Supporting Information for:

A thermoresponsive crosslinker for reversible micelle stabilization

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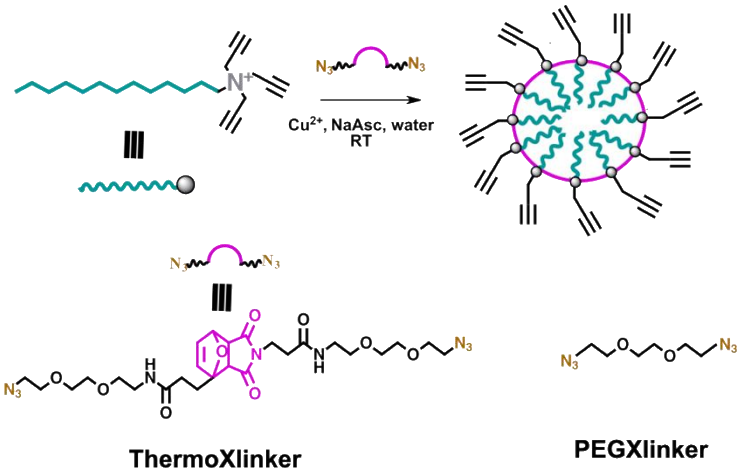
General Method

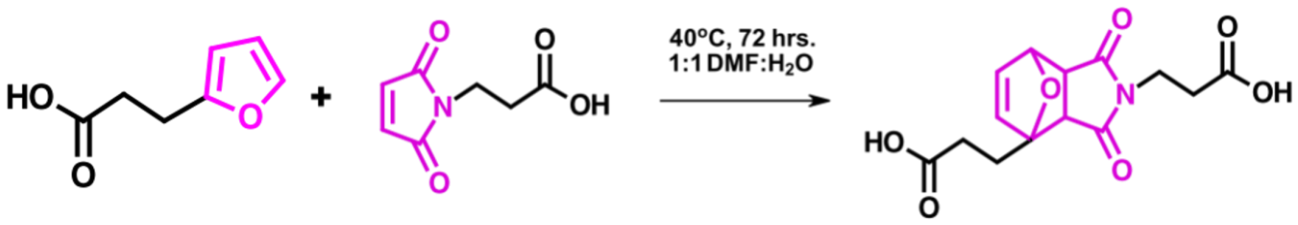
All reagents were used as received from commercial suppliers. HPLC grade methanol, dichloromethane, dimethylformamide, dimethylsulfoxide and chloroform were used for synthetic procedures. Fluorometric maleimide assay kit, *N*,*N*,*N'*,*N'*-Tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU, 98%), *N*,*N*-diisopropylethylamine (DIEA, ≥ 99%) and 3-(2-furyl) propionic acid (97%) were purchased from Sigma-Aldrich. 3-Maleimidopropionic acid (95%) was obtained from Alfa-Aesar. Azide-PEG_3-Azide crosslinker and Azide-PEG_3-Amine were obtained from Lumiprobe. 5'-hexynyl 3'-thiol polythymidine (T20) was obtained from Integrated DNA Technologies (IDT). The 30 nm citrate-stabilized gold nanoparticles were obtained from nanoComposix. The surfactant used in forming surface crosslinked micelles (SCMs) was synthesized as previously reported.[1] Deuterated solvents were purchased from Cambridge Isotope Laboratories. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX-400 spectrometer and spectral processing was carried out using MestReNova version 12.0 (Mestrelab Research S.L.). Mass spectrometry analysis was recorded on a Sciex QSTAR Elite mass spectrometer. Dynamic light scattering (DLS) and zeta potential measurements for SCMs were performed on a Malvern Zetasizer Nano ZS System. UV-VIS spectroscopy was conducted using Double Beam Cary UV-VIS Spectrophotometer (Agilent Technologies).

Representative Scheme



Scheme S1. Synthetic route for the preparation of SCMs.

Syntheses



Scheme S2. Synthesis of Diels-Alder (DA) precursor.

DA precursor. In a clean amber vial, 3-Maleimidopropionic acid (215.1 mg, 1.20 mmol, 1 equiv.) and 3-(2-furyl) propionic acid (349.1 mg, 2.40 mmol, 2 equiv.) were added and dissolved in 1.0 mL of 1:1 DMF: H_2O. The resulting mixture was sonicated for five minutes to ensure complete dissolution of reactants. After which, the solution was stirred at 40°C for 72 hours on a heat block. The solution was then cooled to room temperature and the solvent was removed using high vacuum. The crude product was collected through vacuum filtration after it was precipitated by chloroform. Column chromatography over silica gel was performed using 30:1 dichloromethane: methanol as eluent to obtain the white powder product (255.1 mg, 68% yield). ^{1}H NMR (400 MHz, d_6-DMSO) δ 12.27 (s, 2H), 6.55 (dd, J = 5.7, 1.8 Hz, 1H), 6.46 (d, J = 5.6 Hz, 1H), 5.05 (d, J = 1.7 Hz, 1H), 3.60 – 3.51 (m, 2H), 3.03 (d, J = 6.4 Hz, 1H), 2.84 (d, J = 6.4 Hz, 1H), 2.45 – 2.28 (m, 4H), 2.22 (ddd, J = 14.4, 10.4, 5.7 Hz, 1H), 2.04 (ddd, J = 15.0, 10.5, 5.3 Hz, 1H). ^{13}C NMR (400 MHz, MeOD, δ): 176.7, 175.3, 175.1, 172.9, 138.1, 137.2, 90.8, 80.5, 50.5, 48.9, 34.1, 31.3, 29.3, 24.6. ESI-HRMS (m/z): $[M+H]^+$ calculated for $C_{14}H_{16}NO_7$, 310.0927; found, 310.0926.

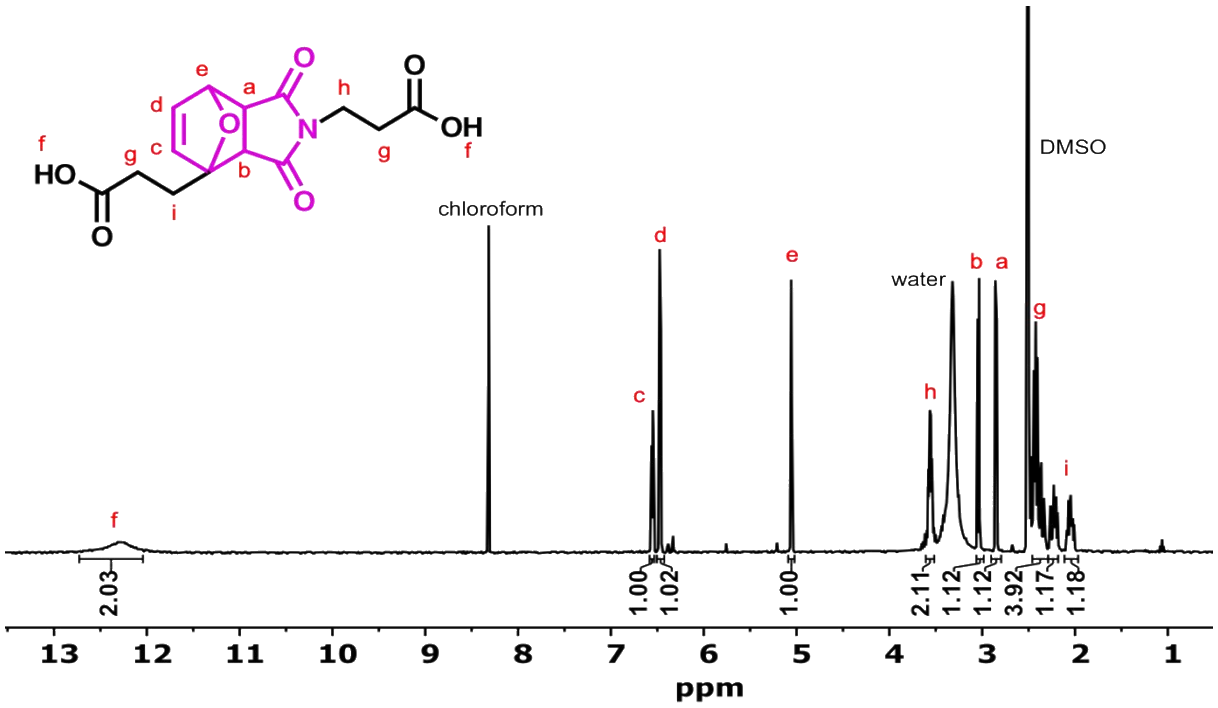


Figure S1: [1]H NMR 400 of the DA precursor in d_6-DMSO.

