after staining. Hydrogels were stained for 18-minutes at 37°C with 0.5 mL of calcein AM and ethidium homodimer-1 solution. After staining, hydrogels were transferred to a chamber slide (Nunc™ Lab-Tek™ II Chamber Slide, Glass, 1 well) for confocal imaging. 200 µm z-stacks were imaged with a Zeiss LSM 800 confocal microscope and orthogonally projected for counting live (green) and dead (red) cells. Three z-stack images were taken per hydrogel, and three hydrogels were imaged per condition. Green and red cells were counted by ImageJ and summed for a total cell count. The percentage of viable cells was calculated by the number of green cells/total cell count x 100%.

Metabolic Activity Assay

AlamarBlue® cell viability reagent (Thermo Fisher) was used to measure MDA-MB-231 metabolic activity in hydrogels at days 1 and 7 time points. AlamarBlue reagent was added 1:10 to complete medium, and 0.5 mL of solution was added per hydrogel and incubated at 37°C for 4 hours. The digested media was collected, and gels were replenished with fresh media for

continued culture. Digested media (0.1 mL) was added to a black 96-well plate, and fluorescence measurements were read on a plate reader (ex/em 560 nm/590 nm). n = 3 for each condition.

Immunostaining and Imaging

Cells were fixed with 4% PFA (Milliore Sigma), 0.25 mL of solution per hydrogel, at room temperature for 15 minutes. After fixation, hydrogels were rinsed 2 times with 0.5 mL PBS and blocked and permeabilized in 0.3 mL of 5% BSA with 0.1% TritonX for 1 h at room temperature. For 3D encapsulation, MDA-MB-231 cells were immunostained for Ki67, a proliferation marker. Mouse anti-human primary monoclonal antibody Ki67 (abcam; Cambridge, MA) was stained in 0.2 mL of 5% BSA + 0.1% TritonX solution (1:100) overnight at 4 °C. The next day hydrogels were washed 3 times in 3% BSA + 0.05% TritonX, for 45 minutes per wash. 2D cell attachment and 3D cell encapsulation hydrogels were stained for cytoskeleton (F-actin) and nuclei (DAPI). Cell cytoskeleton was stained with (1:250) Phalloidin-Tetramethylrhodamine B isothiocyanate (Millipore Sigma; St. Louis, MO) in 5% BSA + 0.1% TritonX overnight at 4 °C. The next day, hydrogel samples were washed 3 times in 3% BSA + 0.05% TritonX, for 45 minutes per wash. The nucleus was stained with 700 nM DAPI for one hour at room temperature followed by 3 times PBS washes before imaging.

Hydrogels were imaged with a Zeiss LSM 800 confocal microscope. Hydrogels were transferred to a chamber slide (Nunc™ Lab-Tek™ II Chamber Slide, Glass, 1 well), and three images were taken per hydrogel at a frame size of 1024 x 1024. For 3D cell encapsulation, 200 μm z-stacks were imaged with a 20x objective. For MDA-MB-231 2D attachment to cyclic RGD, linear RGD, and no peptide hydrogels, images were taken with a 10x objective. Cells were counted per field of view and averaged for each hydrogel condition to obtain the average number of MDA-MB-231 cells attached per field of view to the hydrogel surface.

Fluorescent MDA-MB-231 Labeling

The AF488 conjugated cyclic RGD peptide was added to MDA-MB-231 cells to evaluate labeling of the cell surface. Cells were seeded in a 96-well plate at 90,000 cells per cm² (30,000 cells per well) two days prior to adding the AF488 cyclic RGD peptide, allowing for sufficient confluency (~70%) for the experiment. Either 20 µM AF488 cyclic RGD or 20 µM AF488 DIBO (control) diluted in phenol red-free FBS-free DMEM culture medium (Corning; Tewksbury, MA) was added to cells (0.1 mL per well) and incubated for 4 hours at 37°C and 5% CO₂ (n = 3, 3 images per sample). After incubation, wells were rinsed 4 times with 0.1 mL PBS, fixed with 4% PFA for 15 minutes at room temperature, rinsed 2 times with PBS, stained with DAPI (700 nM) 30 minutes on a rocker, and rinsed 2 times with PBS before imaging.

Labeled cells were imaged with a Zeiss LSM 800 confocal microscope with a 10x objective. Bright field images with DAPI and AF488 channels were taken to image MDA-MB-231 cells for AF488 cyclic RGD binding. Three images were taken per well. AF488 fluorescence and DAPI counts were analyzed by ImageJ for each image to normalize total fluorescence on a per cell basis. Percentage of fluorescent cells reported was calculated as the average of normalized fluorescence per cell x 100% from three images per well (n = 3, per sample) for AF488 cyclic RGD and AF488 DIBO (control) samples.

Statistical Analysis

All values are reported as average and standard error (n = 3 for each condition). Unless otherwise stated, statistical significance was determined by a two-sided Student T-test.

