Enzyme-Regulated Topology of a Cyclic Peptide Brush Polymer for Tuning Assembly

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General Methods

All reagents were purchased from commercial sources and used without further purification.Peptides were synthesized using an AAPPTEC Focus XC automated synthesizer. Amino acids were purchased from AAPPTEC and NovaBiochem. N-(Glycine)-cis-5-norbornene-exo dicarboximide (Nor-Gly) and $(IMesH_2)(C_5H_5N)_2(Cl)_2Ru{=}CHPh$ ('Ru') were prepared according to published protocols.[1, 2] All enzymatic reactions were performed in PBS buffer at pH 6 with 50 mM $CaCl_2$. HPLC analysis was performed on a Jupiter Proteo90A Phenomenex column (150 x 4.60 mm) using a Hitachi-Elite LaChrom L-2130 pump equipped with a UV-Vis detector (Hitachi-Elite LaChrome L-2420). Purification was done using a Jupiter Proteo 90A Phenomenex column (2050 x 25.0 mm) on a Armen Spot Prep II. Buffer A was 0.1% TFA in water and Buffer B was 99.9% ACN and 0.1 % TFA. The sequence identities of purified peptide-monomers were confirmed using ESI-MS in the UCSD Chemistry and Biochemistry Molecular Mass Spectrometry Facility. ^1H (400 MHz) spectra were recorded on a Varian Mercury Plus spectrometer. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra were acquired on a Bruker Ultraflex-III TOF/TOF mass spectrometer (Bruker Daltonics, Inc., Billerica, MA) equipped with a Nd:YAG laser (355 nm) was used at the university of akron mass spectrometry center. The spectrum was measured in linear mode. Polymer dispersities and molecular weights were determined by size-exclusion chromatography (phenomenex Phenogel 5u 10, 1k- 75k, 300 x7.80 mm in series with a Phenomex Phenogel 5u 10, 10K-1000K, 300 x 7.80 mm (0.05 M LiBr in DMF)) using a Shimadzu pump equipped with a multi-angle light scattering detector (DAWN-HELIOS: Wyatt Technology) and a refractive index detector (Wyatt OptiLab T-rEX) normalized to a 30,000 MW polystyrene standard. Snakeskin^{TM} dialysis tubing was purchased from Thermoscientific, Inc. with a molecular weight cut off (MWCO) of 3500 g/mol. LCST analysis was conducted using a Cary Series 100 UV-Vis spectrophotometer equipped with a Cary temperature controller. Scattering intensity of micelles was measured via DLS using a DynaPro NanoStar (Wyatt Technology). TEM samples were deposited on carbon/formvar-coated copper grids (Ted Pella Inc.), stained with 1% w/w uranyl acetate, and imaged using a Technai G2 Sphera operating at an accelerating voltage of 200 kV. Zeta potential was determined using a Zetasizer ZS90 Dynamic Light Scattering instrument (Malvern Instruments, Worcestershire, UK). Gd(III) ion concentration was quantified by inductively coupled plasma-optical emission spectrometry

(ICP-OES) using a Perkin Elmer Optima 3000DV spectrometer. Relaxivity was calculated via Bruker 7.0 T magnet with Avance II hardware equipped with a 72 mm quadrature transmit/receive coil.

Peptide Synthesis

The linear peptide monomer (**L1**) shown in Figure S1 was synthesized via standard Fmoc-based solid phase synthesis using Wang resin (AAPPTEC) via automated synthesis. Fmoc deprotection was performed using 20% 4-methylpiperidine in DMF. Amino acid couplings were carried out using HBTU and DIPEA (resin/amino acid/HBTU/DIPEA 1:3.5:3.4:4). The final peptide monomers were cleaved from the resin using a mixture of TFA/H_2O/TIPS (95:2.5:2.5) for 45 minutes. The peptides were precipitated and washed with cold ether and purified using preparative HPLC. The cyclization reaction (Figure S1) was performed by dissolving (200 mg, 175µmol) linear peptide monomer in 200 mL DMF. HBTU (200mg, 525µmol) and DIPEA (92µL, 525µmol) was dissolved in 40 mL DMF and slowly add to the reaction mixture while stirring at room temperature. The reaction mixture was left to stir for 3 days. The same amounts of HBTU and DIPEA were added to the reaction, which was left stirring for another two days at rt. The DMF solution was concentrated in vacuo at 55 °C. The cyclic peptides were precipitated and washed with cold ether, purified by preparative HPLC then lyophilized to give a white solid (yield 75%). The ^{1}H (400 MHz) NMR spectrum of the monomer (Figure S8b) shows the presence of the olefinic proton resonances indicative of the norbornene (peak "a" in the figure). Peptide identities and purities were also confirmed using ESI-MS and RP-HPLC monitoring at λ_{abs} = 214 nm (Figure S2).

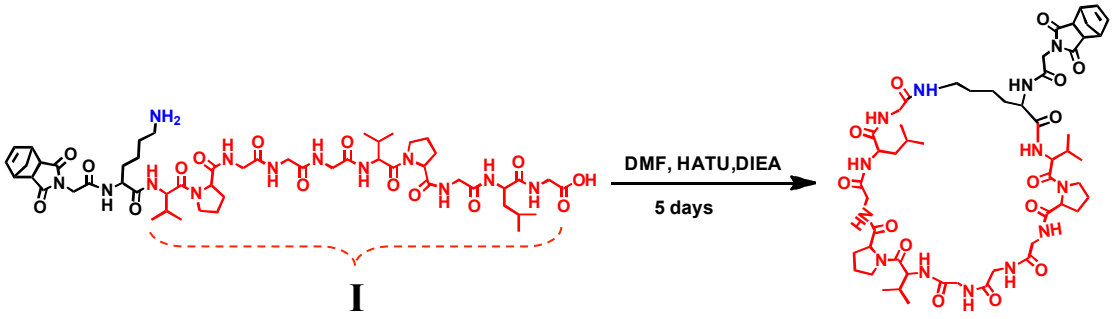

Figure S1. Cyclization reaction of linear peptide monomer (**L1**) to yield the cyclic monomer (**1**). Range I is the elastin-based sequence VPGGGVPGLG (red color).