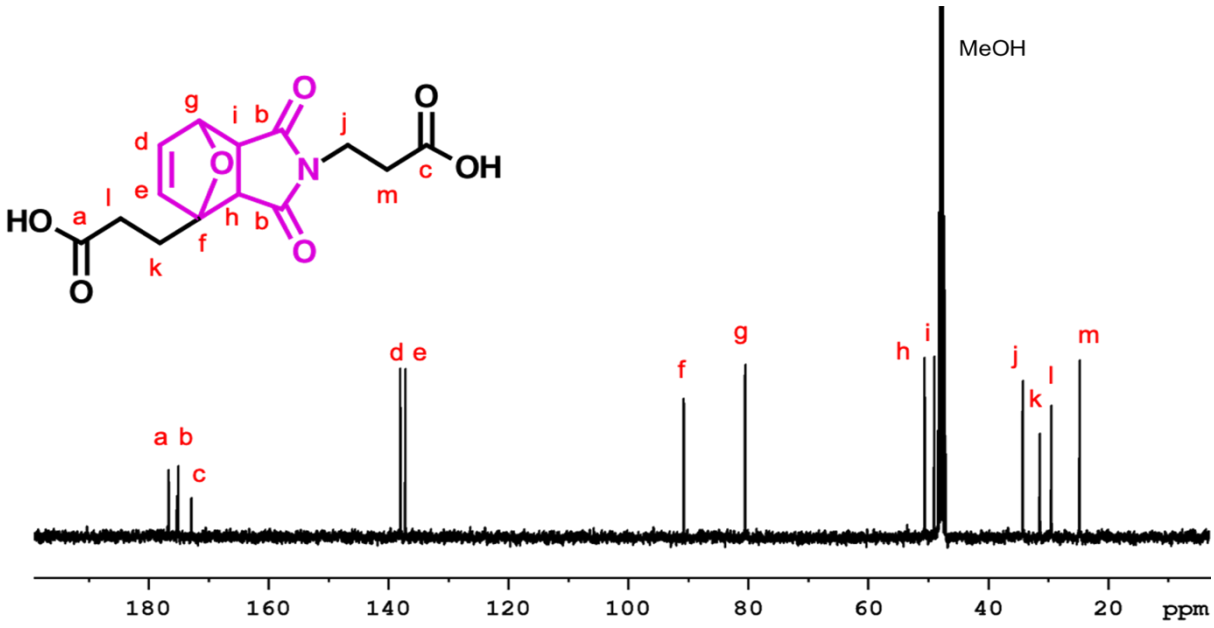


Figure S2. [13]C NMR 400 of the DA precursor in MeOD.

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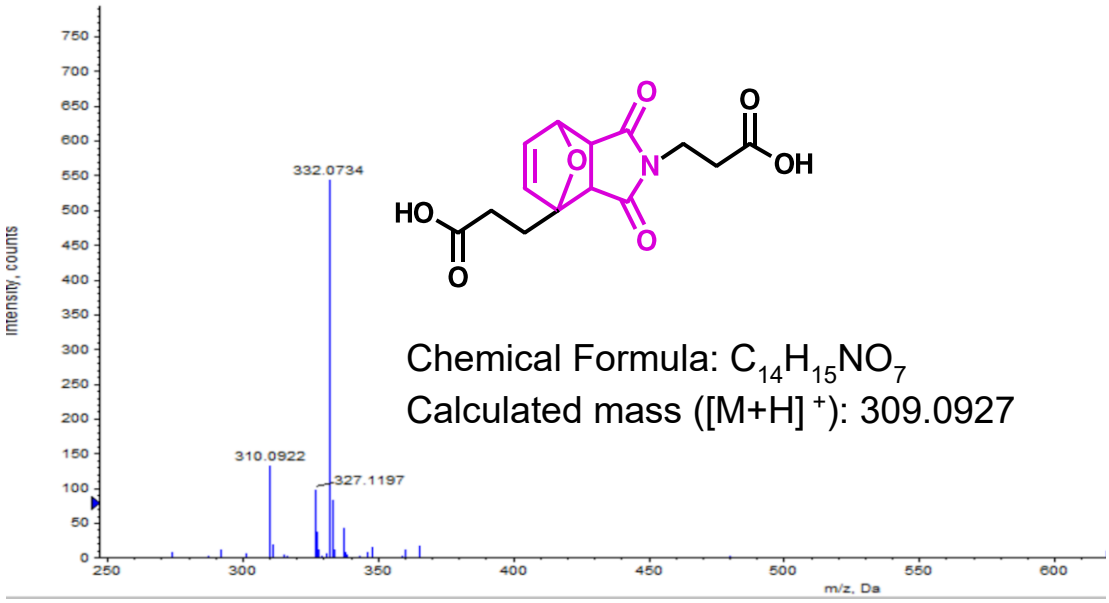
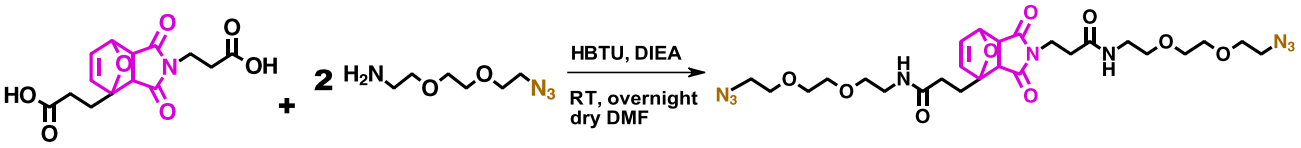


Figure S3. ESI mass spectrum (positive mode) of the DA precursor.

Table S1. Mass spectrometry analysis of the DA precursor

Species	Found (m/z)
$[M+H]^+$	310.0926
$[M+Na]^+$	332.0735



Scheme S3. Synthesis of Thermoresponsive Crosslinker (ThermoXlinker)

ThermoXlinker. To a solution of DA precursor (74 mg, 0.24 mmol, 1 equiv.) in 1.0 mL of dry DMF, HBTU (227 mg, 0.60 mmo, 2.5 equiv.), DIPEA (108 mg, 0.84 mmol, 3.5 equiv) and Azide-PEG_3-Amine (110 mg, 0.60 mmol, 2.5 equiv.) were added and the resulting solution was stirred at room temperature overnight. After this period, DMF was removed using high vacuum and the crude reaction mixture was dissolved in ~1 mL of DCM. Column chromatography over silica gel was then performed starting with 50:1 until 25:1 dichloromethane: methanol as eluent, to afford a yellow viscous product (72.4 mg, 49%yield). ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.96 (s, 2H), 6.47 (s, 1H), 6.35 (s, 1H), 5.12 (s, 1H), 3.72 (t, J = 1.80 Hz, 2H), 3.63 (m, 8H), 2.97 (t, J =12.1 Hz, 6H), 2.84 (s, 4H), 2.76 (s, 2H), 2.44 (m, 4H), 2.34 (m, J = 1.24 Hz, 2H), 2.25 (t, J =1.45 Hz, 2H). ^{13}C NMR (400 MHz, $CDCl_3$, δ): 176.1, 174.9, 172.6, 170.3, 138.5, 138.5, 137.2, 91.1, 80.5, 70.4, 70.3, 70.1, 70.0, 55.0, 50.6, 48.9, 44.9, 39.7, 36.5, 35.2, 31.9, 23.3. ESI-HRMS (*m/z*): $[M+H]^+$ calculated for $C_{26}H_{40}N_9O_9$, 622.2949; found, 622.2937.