Supplemental Figures

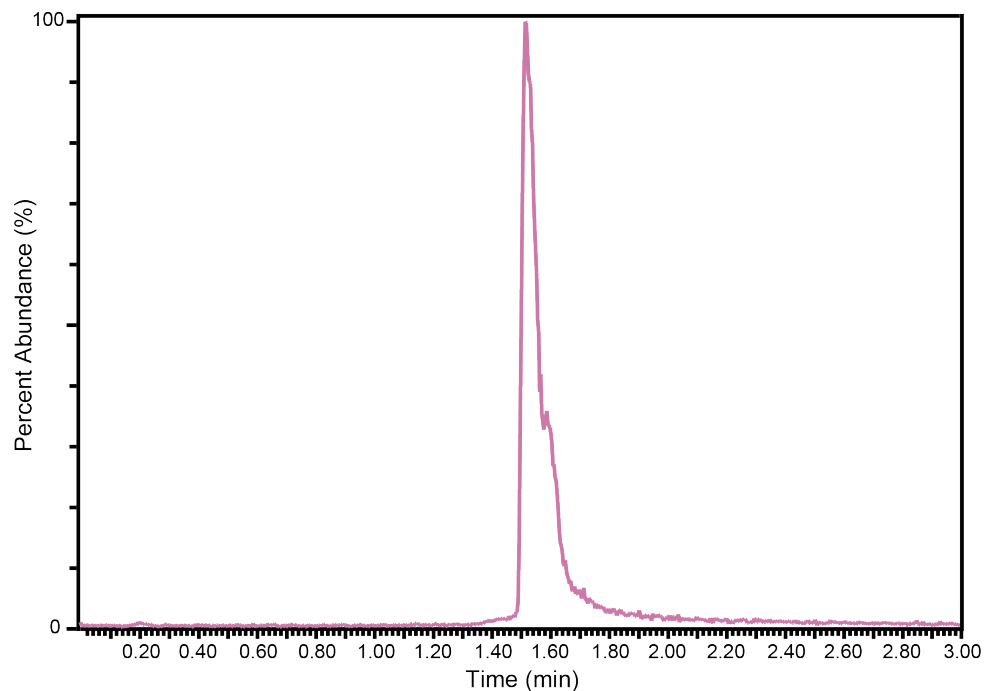


Figure S1. *Linear RGD UPLC Trace.* Linear RGD was purified using reverse phase HPLC, and the desired fraction was collected and characterized using LC-MS. A single peak was observed in the UPLC trace for the purified linear fraction, indicating a pure peptide that had a mass corresponding to the desired product (Fig. 2a).

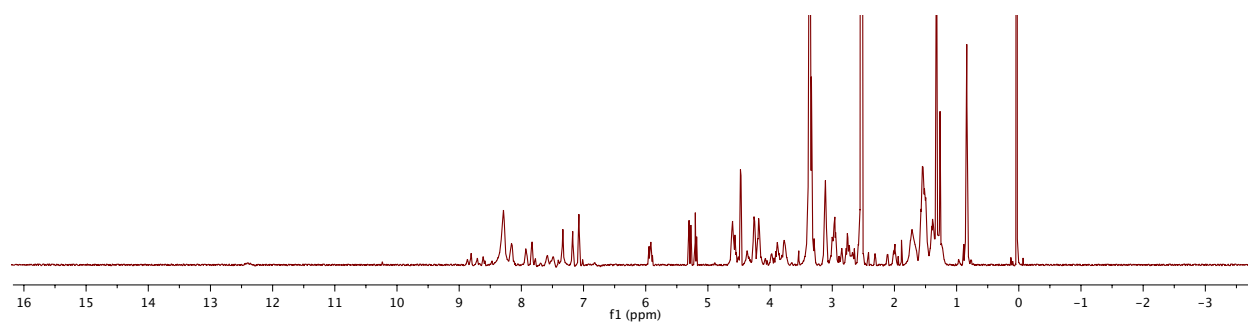


Figure S2. *Linear RGD ¹H NMR Spectrum.* Complete ¹H spectrum of linear RGD sequence (DMSO, 600 MHz).

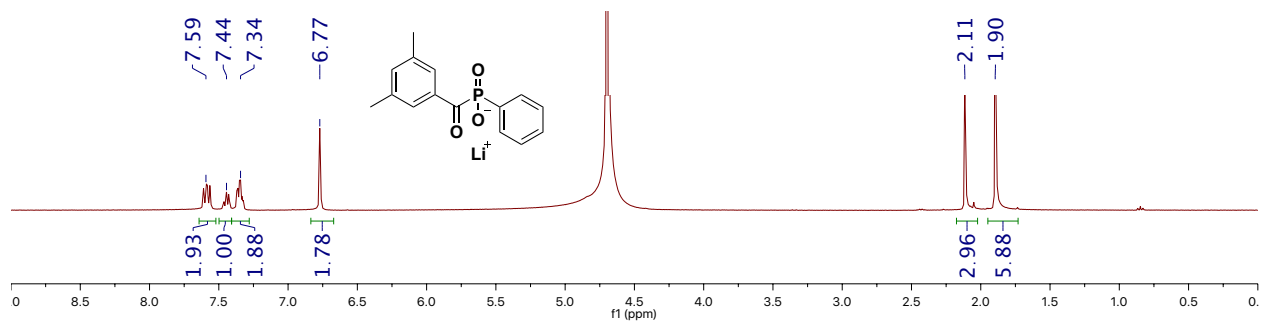


Figure S3. LAP ^1H NMR Spectrum. Complete ^1H spectrum of LAP confirming product formation (D₂O, 600 MHz).