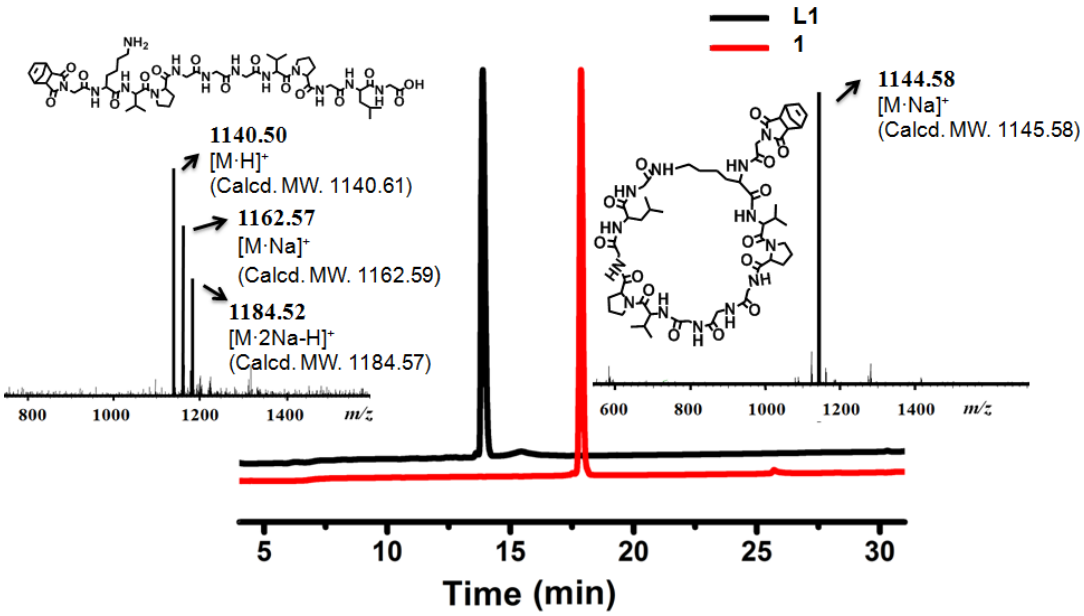

Figure S2. ESI-MS data and HPLC traces of linear peptide monomer (**L1**) and cyclic peptide monomer (**1**). HPLC gradient: 20%-60% ACN over 30mins.

Gd-TA synthesis (see Figure S3, S4 and S5)

To a stirred solution of DOTA – tris (*tert* - butyl ester) - (172 mg, 0.30 mmol) in dry DMF (0.6 mL) was added DIPEA (242 µL, 1.40 mmol) and HATU (132 mg, 0.35 mmol) followed by a solution of the diamine (80 mg, 0.145 mmol) and DIPEA (242 µL, 1.40 mmol) in dry DMF (1 mL). The reaction was stirred under a nitrogen atmosphere for 48 hrs and dried under vacuum. The compound was the redissolved in a 1:1 TFA:CH_2Cl_2 solution (20 mL) and stirred at room temperature for 6 hrs to deprotect the carboxylic acid groups. The reaction was concentrated to dryness and purified by Prep-HPLC (40%-60%B) followed by lyophylization to give 113mg (71%) of **2** as a white solid. ^{1}H NMR (DMF-d_7): δ (ppm) 7.2 (m, 4H), 6.95 (m, 4H), 5.92 (m, 2H), 4.78 (d, 4H), 4.02-3.23 (m, 44H). ^{13}C NMR (DMF-d_7): δ (ppm) 172.97, 160.12, 158.17, 132.76, 130.86, 129.62, 122.05, 119.68, 117.31, 115.62, 114.95 65.13, 56.51, 55.60, 54.91, 52.36, 50.65, 41.85. ESI, 1097.55 [M-H]$^-$, found: 1097.87.

Figure S3 Synthesis of **2**.