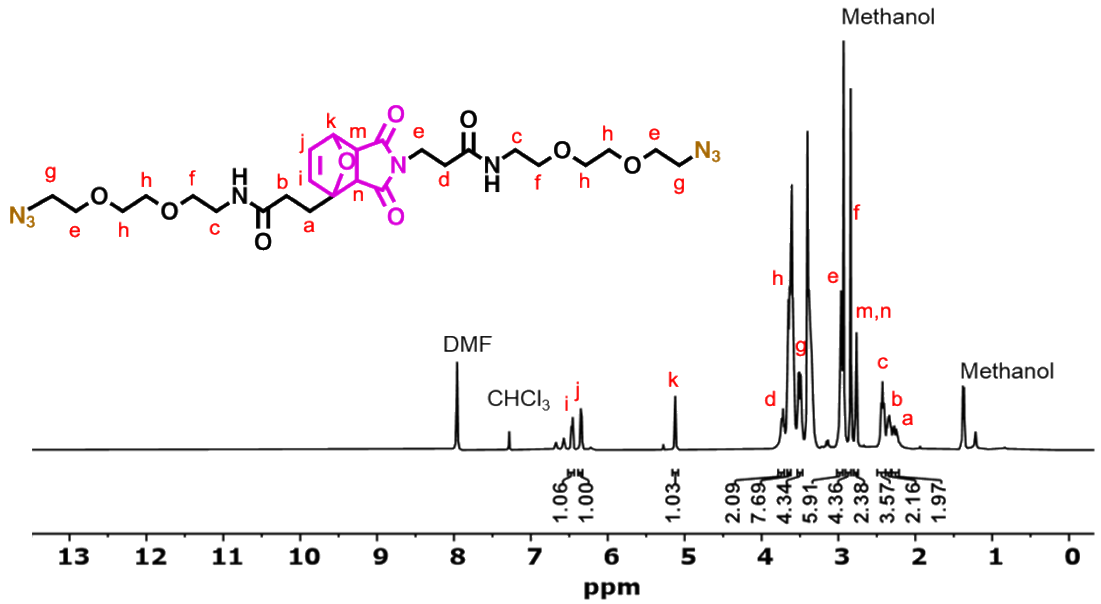


Figure S4: ^{1}H NMR 400 of ThermoXlinker in $CDCl_3$.

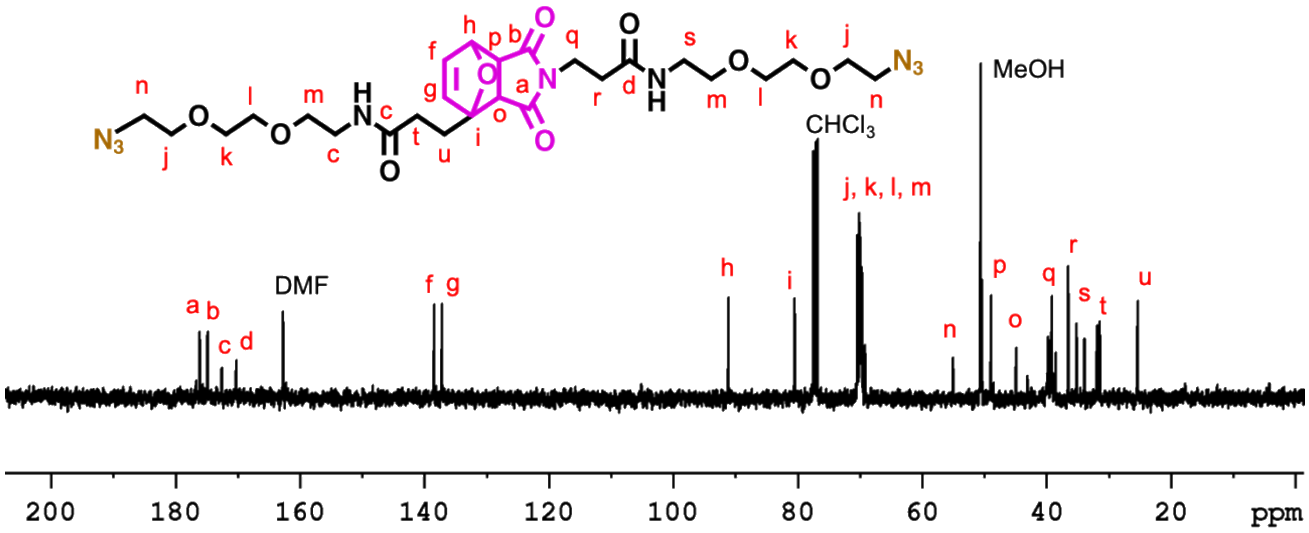


Figure S5. ^{13}C NMR 400 of ThermoXlinker in $CDCl_3$.

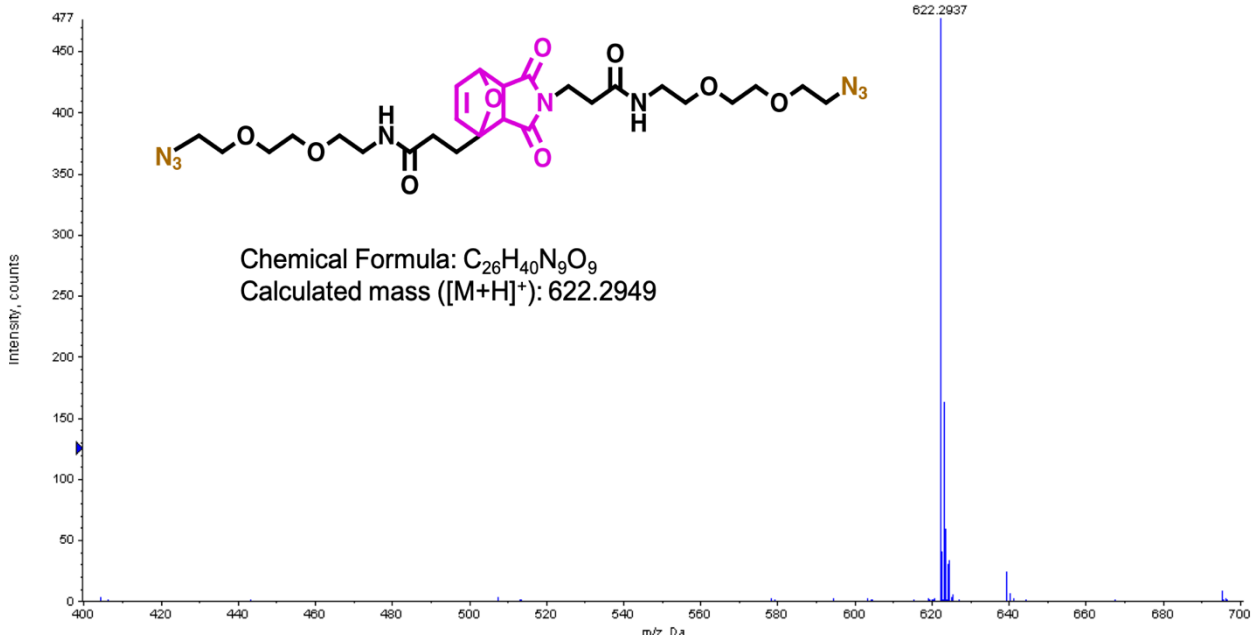


Figure S6. Mass spectrum (positive mode) of the ThermoXlinker.

Table S2. Mass spectrometry analysis of the ThermoXlinker

Species	Found (m/z)	Mass Accuracy (ppm)
$[M+H]^+$	622.2937	1.9274

Temperature Response Analysis of ThermoXlinker and DA Precursor via ^{1}H-NMR. A 1.5 mM solution of ThermoXlinker in d_6-DMSO (1 mL) was prepared in a 2-mL vial. The solution was then placed on a 70°C heat block and the ^{1}H-NMR spectrum at different time points (0, 2 hrs., 6 hrs. and 24 hrs.) were recorded. For the DA precursor, two 1 mM solutions in d_6-DMSO (1.0 mL) were prepared in 2-mL vials. Each solution was heated for 30 minutes at different temperatures- 60°C and 70°C- prior to ^{1}H-NMR spectrum acquisition. Relative concentrations of ThermoXlinker and decomposition products were determined through proton integration and percent dissociation for each spectrum was calculated.