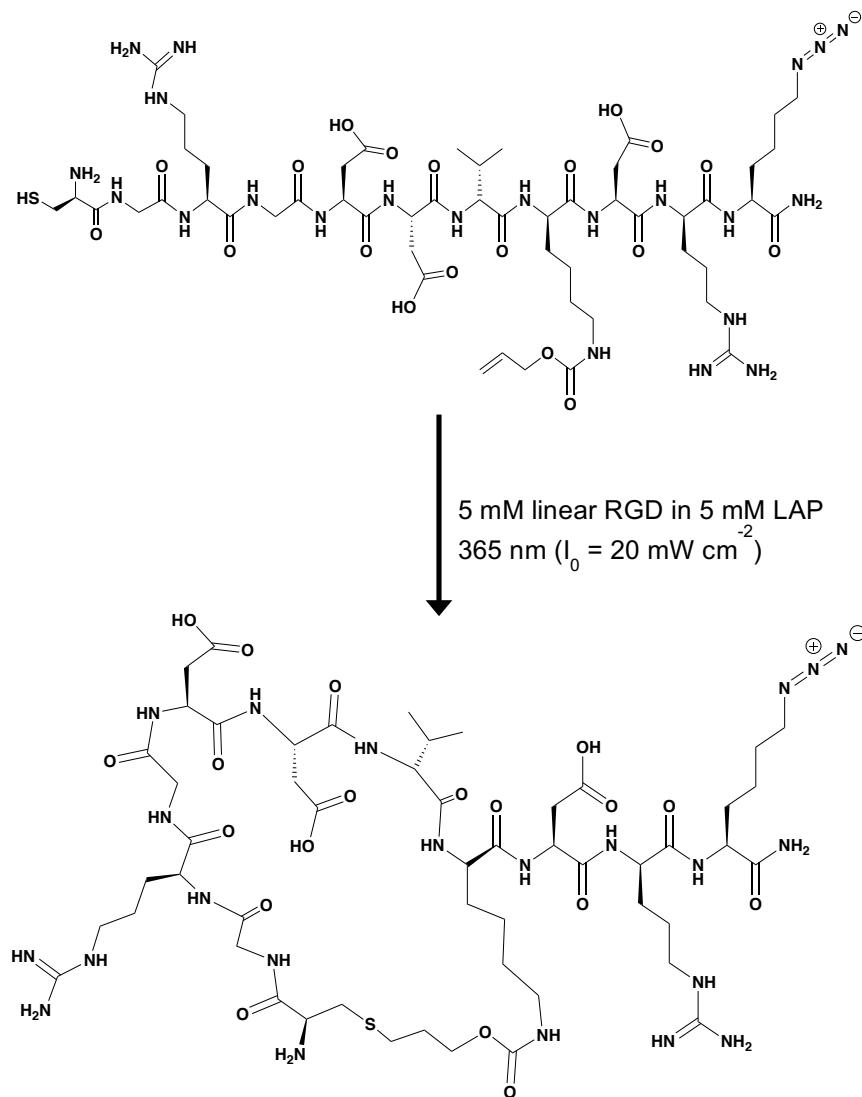


Figure S4. Chemical structures of linear and cyclic RGD peptides. Linear RGD (top) was cyclized using a photoinitiated thiol-ene reaction between the free thiol of the N-terminus Cys and the alloc group of alloc-protected Lys found in the 8th position on the peptide backbone. The thiol-ene reaction was conducted in an aqueous solution using dilute linear RGD (5 mM) in the presence of LAP (5 mM) irradiated for 10 min (20 mW cm⁻² at 365 nm) to form cyclic RGD (bottom).

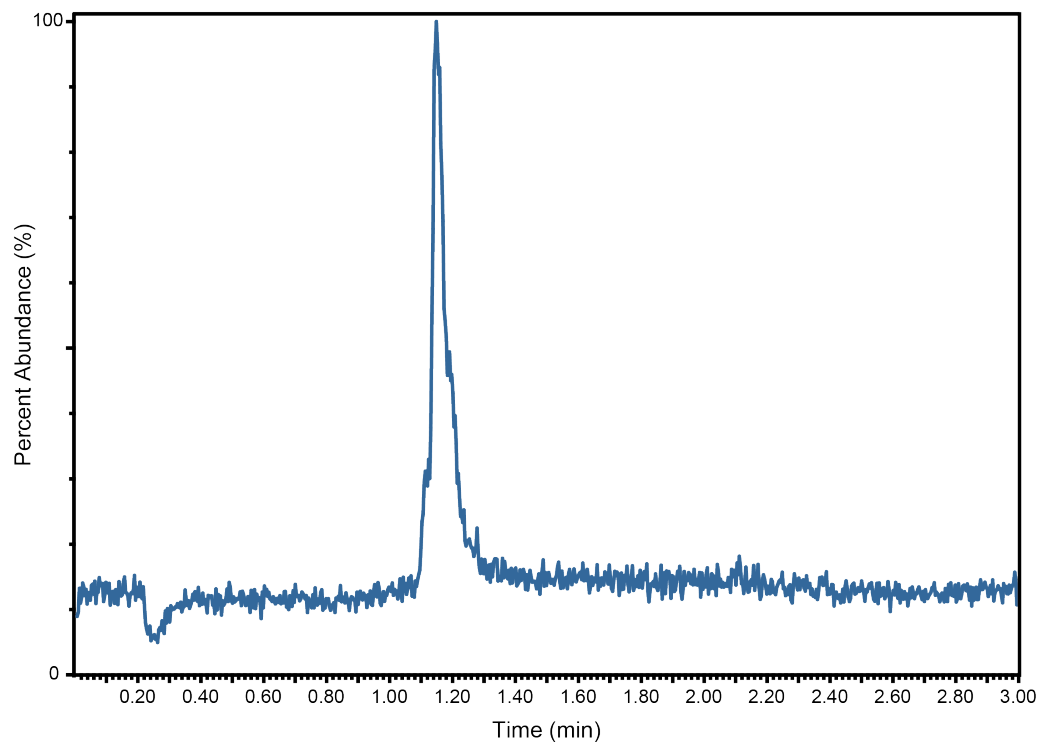


Figure S5. *Cyclic RGD UPLC Trace.* Cyclic RGD was purified using reverse phase HPLC and the desired fraction was collected and characterized using LC-MS. A single peak was observed in the UPLC trace for the purified linear fraction, indicating a pure peptide that had a mass corresponding to the desired product (Fig. 2b).

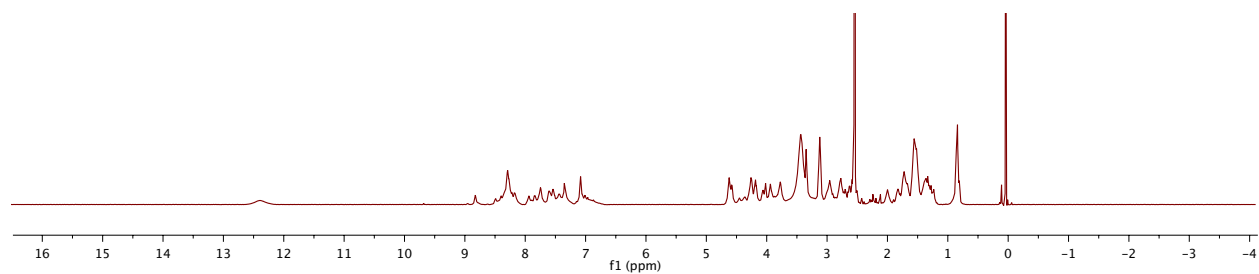


Figure S6. *Cyclic RGD ¹H NMR Spectrum.* Complete ¹H spectrum of cyclic RGD sequence (DMSO, 600 MHz).