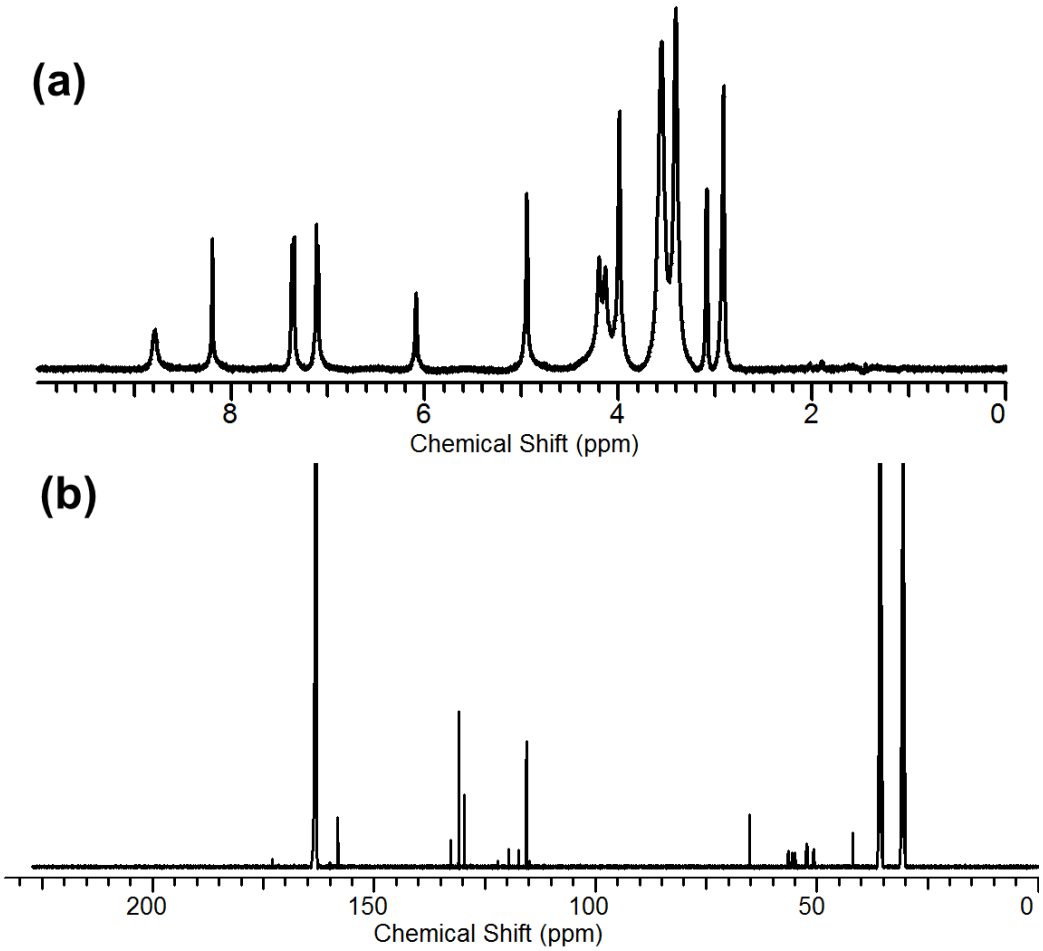

Figure S4 (a) ^{1}H NMR and (b) ^{13}C NMR of **2** in DMF-d_7.

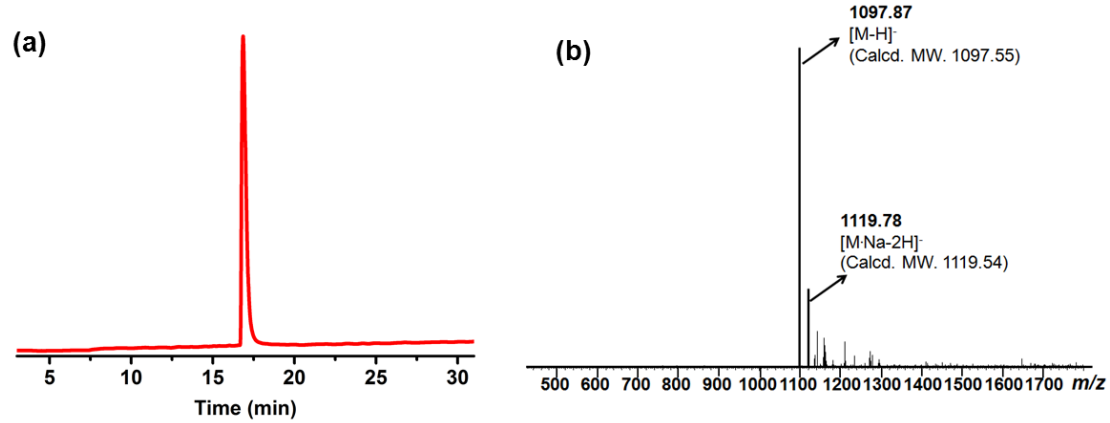

Figure S5 (a) HPLC trace of **2** (10%-50% ACN over 30 mins) and (b) ESI-MS of **2**.

To complete the synthesis of **Gd-TA**, **2** (113mg) was dissolved in water (5 mL) and an excess of $GdCl_3$ (100 mg, 0.37 mmol) was added. The pH was adjusted to 6 with 1M NaOH and stirred at room temperature for 24 hrs. The final product was purified by Prep-HPLC (10%-50% B) followed by lyophylization to give 129mg (89%) **Gd-TA** as a white solid. ESI, 1407.45 $[M-H]^-$, found: 1407.36 (see Figure S7).

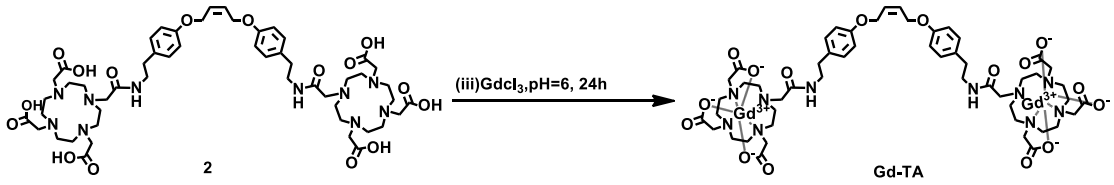

Figure S6 Synthesis of **Gd-TA**.

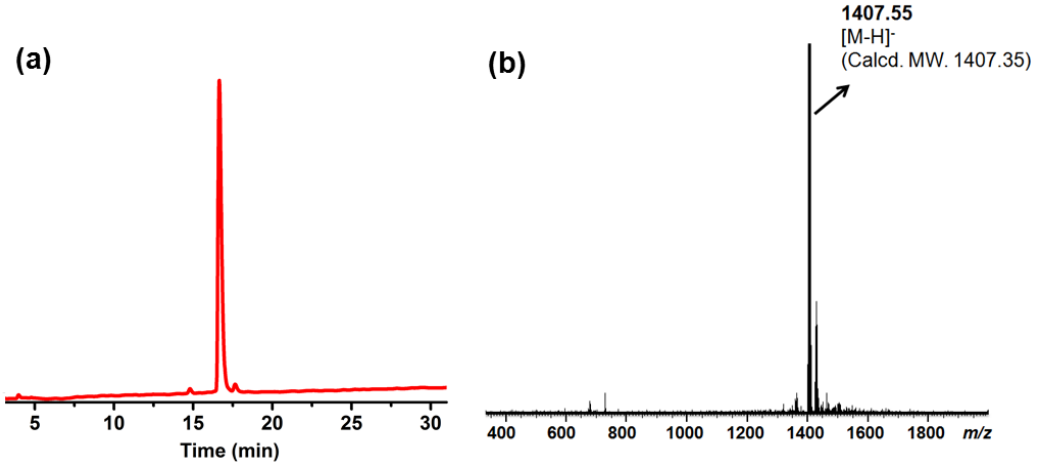

Figure S7 (a) HPLC trace of **Gd-TA** (10%-50% ACN over 30 mins) and (b) ESI-MS of **Gd-TA**.