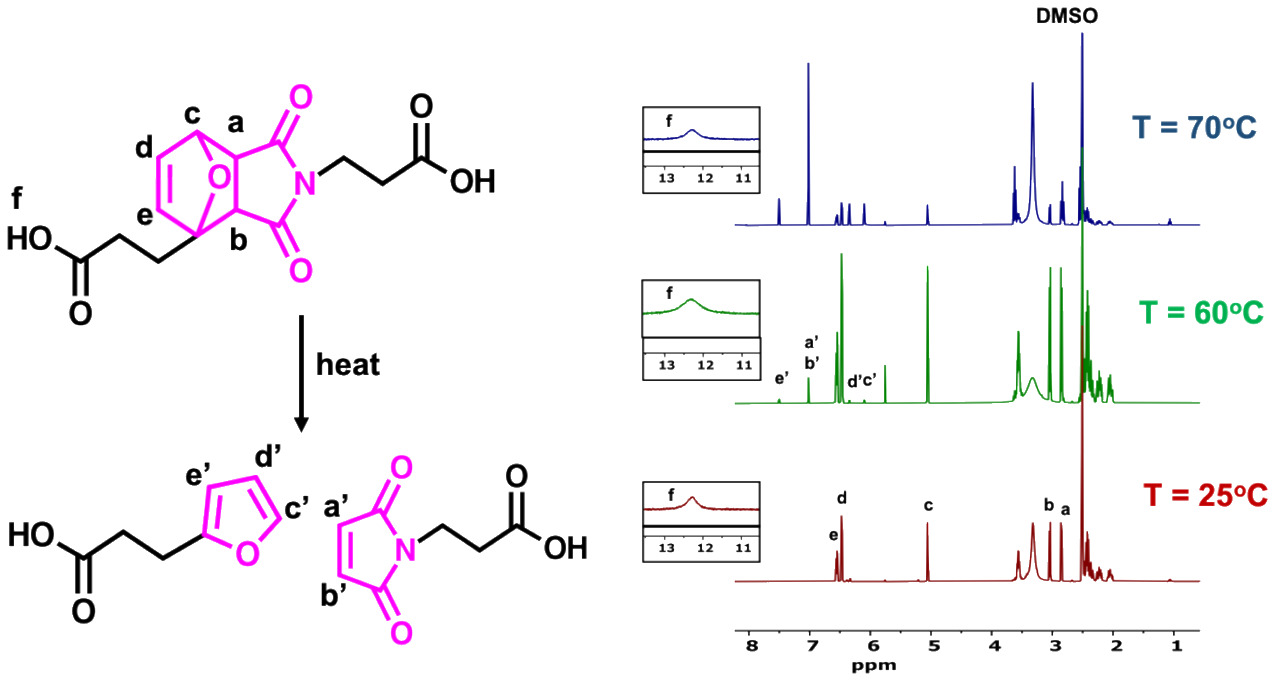


Figure S7. H-NMR study demonstrates the temperature responsiveness of DA precursor, indicating a greater extent of retro-Diels-Alder (rDA) reaction at 70°C (50 % dissociation) compared to 60°C (7.5 % dissociation) within 30 minutes of heating.

ThermoXlinker Decomposition Analyzed via Mass Spectrometry. A 250 µM solution of ThermoXlinker was prepared in 500 µL of distilled water, and subsequently heated at 70ºC for 24 hrs. Afterwards, the mass spectrum was recorded.

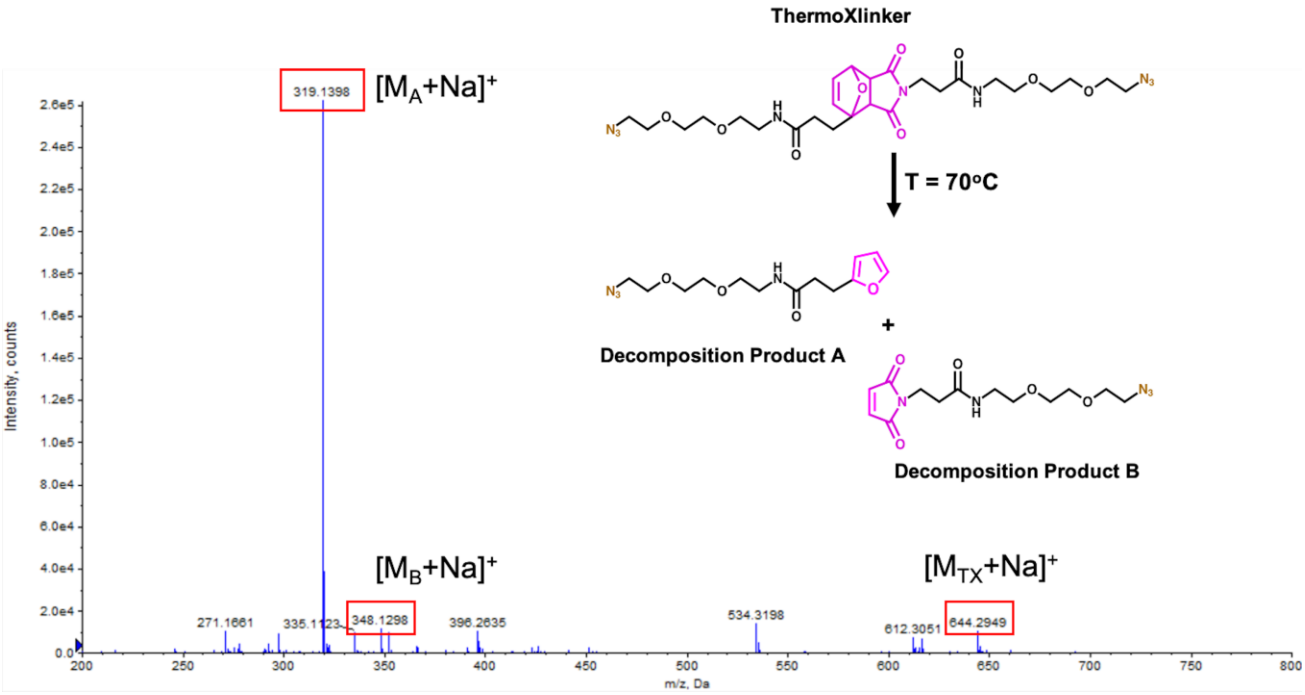


Figure S8. Mass spectrum (positive mode) of ThermoXlinker after 24 hrs. of heating at 70ºC, showing the decomposition products from retro-Diels-Alder (rDA) reaction.

Table S3. Mass spectrometry analysis of the ThermoXlinker decomposition products

Species	Calculated	Found (m/z)	Mass Accuracy (ppm)
$[M_A+Na]^+$	319.1382	319.1398	4.9
$[M_B+Na]^+$	348.1284	348.1298	4.1

Preparation of Empty Surface-Crosslinked Micelles (SCMs). The micelle solution was prepared by dissolving the surfactant (1.0 mg, 0.003 mmol) in 235 µL of distilled water. To this solution, ThermoXlinker (0.9 mg dissolved in 5.0 µL DMSO, 0.0015 mmol), THPTA-Cu complex (5.0 µL of 25 mM stock, 0.00025 mmol), and sodium ascorbate (5. 0 µL of a 25 mg/mL solution in water, 0.0025 mmol) were added. The resulting mixture was gently shaken for 4 hours at room temperature. The SCMs were purified by centrifuging at 10,000 rpm for 5 minutes and washing with distilled water (2x). For the non-thermoresponsive SCMs, Azide-PEG_3-Azide (in 5.0 µL DMSO, 0.0036 mmol) crosslinker was added instead and purification was performed by running the final crude mixture over a G25 Sephadex column. The size and surface charge of SCMs were determined by dynamic light scattering and zeta potential measurement, respectively.