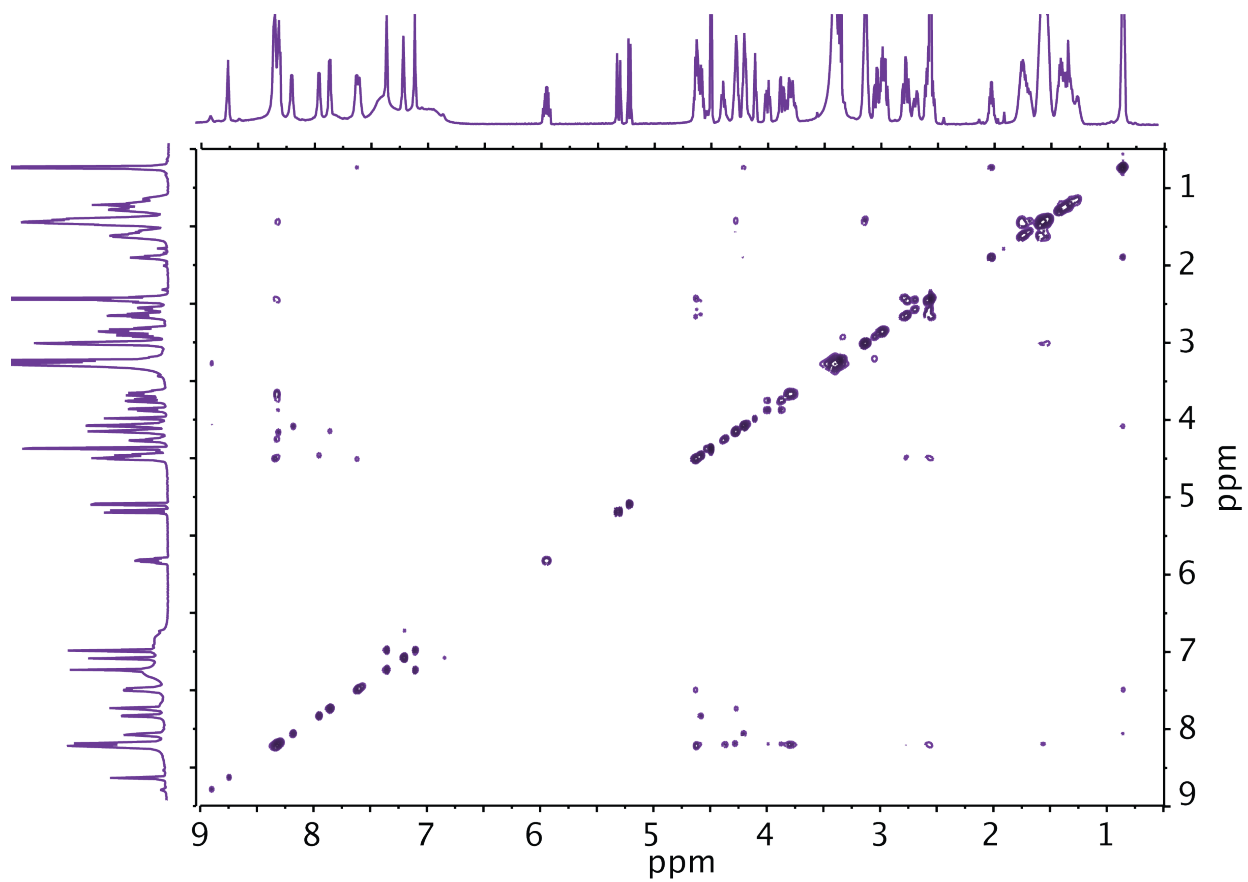


Figure S7. NOSEY for Linear RGD. NOSEY spectrum used to help assign proton shifts associated with the linear RGD sequence (DMSO, 600 MHz).