Polymer Synthesis

Homopolymer: The peptide monomer **1** was polymerized via ROMP using Grubbs' modified second generation initiator $(IMesH_2)(C_5H_5N)_2(Cl)_2Ru{=}CHPh$. The initiator (1 equiv.) was dissolved in DMF and added to **1** (5 equiv.) in DMF to yield a final volume of 500 µL under inert atmosphere. The resulting mixture was left to stir under N_2 for 3 hrs followed by quenching with **Gd-TA** (1.2 equiv.) for about 1 hour. The solution was then transferred to a 3500 MWCO dialysis tubing and dialysis against water for 3 days to remove excess **Gd-TA**. ^{1}H (400 MHz) NMR spectra of the polymer (**P0**) (i.e. NMR performed prior quenching with paramagnetic **Gd-TA**) confirmed the absence of monomer (no olefin peak at 6.30 ppm) and the presence of broad trans and cis olefin peaks of the polymer backbone at 5.80 and 5.55 ppm, respectively (Figure S8). SEC-MALS of **P1**: $M_{n,\,SEC}$ = 7,000 g/mol, $M_{w,\,SEC}$ = 7,200 g/mol, $Đ$ = 1.03.

Block copolymer: The initiator, as above (1 equiv.), was dissolved in DMF and added to norbornene phenyl monomer **2** (10 equiv.) in DMF to a final volume of 200 µL under inert atmosphere. The resulting mixture was left to stir under N_2 for 10 mins, at which point a small aliquot was removed, quenched with ethyl vinyl ether, and taken for analysis of the first block by SEC-MALS (data below), followed by addition of **1** (5 equiv.) in DMF to the main solution giving a final volume of 500 µl. The resulting mixture was left to stir under N_2 for 3 hrs followed by quenching with **Gd-TA** (1.2 equiv.) for approx. 1 hour. The solution was then transferred to a 3500 MWCO dialysis tubing and dialysis against water for 3 days to remove excess **Gd-TA**. SEC-MALS of the phenyl block (norbornene phenyl monomer **2**): $M_{n,\,SEC}$ = 2,600 g/mol, $M_{w,\,SEC}$ = 2,800 g/mol, $Đ$ = 1.07, DP(**2**) = 10. SEC-MALS of **P2**: $M_{n,\,SEC}$ = 9,200 g/mol, $M_{w,\,SEC}$ = 9,400 g/mol, $Đ$ = 1.03, DP(**1**)= 5 (see Figure S17). DP = Degree of polymerization

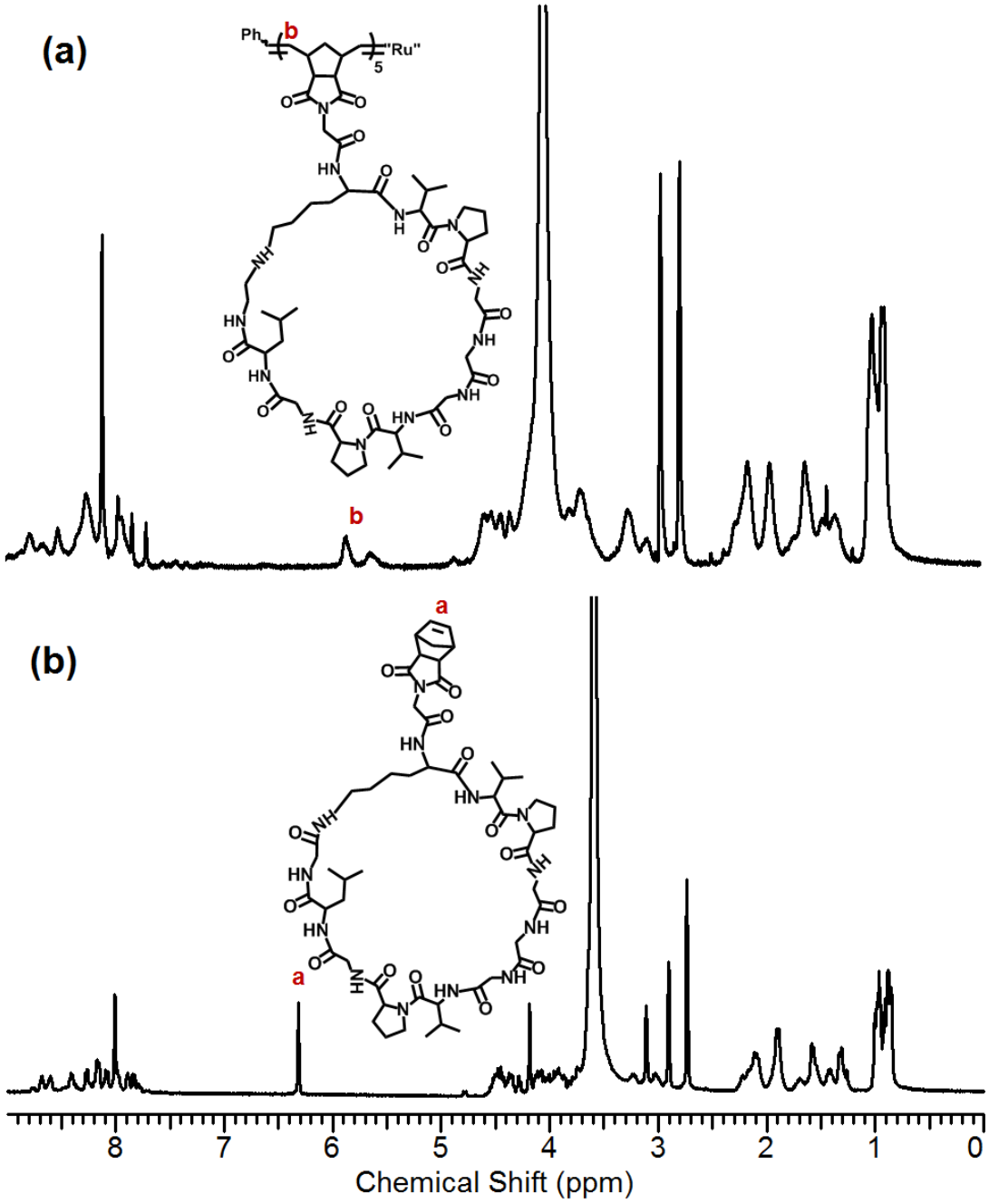

Figure S8 ^{1}H NMR spectra of (a) **P0** and (b) **1** in DMF-d_7.