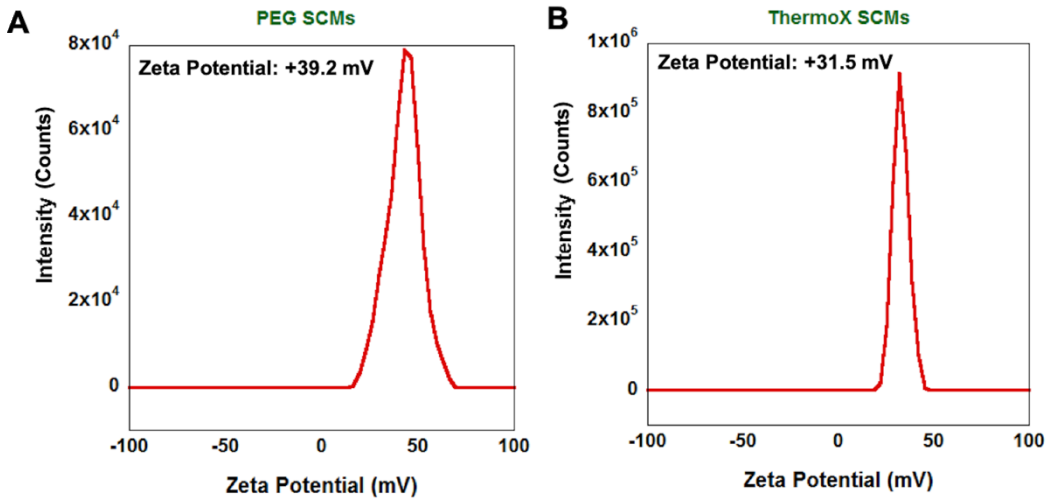


Figure S9. Zeta potential of **A)** PEG SCMs (non-thermoresponsive) and **B)** Thermoresponsive SCMs, confirming the positive surface charge of the micelles.

Temperature Response of SCMs investigated via Dynamic Light Scattering. From the stock SCM solutions, 400 µL of 1 mM (by surfactant) solutions were prepared in distilled water, one for each temperature – 25ºC, 30ºC, 35ºC, 40ºC, 50ºC, 60ºC, 70ºC and 80ºC. The solutions were placed in quartz cuvette and heated at the desired temperature for 30 minutes using a temperature controller accessory. The particle sizes were measured through dynamic light scattering (dispersant: micro rheology optimization) and the experiment was repeated for two more trials.

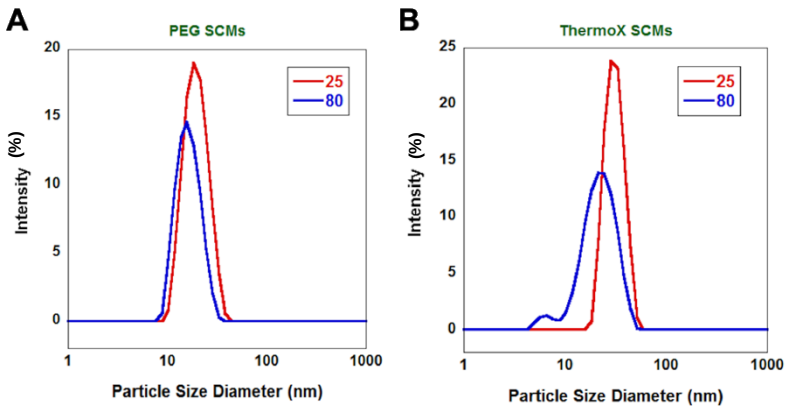


Figure S10. Comparison of the particle size distribution of **A)** PEG SCMs (non-thermoresponsive) and **B)** ThermoSCMs before (at 25ºC) and after heating at 80ºC for 30 minutes.

Table S4. Particle size distribution analysis of PEG and ThermoSCMs

Micelles	**Polydispersity Index (PDI)**	
	25ºC	**80ºC**
PEG SCMs	0.174	0.183
ThermoX SCMs	0.067	0.269

Transmission Electron Microscope (TEM) Imaging of SCMs. The SCMs were visualized using a ThermoFisher Tecnai G2 Spirit BioTWIN Microscope operated at 80 keV. Samples were prepared by placing 3.0 μL of SCM solution (stock: 10 mM by surfactant) onto a plasma-cleaned and carbon-coated copper grid, and adding 100.0 μL of 0.5% uranyl acetate (UA). The grids were air-dried for at least an hour prior to imaging. For the thermal decomposition study, an empty thermoresponsive SCM solution (1.0 mM by surfactant) was prepared in 100.0 μL distilled water and heated at 70ºC on a heat block. After 2 and 24 hrs., 3.0 μL aliquots were collected and imaged under the TEM microscope.

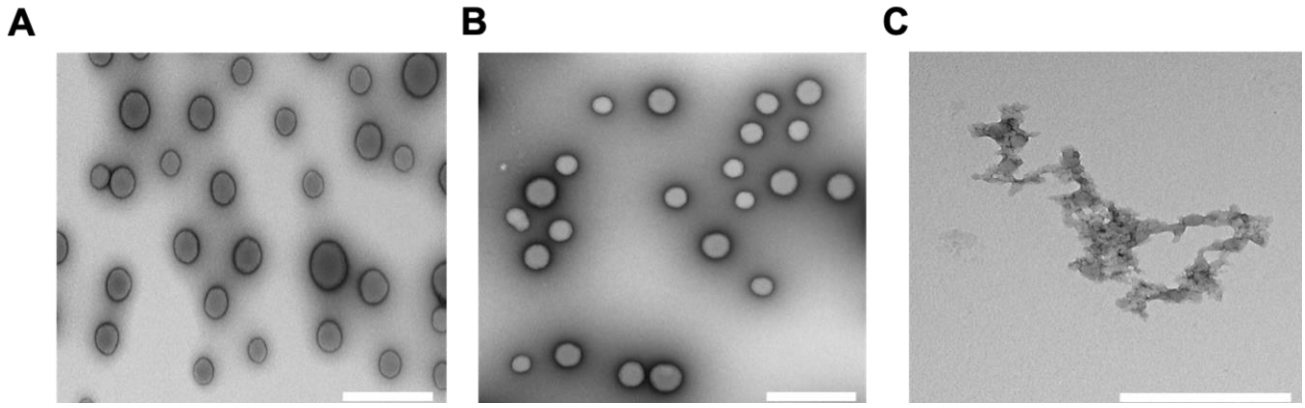


Figure S11. Decomposition of ThermoSCMs upon heating at 70ºC as monitored by TEM. **A)** t = 0 hrs. showing intact SCMs **B)** t = 30 mins. showing minor reduction in SCM size and **C)** t = 6 hrs. showing complete breakdown of SCMs. All samples were stained with 0.5% uranyl acetate. Scale bars are 500 nm.

Kinetics of Thermoresponsive SCM Degradation. From the stock of the empty SCM solution, 50 µL of aliquot was diluted to 125 µL using distilled water, resulting in a final concentration of 2.4 mM with respect to the ThermoXlinker. The SCM solution, contained in a tightly sealed tube, was then heated at 70°C on a heat block to induce the retro-Diels-Alder (rDA) reaction. After 10 minutes, the SCM solution was placed in an ice bath for 30 minutes to let the water vapor condense and prevent the forward DA reaction. Any volume changes were noted prior to the maleimide quantitation and a similar heating protocol was applied to all other kinetic time points (30, 60, 120 and 360 minutes). 10 µLs aliquot from each SCM solution was collected to determine the concentration of maleimide produced from ThermoXlinker dissociation using a fluorometric maleimide assay kit (Sigma Aldrich). The fluorescence intensity (excitation: 490 nm, emission: 520 nm) was measured using a Biotek Cytation 5 microplate reader.