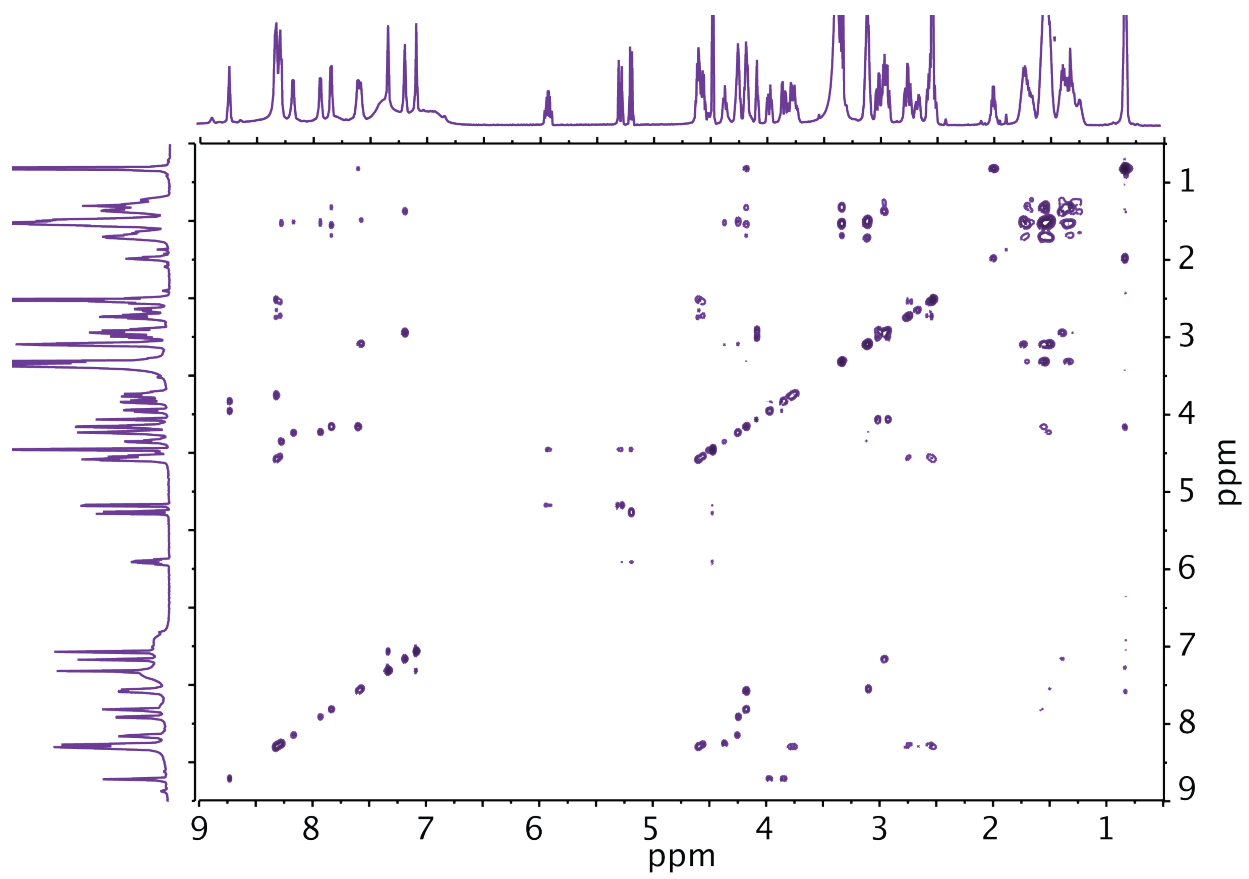


Figure S8. TOCSY for Linear RGD. TOCSY spectrum used to help assign proton shifts associated with the linear RGD sequence (DMSO, 600 MHz).

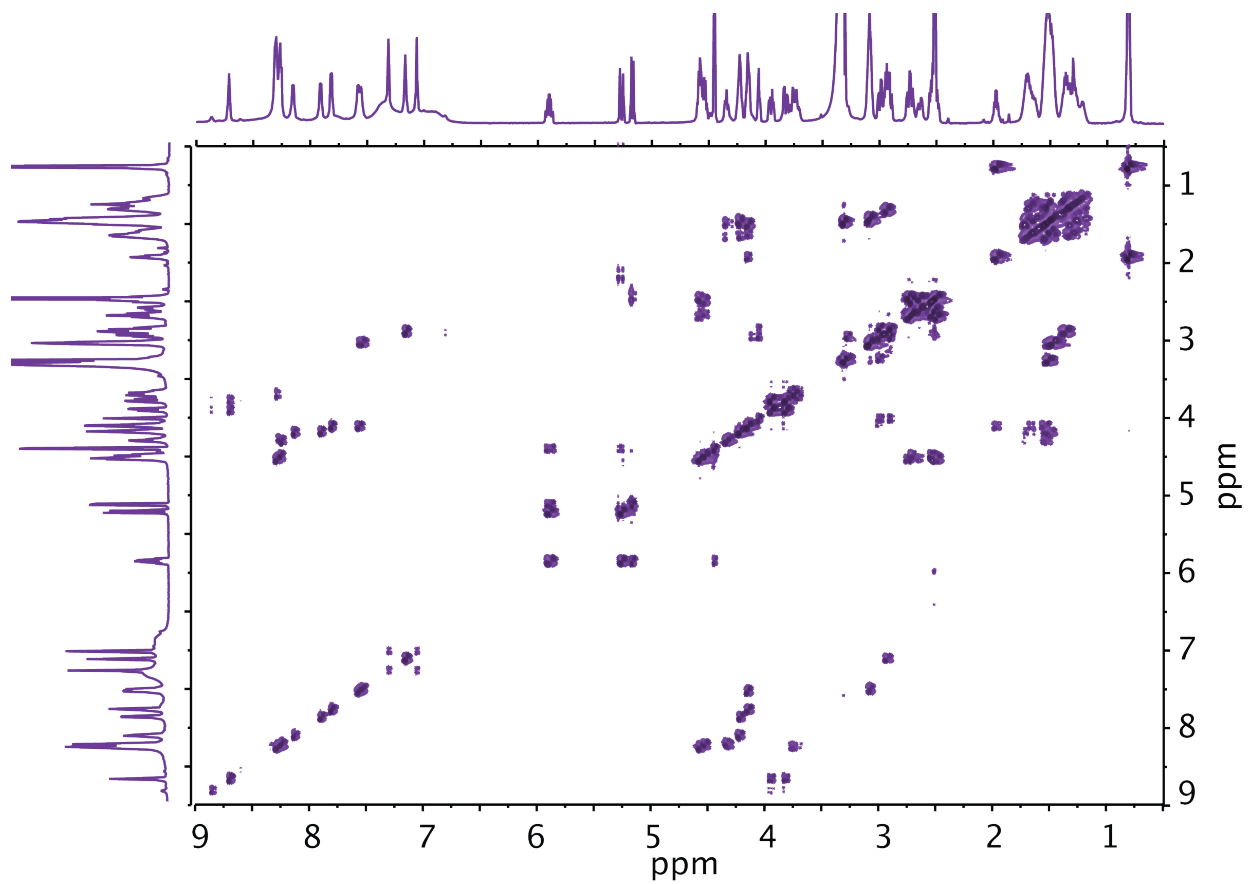


Figure S9. COSY for *Linear RGD*. COSY spectrum used to help assign proton shifts associated with the linear RGD sequence (DMSO, 600 MHz).