Micelle Preparation and Characterization.

The block copolymer was first dissolved in DMF (1 mg/mL) as the common solvent and stirred at room temperature overnight to ensure complete dissolution of the polymer. The solution was filtered through a 0.22 μm filter to remove any dust. Deionized water was filtered through a 0.22 μm filter and added dropwise at a rate of 10 μL/ hour using a syringe pump into a vial containing 1 mL of the stock solution. Water addition was continued until reaching a final water content of 85 % (wt). Then the solution was dialyzed against deionized water for three days to remove the common solvent.

Micellar solutions were heated using microtube incubators (Denville Scientific Inc). Hydrodynamic diameter (D_h) was determined by Dynamic Light Scattering (DLS) using a Wyatt Dynapro NanoStar. Transmission Electron Microcopy (TEM) was performed on a FEI Sphera microscope operating at 200 keV. TEM grids were prepared by depositing small (3.5 μL) aliquots of sample onto grids (~ 2 min, Formvar stabilized with carbon (5-10 nm) on 400 copper mesh, Ted Pella Inc.) that had previously been glow discharged using an Emitech K350 glow discharge unit and plasma-cleaned for 90 s in an E.A. Fischione 1020 unit. The sample grid was then stained with a 1% uranyl acetate solution, rinsed with water (~ 5 μL), and excess liquid removed. Micrographs were recorded on a 2 K X 2 K Gatan CCD camera.

LCST measurement and Morphology Characterization

The LCST of **P1** was obtained by taking the midpoint of the UV spectrum obtained on a Cary Series 100 UV-Vis spectrophotometer equipped with a Cary temperature controller. The samples were heated at 1 °C·min^{-1} and ranged in concentration from 0.1 to 0.6 mg·mL−1 in PBS buffer (pH = 6). The data interval was 0.06 °C. The LCST of **P2** was measured by heating the solution at 0.5 °C·min^{-1} in water (0.6 mg·mL^{-1}). The data interval was 0.06 °C.

The morphology of **P1**in solution above LCST was characterized by TEM using the same TEM grid preparation method as described before.

Thermolysin cleavage protocol

Thermolysin stock solution: 25 μM thermolysin in water.

Conditions for thermolysin cleavage efficiency of 1 as shown in Figure S10:

1 (1 mg, 890 μmol) was dissolved in 1.6 mL 1xPBS buffer solution (0.5 mM $CaCl_2$, pH = 6), followed by adding 40 μL thermolysin stock solution and incubated at 55 °C for 30 min.

Control experiment:

Thermolysis was denatured by adding 40 μL EDTA solution (100 mM) to 40 μL thermolysin stock solution. **1** (1 mg, 890 μmol) dissolved in 1.6 mL 1xPBS buffer solution (0.5 mM $CaCl_2$, pH = 6). The denatured thermolysin was then added to the **1** solution.

Conditions for thermolysin cleavage of P1 as shown in Figure 3, Figure S14 and Figure S16:

P1 (1 mg, 780 μmol) was dissolved in 1.6 mL 1xPBS buffer solution (0.5 mM $CaCl_2$, pH = 6), followed by adding 40 μL thermolysin stock solution and incubated at 55 °C for 30 min. The solution was then diluted to various concentrations for LCST testing.

Control experiment:

Thermolysis was denatured by adding 40 μL EDTA solution (100 mM) to 40 μL thermolysin stock solution. **P1** (1 mg, 780 μmol) was dissolved in 1.6 mL 1xPBS buffer solution (0.5 mM $CaCl_2$, pH = 6). The denatured thermolysin was then added to the **P1** solution. The solution was then diluted to various concentrations for LCST testing.

Conditions for thermolysin cleavage kinetic of P1 as shown in Figure S15:

P1 (1 mg, 780 μmol) was dissolved in 1.6 mL 1xPBS buffer solution (0.5 mM $CaCl_2$, pH = 6), 80 μL was incubated at 55 °C until the UV and DLS signals were stable with addition of 4 μL thermolysin stock solution 5 mins after starting the measurement.

Control experiment:

P1 (1 mg, 780 μmol) was dissolved in 1.6 mL 1xPBS buffer solution (0.5 mM $CaCl_2$, pH = 6), 80 μL was incubated at 55 °C until the UV and DLS signals were stable with addition of 4 μL

thermolysin stock solution and 4 μL EDTA solution (100 mM) 5 mins after starting the measurement.

Conditions for thermolysin cleavage of P2 micelles as shown in Figure 5 and Figure S20:

4 μL thermolysin stock solution and 6 μL $CaCl_2$ (50 mM) to 600 μL **P2** micelles (20 μM). Incubate at 55 °C for 4 hours.

Control experiment:

4 μL thermolysin stock solution, 4 μL EDTA (100 mM) and then 6 μL $CaCl_2$ (50 mM) to 600 μL **P2** micelles (20 μM). Incubate at 55 °C for 4 hours.

In vitro MRI study

The longitudinal relaxation time (T_1) measurements were acquired with a Bruker 7.0 T magnet with Avance II hardware equipped with a 72 mm quadrature transmit/receive coil. T_1 contrast was determined by selecting regions of interest (ROI) using Software ParaVision Version 5.1. The parameters for 7 T MRI of **P1**: TR = 500.0 ms, TE = 12.6 ms, Echo length = 1, FOV = 8.31 x 3.99 cm^2, slice thickness = 1 mm, NEX = 1 mm, matrix = 256*123. The parameters for 7 T MRI of **P2** micelles: TR = 500.0 ms, TE = 12.6 ms, Echo length = 1, FOV = 7.31 x 3.12 cm^2, slice thickness = 1 mm, NEX = 1 mm, matrix = 272*116. r_1 value was calculated after the curve fitting of 1/ T_1 (s^{-1}) *versus* Gd(III) ion concentration (μM). The Gd(III) ion concentration was calibrated by Inductively coupled plasma-optical emission spectrometry (ICP-OES).