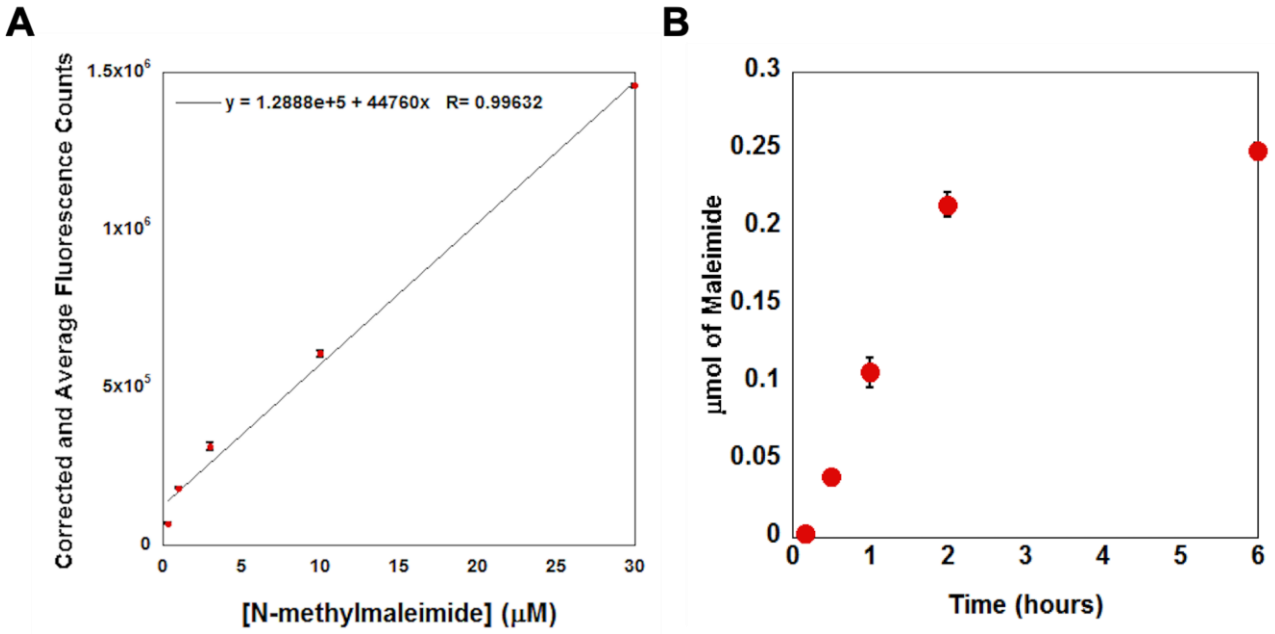


Figure S12. A) Calibration curve for *N*-methylmaleimide, $n = 3$ **B)** Monitoring the maleimide concentration (in µmol) as function of heating (T = 70°C) time, $n = 3$.

Pyrene Encapsulation. The surfactant (1.0 mg, 0.003 mmol) and pyrene (0.37 μmol) were first dissolved in 20.0 μL DCM. The resulting mixture was dried overnight under nitrogen gas at room temperature. After adding 235 μL of distilled water, the solution was sonicated for 5 minutes followed by the sequential addition of ThermoXlinker (0.9 mg dissolved in 5.0 μL DMSO, 0.0015 mmol), THPTA-Cu complex (5.0 μL of 25 mM stock, 0.00025 mmol), and sodium ascorbate (5. 0 μL of a 25 mg/mL solution in water, 0.0025 mmol). The reaction mixture was gently shaken for 4 hours at room temperature. For the non-thermoresponsive or PEG SCMs, Azide-PEG_3-Azide (in 5.0 μL DMSO, 0.0036 mmol) crosslinker was added instead. The purification of the pyrene-loaded SCMs was carried out in the same manner as that of empty SCMs. The size and surface charge of the nanoparticles were determined by dynamic light scattering and zeta potential measurement, respectively.

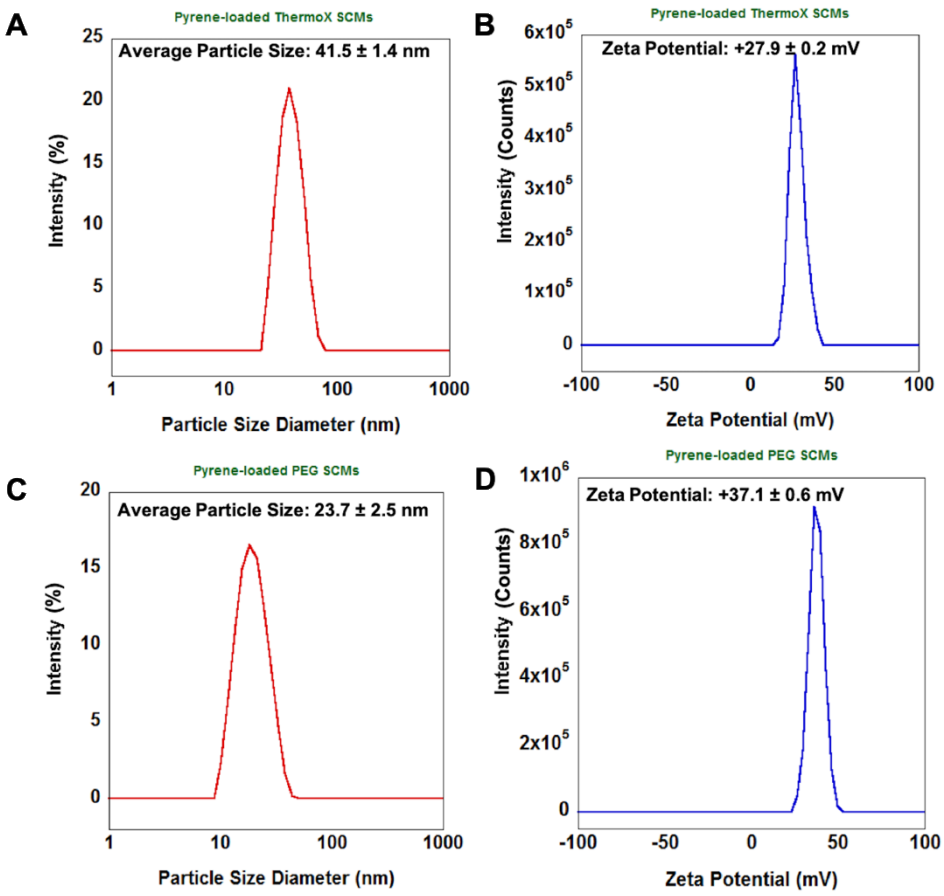


Figure S13. Particle size (**A** and **C**) and zeta potential (**B** and **D**) of pyrene-loaded ThermoX and PEG SCMs.

Analysis of Pyrene Encapsulation. 100 μM pyrene in ethanol was prepared as a stock solution from which different external standard solutions (0.05, 0.1, 0.2, 0.3, 0.4, 0.5 μM) were prepared in distilled water for a total volume of 800 μL. The aqueous SCM sample solution was prepared by diluting a 0.5 μL aliquot of the 10 mM stock SCM solution to a total volume of 800 μL. Fluorescence spectra (excitation: 334 nm, scan: 360-550 nm) were recorded using a Jobin Yvon Fluorometer at 25°C. The fluorescence intensity at 384 nm was considered in constructing the calibration curve and in quantifying the amount of pyrene loaded in the SCM. At this wavelength, the fluorescence emission of pyrene is invariable to the polarity of the environment.[2,3]