Table S1. Proton chemical shift assignment of linear RGD sequence as determined using NOESY, TOCSY, and COSY 2D NMR spectra. All reported proton chemical shifts are referenced to TMS.

| Amino Acid | Amide | α -H | β -H | γ -H | δ -H | ϵ -H | Other |
|------------|-------|--------------|--------------|-------------|-------------|---------------|--|
| c1 | -- | 4.06 | 2.97 2.91 | -- | -- | -- | SH – 1.36 |
| G1 | 8.86 | 3.85 3.83 | -- | -- | -- | -- | -- |
| R1 | 8.13 | 4.52 | 1.72 | 1.53 | 3.75 | -- | Side Chain Amide – 7.06 |
| G2 | 8.71 | 3.95 3.82 | -- | -- | -- | -- | -- |
| D1 | 8.26 | 4.34 | 2.72 2.54 | -- | -- | -- | -- |
| d2 | 8.3 | 4.57 | 2.73 2.51 | -- | -- | -- | -- |
| v1 | 7.57 | 4.15 | 1.98 | 0.81 | -- | -- | -- |
| K1 | 7.81 | 4.15 | 1.68 | 1.37 | 1.31 | 1.55 | Side Chain Amide – 7.17 Alloc OCH ₂ – 4.45 Alloc CH – 5.9, 5.26, 5.17 |
| R2 | 7.91 | 4.23 | 1.72 | 1.54 | 3.09 | -- | Side Chain Amide – 7.31 |
| D3 | 8.29 | 4.78 | 2.68 2.64 | -- | -- | -- | -- |
| K2 | 8.15 | 4.23 | 1.64 | 1.37 | 1.23 | 1.51 | -- |

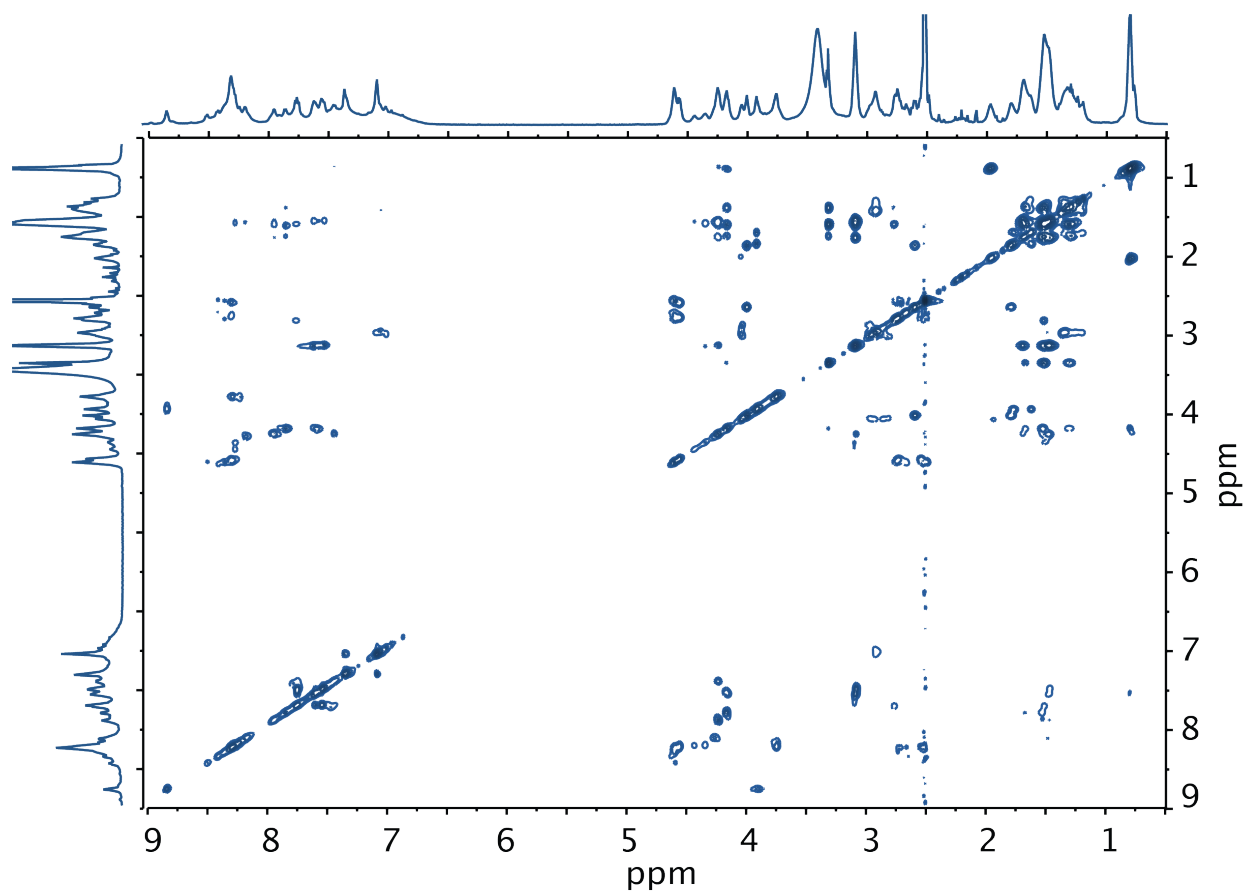


Figure S10. TOCSY for Cyclic RGD. TOCSY spectrum used to help confirm the alkyl-sulfide bond formation through the cyclization process for cyclic RGD formation (DMSO, 600 MHz).