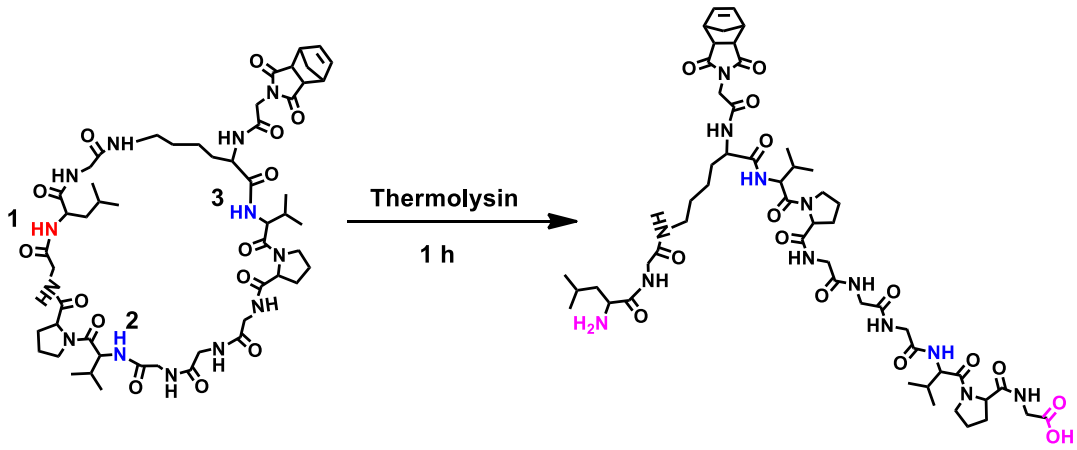

Figure S9. Thermolysin cleavage of **1** at position 1 (red color). Position 2 and 3 (blue color) are not the cleavage sites as Proline residues beside the cleavage sites block the thermolysin cleavage. After the cleavage, one carboxylic acid and amine group were released (pink color).

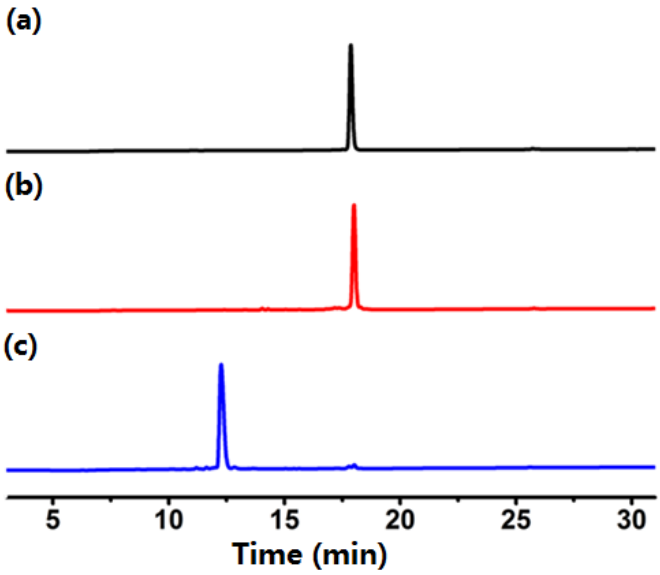

Figure S10 HPLC trace of (a) **1**; (b) **1** treated with denatured thermolysin at 55 °C for 1 h; (c) **1** treated with thermolysin at 55 °C for 1 h. HPLC gradient: 20%-60% ACN over 30mins.

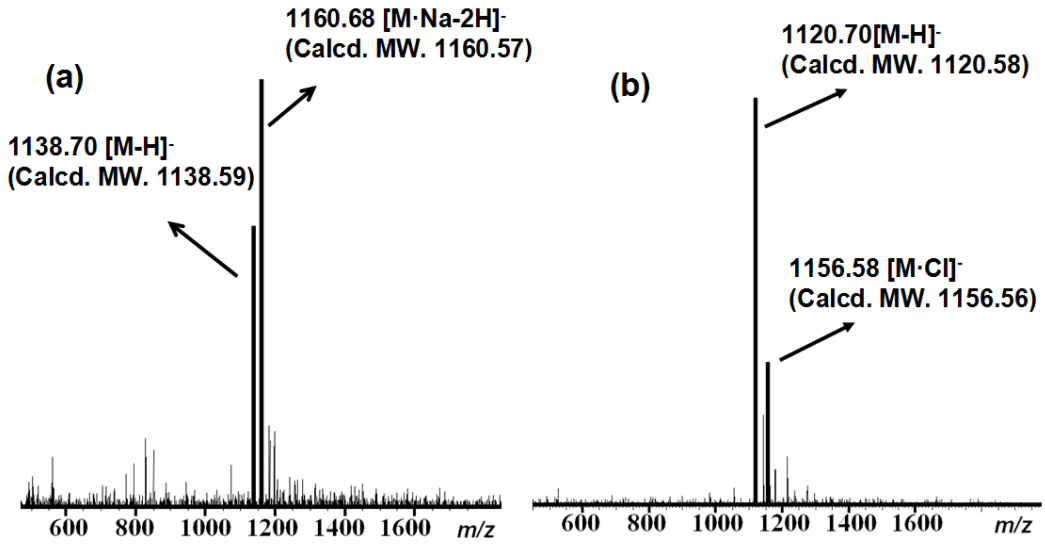

Figure S11. ESI-MS of (a) **1** treated with thermolysin for 1 h, and (b) **1** treated with denatured thermolysin for 1 h.

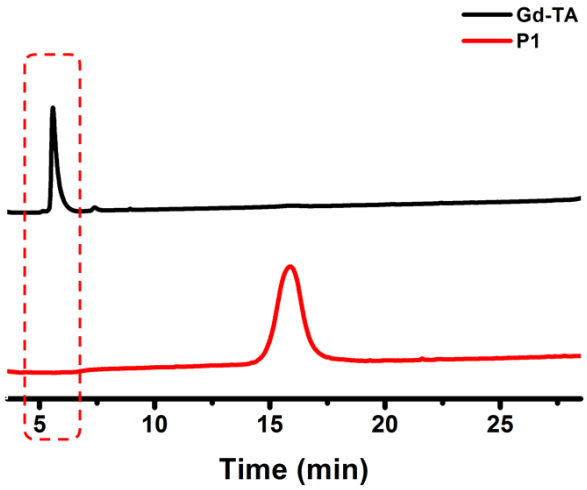

Figure S12 HPLC trace of (a) **Gd-TA** and (b) **P1** after dialysis. HPLC gradient: 20%-100% ACN over 30 min.