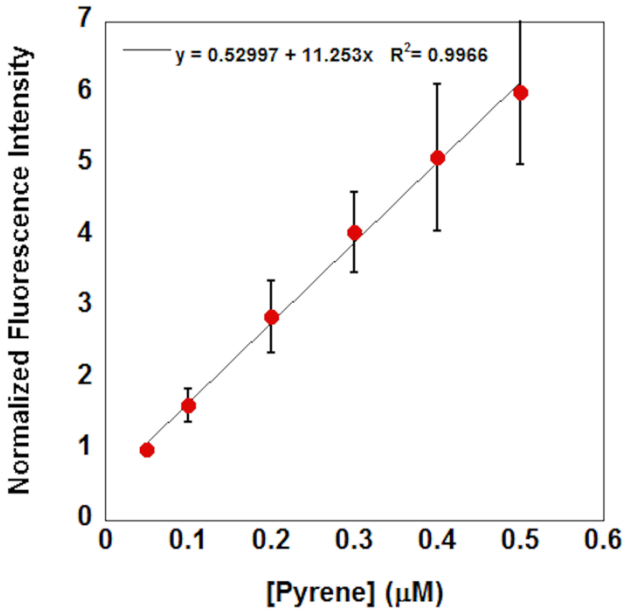
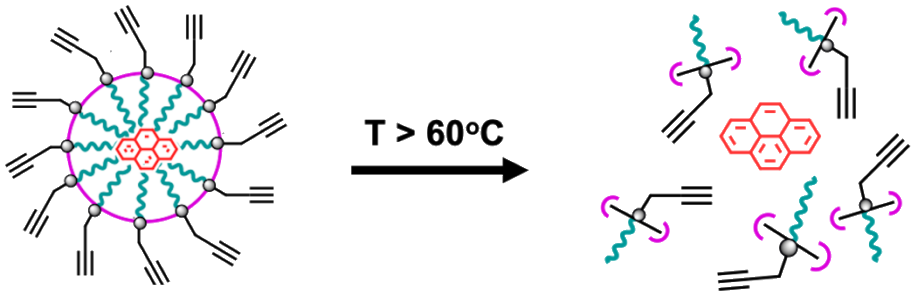


Figure S14. Calibration curve for pyrene, n = 3.

Pyrene Release Assay. In a typical assay, a sample solution was prepared by diluting 15 μL of stock thermoresponsive SCM ($[pyrene]_{stock}$ = 142 μM) solution to 2 mL using distilled water, giving a final pyrene concentration of 1.1 μM. This solution was then placed in the sample holder of a Jobin Yvon Fluorometer that was maintained at the target temperature (T = 25, 45, 60, 70°C). The fluorescence spectra (excitation: 334 nm, scan: 360-550 nm) were recorded at different time points (t = 0, 10, 30, 60, 120 and 360 minutes). The control experiments were only done at 70°C, wherein 1.1 μM solutions of free pyrene and PEG SCM ($[pyrene]_{stock}$ = 113 μM) were prepared and the fluorescence spectra were collected at similar time points.



Scheme 4. Temperature-induced breakdown of thermo SCMs and pyrene release.

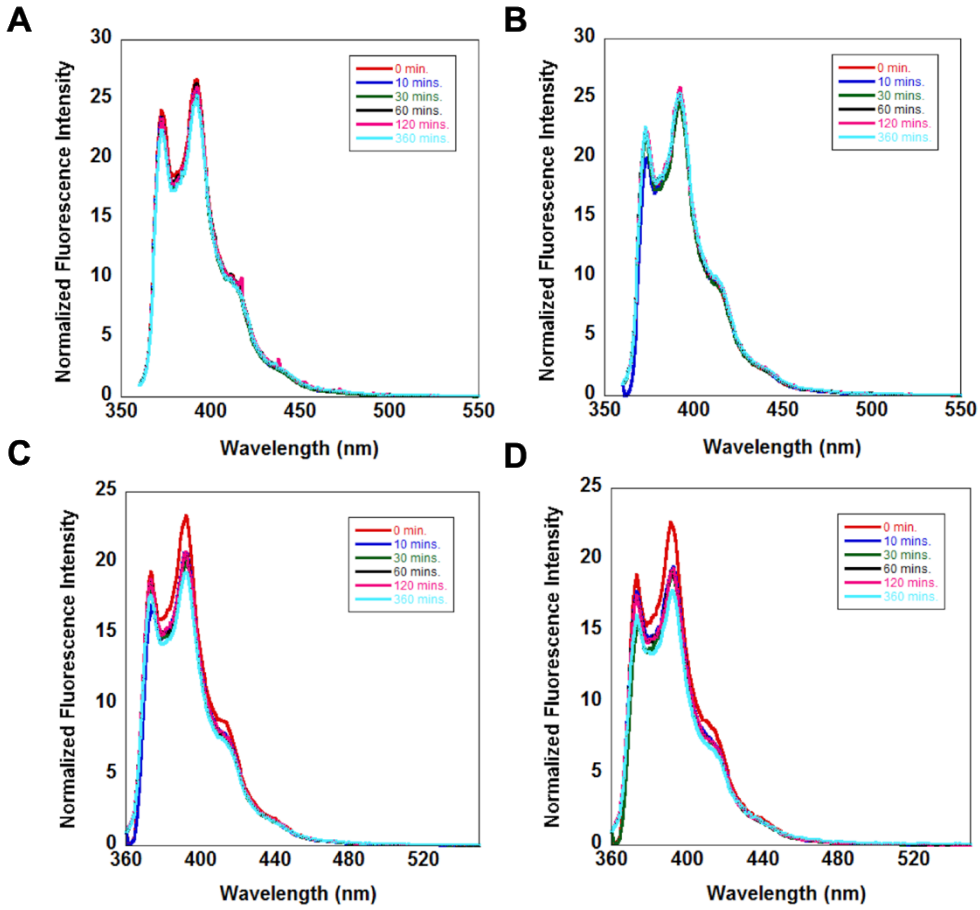


Figure S15. Normalized emission spectra of pyrene (1.1 μM) encapsulated in thermoresponsive SCMs at different time points and heating temperatures. **A)** 25°C **B)** 45°C **C)** 60°C and **D)** 70°C

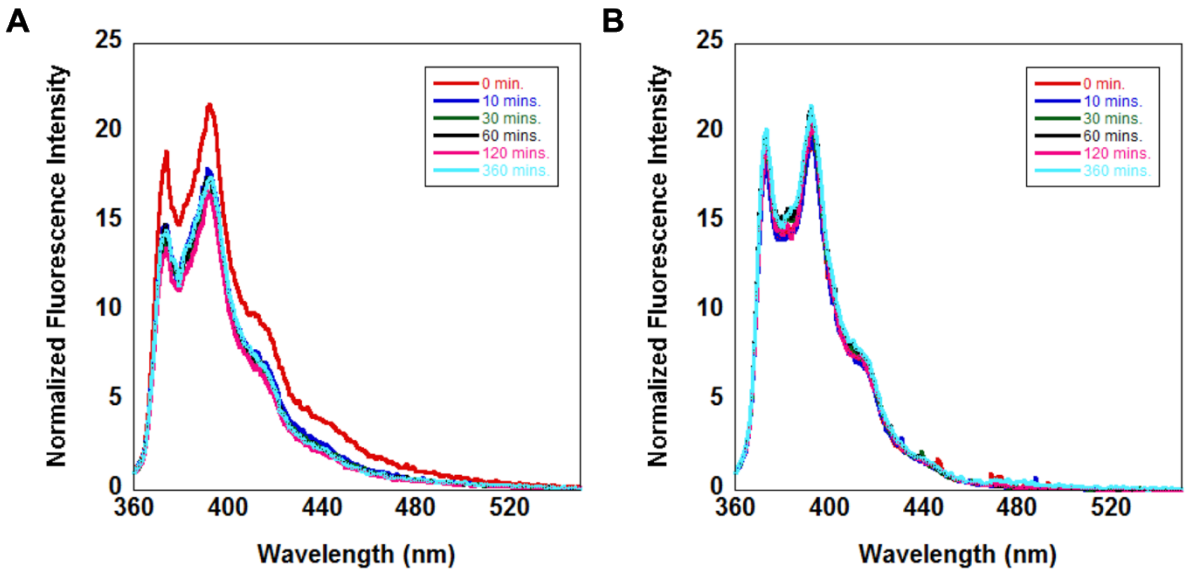


Figure S16. Normalized emission spectra of **A)** pyrene (1.1 μM) encapsulated in PEG-crosslinked SCMs and **B)** free pyrene (1.1 μM) in aqueous solution. Both samples were heated at 70°C.