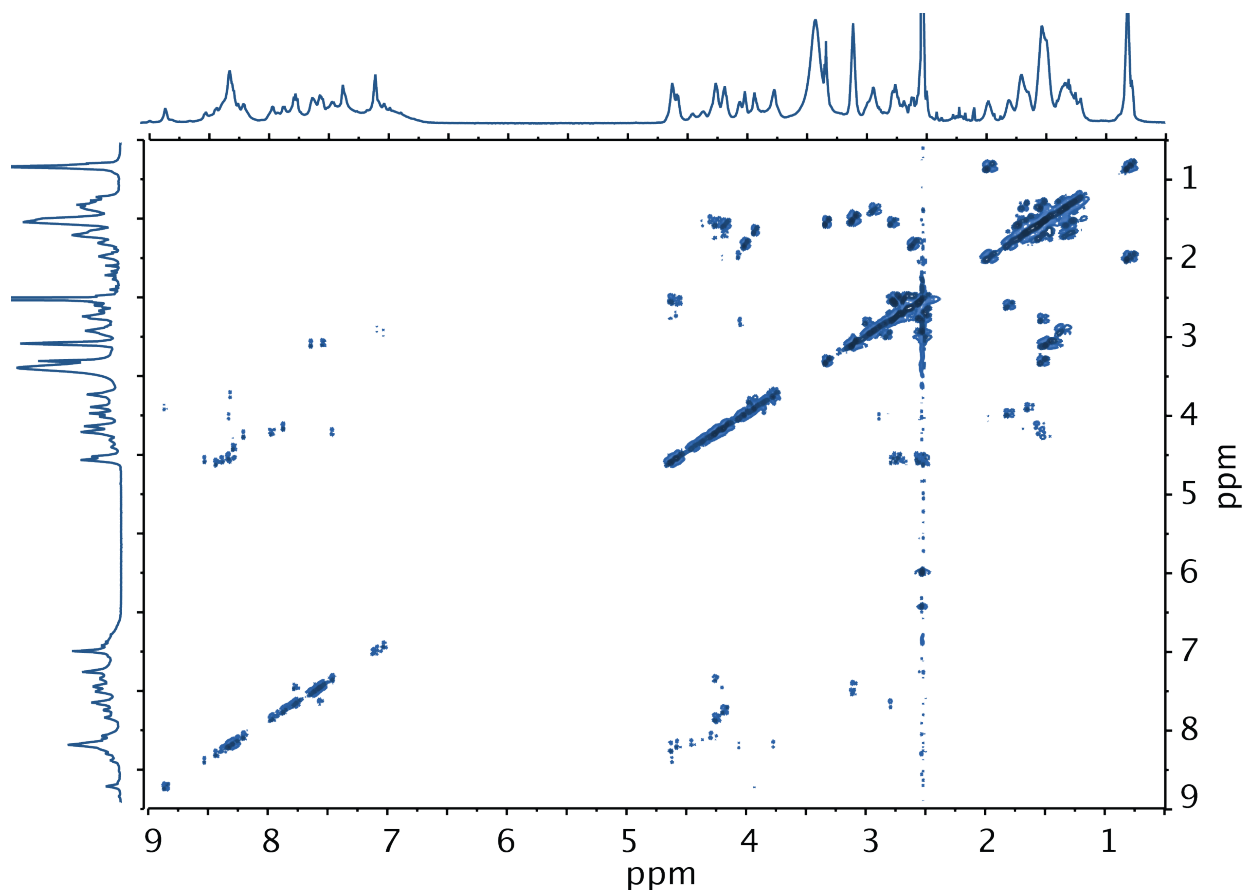


Figure S11. COSY for Cyclic RGD. COSY spectrum used to help confirm the alkyl-sulfide bond formation through the cyclization process for cyclic RGD formation (DMSO, 600 MHz).

Table S2. Proton chemical shift assignment of the linear and cyclic RGD sequence as determined by 2D NMR spectra. All reported proton chemical shifts are referenced to TMS.

| Amino Acid | Amide | α -H | β -H | Other |
|--------------------|-------|-------------|--------------|--|
| c1 - linear | -- | 4.06 | 2.97 2.91 | SH – 1.36 |
| c1 - cyclic | -- | 3.91 | 1.77 1.65 | SH – not observed |
| K1 -linear | 7.81 | 4.15 | 1.68 | Side Chain Amide – 7.17 Alloc OCH ₂ – 4.45 Alloc CH – 5.9, 5.26, 5.17 |
| K1 - cyclic | 8.14 | 4.25 | 1.64 | Side Chain Amide – 7.72 Alloc OCH ₂ – 3.99 Alloc CH ₂ – 2.59, 1.79 |

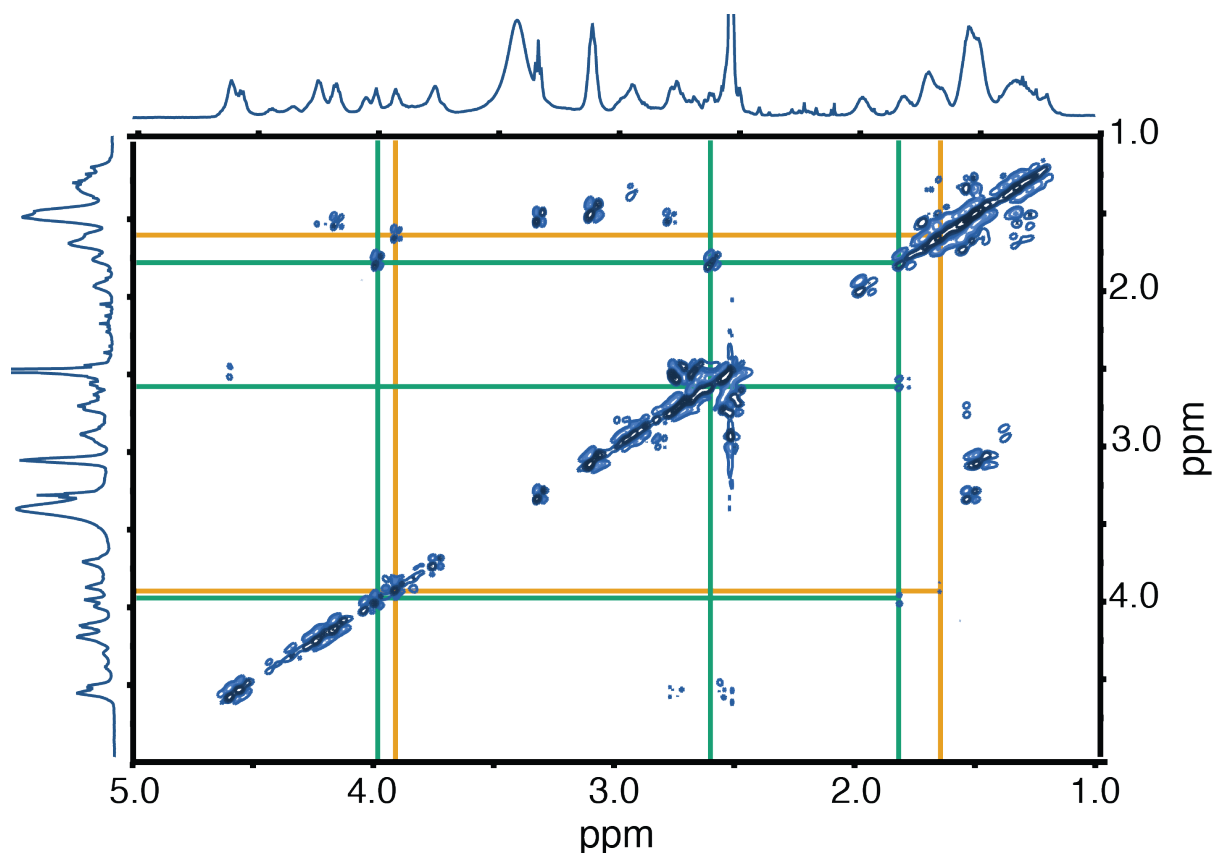


Figure S12. Cyclic RGD COSY Spectrum. Zoomed in version of COSY spectrum for cyclic RGD found in main text included for improving visualization (DMSO, 600 MHz).