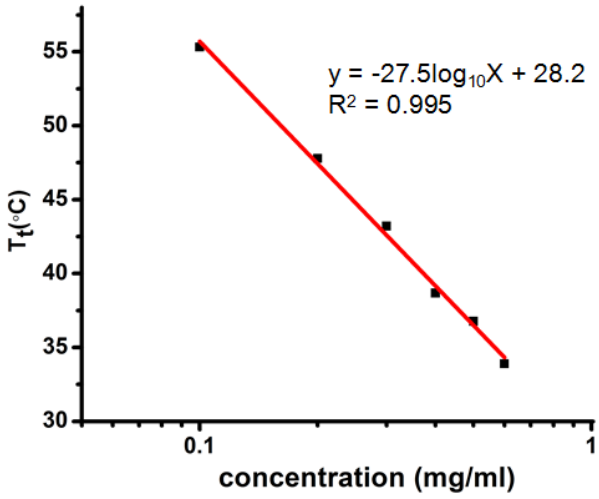

Figure S13. LCST *versus* concentrations of **P1** in PBS at pH = 6. LCST was taken as the midpoint on the UV-Vis spectrum.

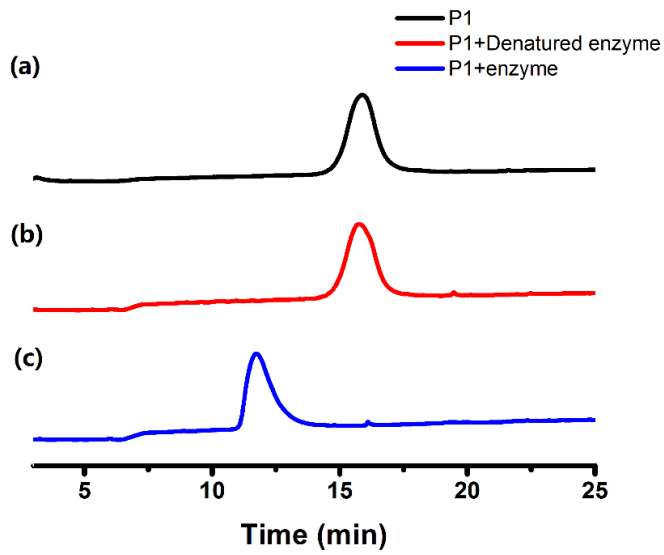

Figure S14 HPLC trace of (a) **P1**, (b) **P1** with denatured enzyme at 55 °C for 1 h, (c) **P1** treated with thermolysin at 55 °C for 1h. HPLC gradient: 20%-100% ACN over 30 min.

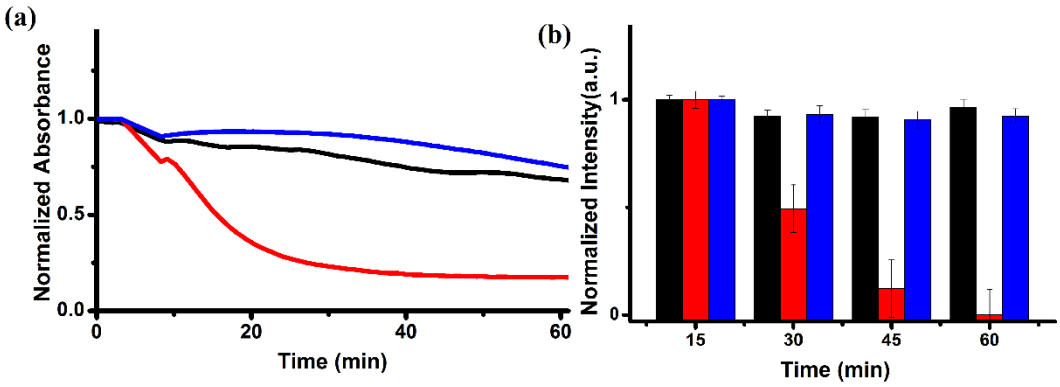

Figure S15. (a) UV-Vis spectroscopy of the enzyme-driven disassembly process at 550 nm and (b) Scattering intensity of the polymers during the enzyme-driven disassembly process as measured by DLS. All solutions were pre-heat to 55 °C and equilibrant for 5 min before starting. **P1** (black), **P1** with enzyme (red) and **P1** with denatured enzyme (blue).

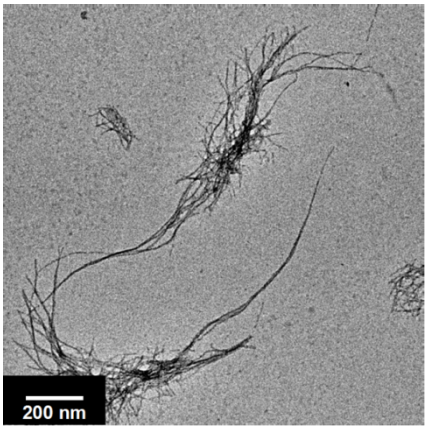

Figure S16. TEM image of the cylindrical structure formed by **P1** with denatured enzyme above the LCST.

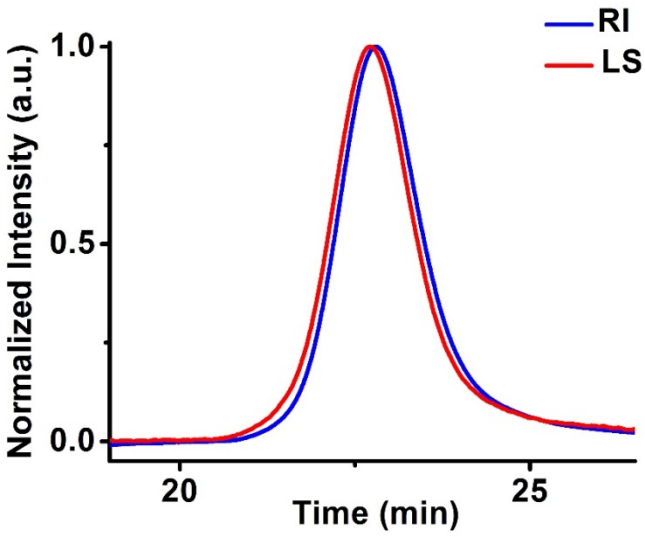

Figure S17. SEC trace of **P2**.