Synthesis of Spherical Nucleic Acids (SNAs). In a typical synthesis, 20 nmol of 5'-hexynyl polyT20 DNA was treated with 300 µL of 0.1M DTT in pH 8 phosphate buffer for 30 minutes. The DNA was then purified using a G25 Sephadex NAP-5 column (GE Healthcare) before adding to 1 mL of 0.33 nM citrate-capped gold nanoparticle (AuNP) (diameter: 30 nm) solution. The resulting mixture was put in a rotisserie and aged in salt solution for four hours, wherein 5 µL of 0.2 M NaCl solution were added every hour. The nanoparticles were purified by repeated (3x) centrifugation and washing with distilled water. The purified nanoparticles were resuspended in 500 µL distilled water prior to characterization using DLS, agarose gel electrophoresis, TEM microscopy and UV-VIS spectroscopy.

Table S5. Particle size and zeta potential measurements of citrate AuNP and SNAs

Nanoparticle	Particle Size Diameter (nm)	Zeta Potential (mV)
Citrate AuNP	29.9 ± 1.6	-57.3 ± 2.1
SNA	43.5 ± 0.9	-43.2 ± 0.5

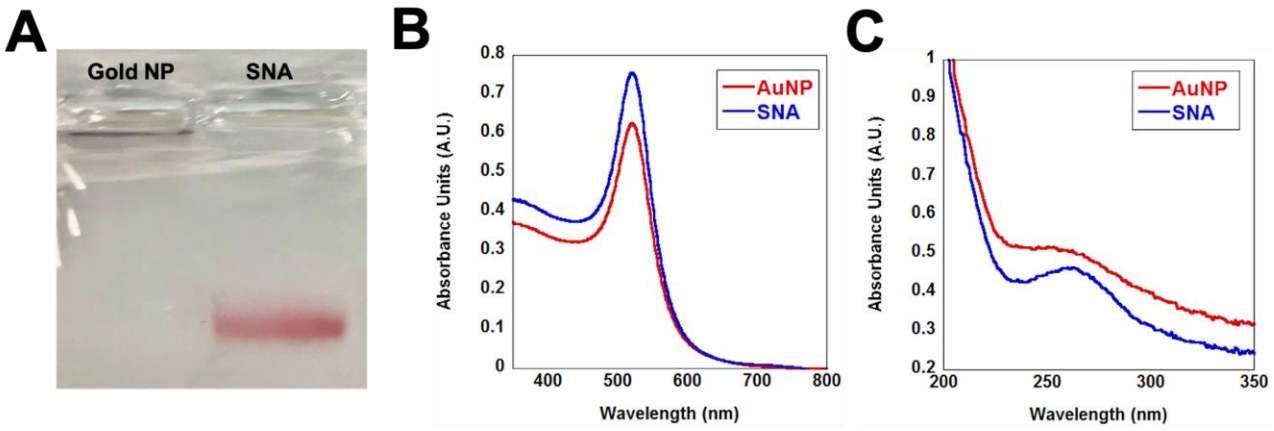


Figure S17. Characterization of SNAs. **A)** 0.5% agarose gel showing the increased electrophoretic mobility of the SNA. The gel was run for 20 minutes at 120 V. **B)** UV-VIS spectrum comparison of AuNP and SNA, indicating the DNA functionalization didn't cause any aggregation. **C)** UV-VIS spectrum of SNA confirms the presence of DNA (peak at 260 nm).

Crosslinking of SNAs. A 250 μL solution containing 0.33 nM SNA (~20 μM of DNA), 20 μM of ThermoXlinker or PEG crosslinker, 0.5 mM Cu-THPTA, 1.0 mM sodium ascorbate, was prepared in water and incubated at room temperature. After the solution turned purple, at around 10 minutes, crosslinked SNAs were purified by repeated (2x) centrifuging and washing with distilled water. The UV-VIS spectrum of the crosslinked SNA solution was recorded and a 5 μL aliquot was taken for TEM microscopy.

Retro DA Disassembly of Crosslinked SNAs. The purified crosslinked SNA solution was heated for 70°C for two hours before recording the UV-VIS spectrum and obtaining a 5 μL aliquot for TEM microscopy. The absorbance at 520 nm was also monitored at different time points (t = 0, 10, 30, 60, 120, 240, 360 minutes).

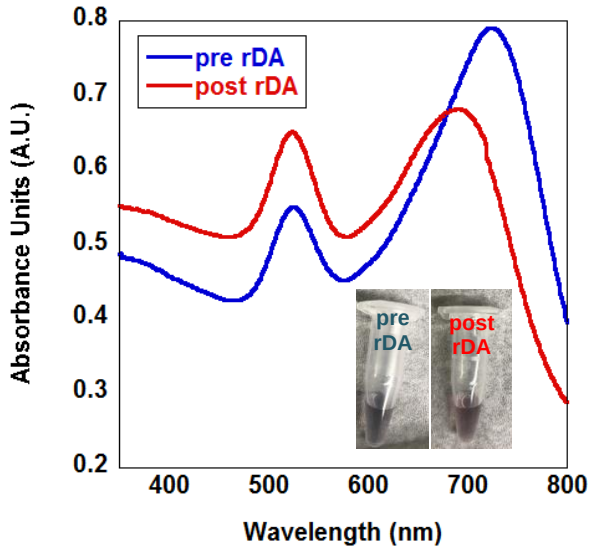


Figure S18. UV-VIS spectrum comparison of SNAs crosslinked with ThermoXlinker before and after rDA-induced disassembly (t = 2 hours, 70°C). The shift to 720 nm peak to 690 nm and the comparable intensity between 520 nm and 690 nm indicates the dissociation of crosslinked SNAs.

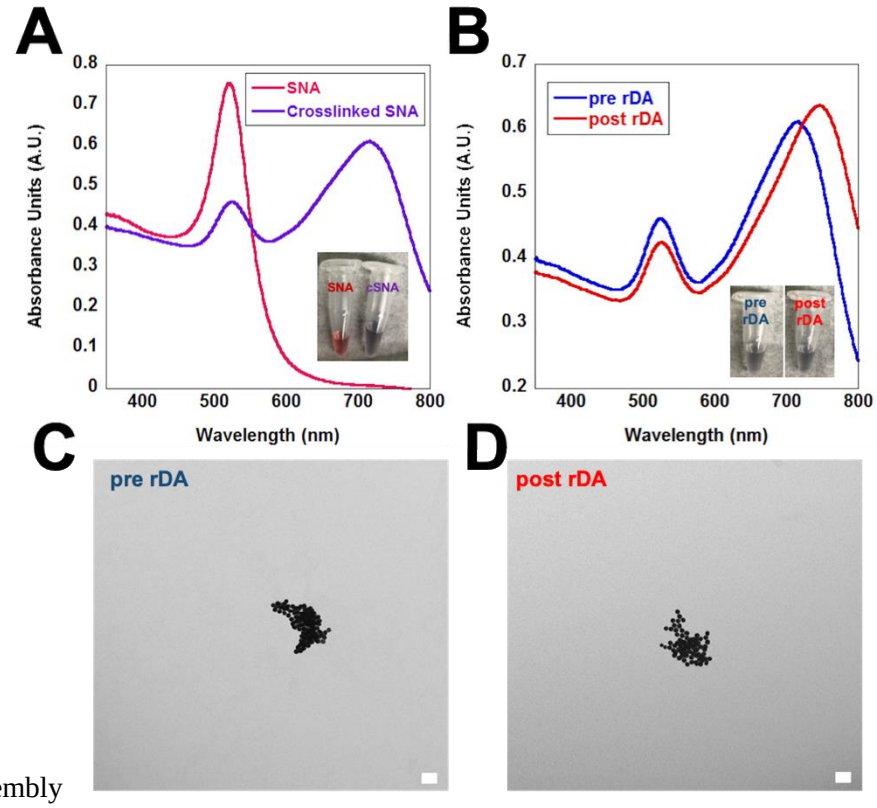


Figure S19. Assembly and disassembly of PEG-crosslinked SNAs. **A)** UV-VIS spectrum and solution comparison between SNAs and PEG-crosslinked SNAs, indicating gold nanoparticle aggregation. **B)** UV-VIS spectrum comparison between PEG-crosslinked SNAs before (25°C) and after rDA (70°C, *t* = 2 hours), showing minimal disassembly. **C)** TEM micrograph of PEG-crosslinked SNAs before rDA and **D)** post rDA. All scale bars are 100 nm.

References

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