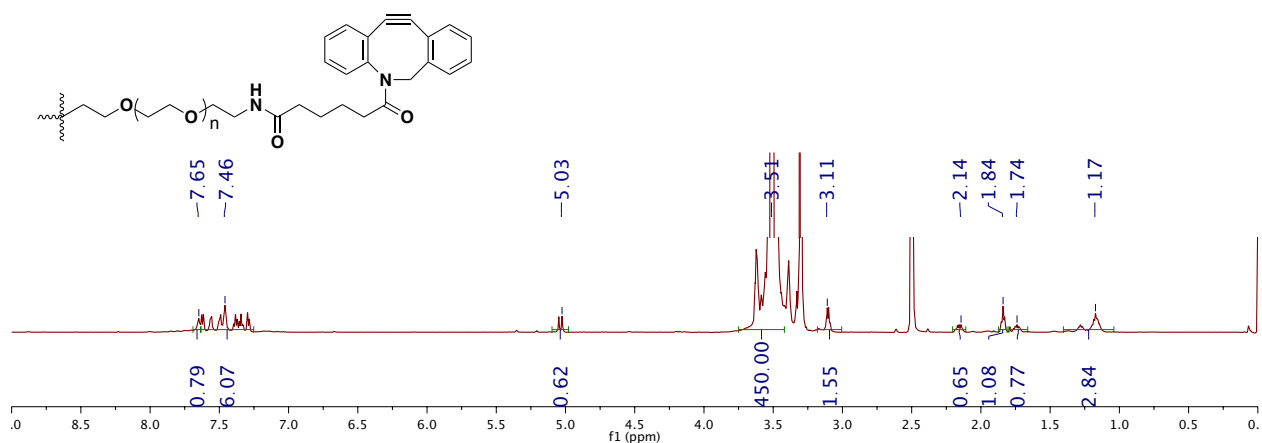


Figure S13. PEG-8-DBCO ^1H NMR Spectrum. Proton NMR used to confirm coupling of DBCO-C6-acid to PEG-8-Amine (DMSO, 600 MHz). Analysis of amide peak ($\delta=7.65$) and cyclooctyne peaks ($\delta=7.46$) were used to determine the number of functional arms of PEG-8-DBCO.

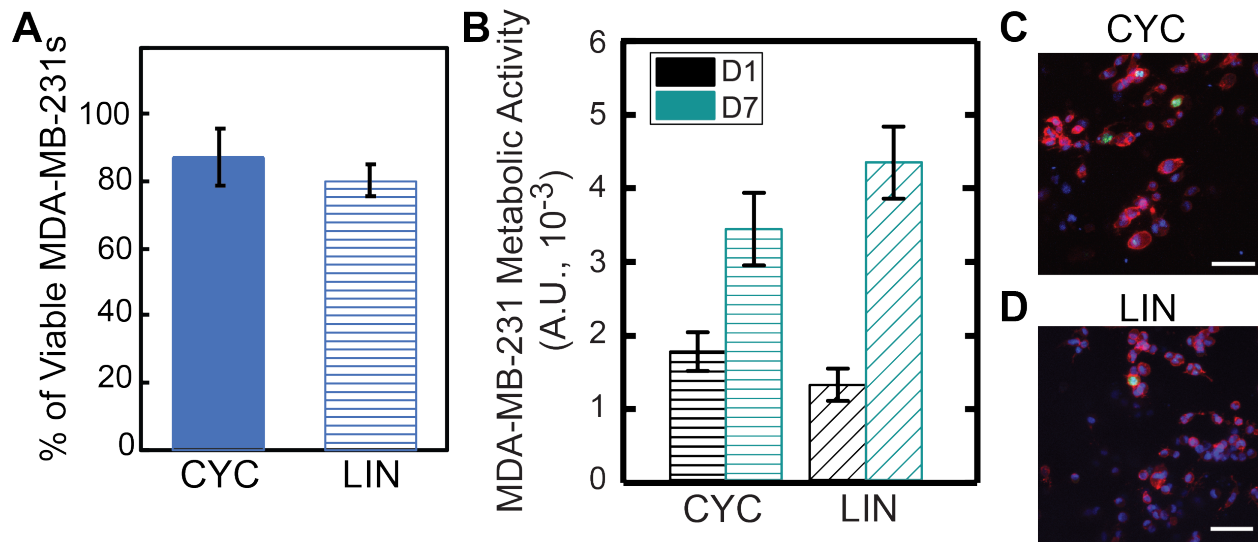


Figure S14. Viability of MDA-MB-231s cultured in hydrogels presenting cyclic or linear RGD. Cell viability and growth in 3D culture were examined in hydrogels containing either the cyclic RGD peptide (CYC) or the linear RGD peptide (LIN). **A)** A Live/Dead cytotoxicity assay revealed high cell viability in both conditions at 7 days in culture. **B)** Metabolic activity of MDA-MB-231s in 3D culture was measured over one week, where increased metabolic activity was observed in both conditions indicating cell growth in 3D culture. To confirm cell proliferation, cells were stained for Ki67, a marker of cell proliferation, after 7 days in culture in hydrogels presenting **C)** cyclic (CYC) or **D)** linear (LIN) RGD. Ki67 was observed for cells in both conditions, indicating both peptides support cell proliferation in these 3D cultures (F-actin (red), DAPI (blue), Ki-67 (green); n = 3; Scale bars, 50 μ m)

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