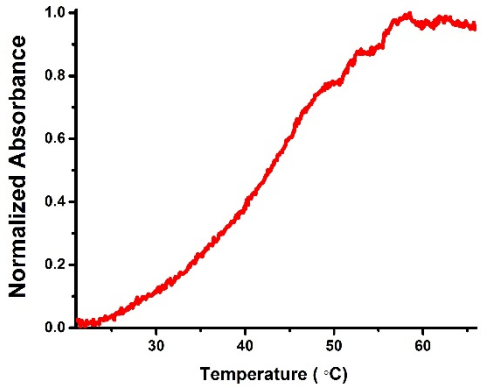

Figure S18. LCST behavior of **P2** micelle solution (0.6 mg/mL) at 550 nm.

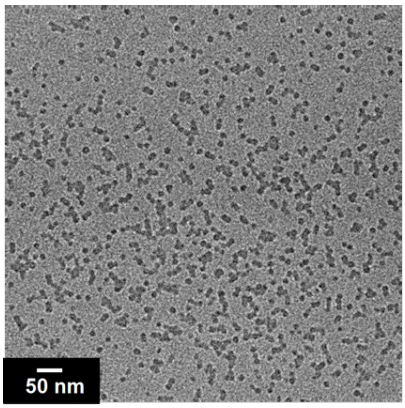

Figure S19. TEM image of **P2** micelles treated at 55°C for 6 h followed by cooling at room temperature for 3days.

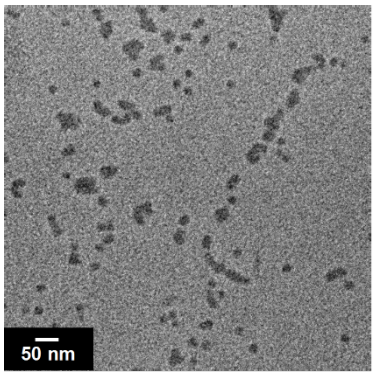

Figure S20. TEM image of **P2** micelles treated with denatured enzyme.

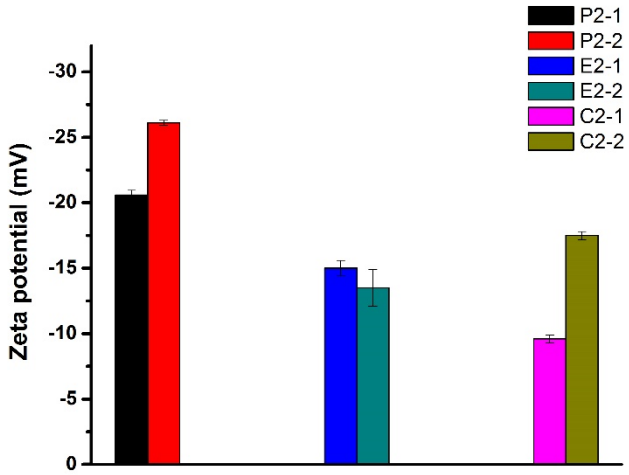

Figure S21. Zeta potential of P2-1: **P2** micelle at r.t., P2-2: **P2** micelle at 55 °C, E2-1: Enzyme treated **P2** micelle at rt, E2-2: Enzyme treated **P1** at 55 °C, C2-1: Denatured enzyme treated **P2** micelle at rt, C2-2: Denatured enzyme treated **P2** micelle above 55 °C.

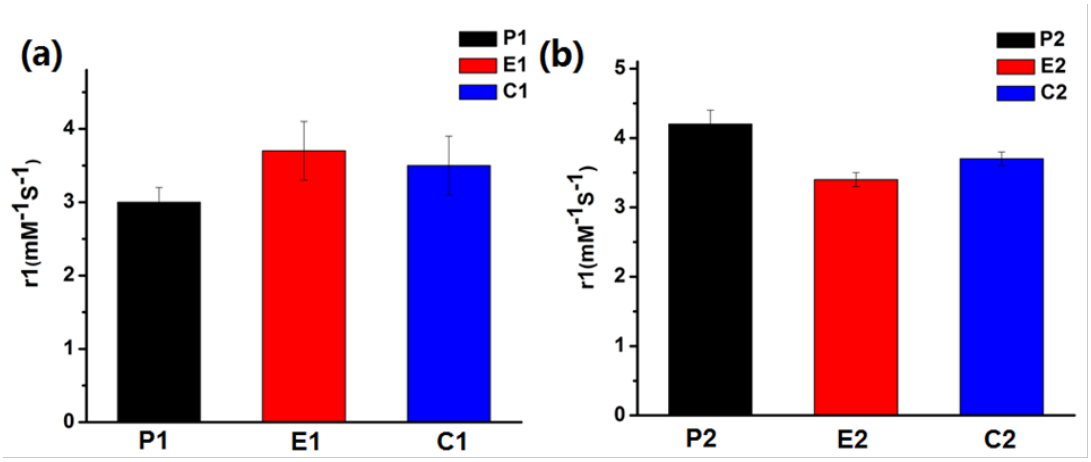

Figure S22. Relaxivity of **P1** and **P2** micelles: (a) P1: untreated **P1,** E1: enzyme treated **P1,** C1: denatured enzyme treated **P1.** (b) P2: untreated **P2** micelle, E2: enzyme treated **P2** micelle, C2: denatured enzyme treated **P2** micelle.

Reference

1. M.-P. Chien, A. M. Rush, M. P. Thompson and N. C. Gianneschi, *Angew. Chem. Int. Ed.*, 2010, 49, 5076-5080.
2. M. S. Sanford, J. A. Love and R. H. Grubbs, *Organometallics*, 2001, 20, 5314-5318.