Supporting Information for

Thiol–Acrylate Michael Addition Strategy for the Templated Synthesis of Water-Soluble Poly(β-Thioester) Nanogel: Superior Encapsulation Stability and UV-Induced Photolysis Mediated On-Demand Guest Release

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Materials and Methods:

All starting materials, reagents, and solvents were purchased from reputed commercial suppliers, including Alfa Aesar, Sigma-Aldrich, TCI, and Avra, and were used as received without any further purification unless otherwise specified. Nuclear Magnetic Resonance (NMR) spectra were recorded at 25°C on a Bruker DPX-300 MHz spectrometer using $CDCl_3$ (purchased from Sigma-Aldrich) as the solvent. Chemical shifts for both ^1H and ^{13}C nuclei are reported in δ (ppm) relative to internal standards. High-resolution mass spectrometry (HRMS) data were acquired using a Q-TOF microYA-263 mass spectrometer equipped with an ESI (electrospray ionization) source. Column chromatography was performed using silica gel (60–120 mesh) as the stationary phase. The molecular weight (M_n) and polydispersity index (PDI) were determined by gel permeation chromatography (GPC) on a Waters system fitted with a Waters 515 HPLC pump and a Waters 2414 refractive index detector, employing DMF as the eluent and calibrated against poly(methyl methacrylate) (PMMA) standards. Additional HRMS

analyses were conducted using XEVO G2-XS Q-TOF and Micromass Q-TOF micro instruments. UV–Vis absorption spectra were collected using a Labtronics LT-291 spectrophotometer, while fluorescence measurements were obtained on a Horiba Jobin Yvon FluoroMax-4 spectrophotometer. Dynamic light scattering (DLS) and zeta potential measurements were performed using a Malvern Nano-Zetasizer. Transmission electron microscopy (TEM) images were captured on a JEOL JEM-2100HR microscope operating at 200 kV and equipped with electron energy loss spectroscopy (EELS) capabilities. Optical fluorescence images were obtained using an Olympus fluorescence microscope. Isothermal titration calorimetry (ITC) data were recorded and analyzed using the MicroCal Origin software (v7.0).

Synthesis of Tertiary amine containing di-acrylate (TADA) amphiphilic monomer (M1):

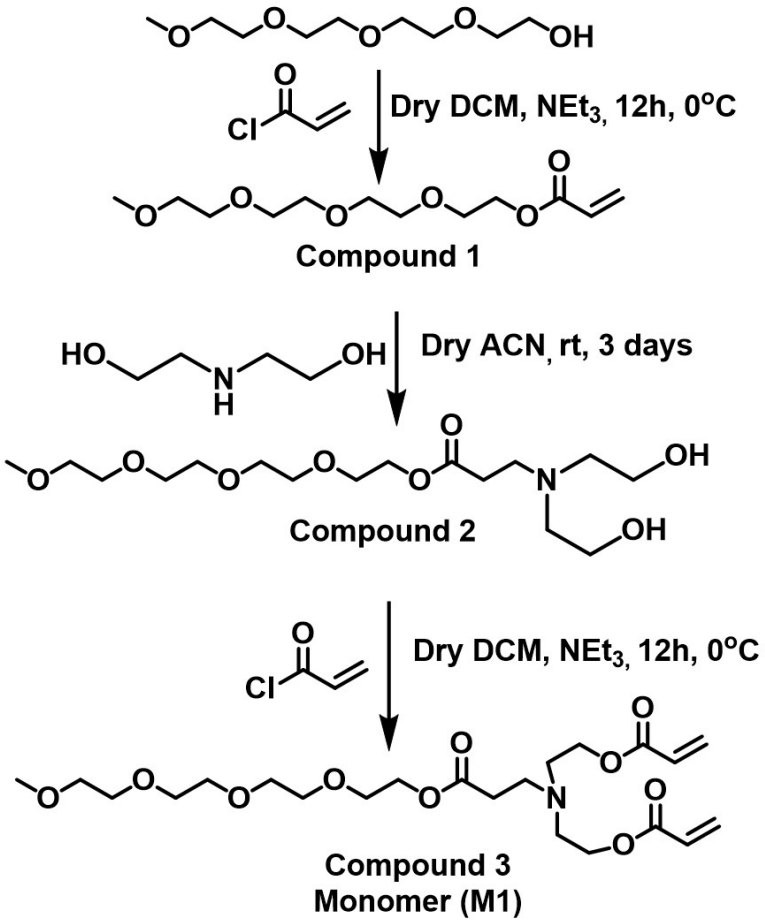
Synthesis of compound 1 and compound 2 is described in the literature[1]. Synthesis of final TADA monomer (M1) compound 3 is explained below.

Compound 3: Initially, compound 2 (250mg, 0.68 mmol) was dissolved in 20 mL of dry DCM under an inert argon atmosphere. To this solution, triethylamine (2.40 mL, 1.7 mmol) was added, and the reaction mixture was stirred while being cooled in an ice bath. A solution of acryloyl chloride (1.31 mL, 1.5 mmol) in 5 mL of dry DCM was then added dropwise to the reaction mixture with constant stirring. After the complete addition, the mixture was allowed to warm to room temperature and stirred further for 12 h to ensure completion of the reaction. Upon completion, the reaction was quenched, and the resulting mixture was washed with water to remove unreacted reagents and salts. The combined organic phase was dried over anhydrous Na_2SO_4, filtered, and the solvent was removed under reduced pressure. The desired product was obtained as a pale yellowish liquid in 95% yield and used in subsequent reactions without further purification.

^{1}H NMR (300 MHz, $CDCl_3$) δ 6.44 (dd, 2H), 6.24 – 6.04 (m, 2H), 5.86 (dd, 2H), 4.33 (t, 4H), 4.24 (t, 2H), 3.75 (t, 2H), 3.67 (12H), 3.56 (t, 2H), 3.39 (s, 3H), 2.99 (t, 4H), 2.64 (t,2H).

^{13}C NMR (75 MHz, $CDCl_3$) δ 207.08, 165.90, 131.05, 128.28, 71.92, 70.59, 69.12, 63.72, 59.04, 52.50, 50.25, 30.96.

ESI-MS: m/z calculated for $C_{22}H_{37}NO_{10}$ $(M+H)^+$ = 476.2417, observed = 476.2460



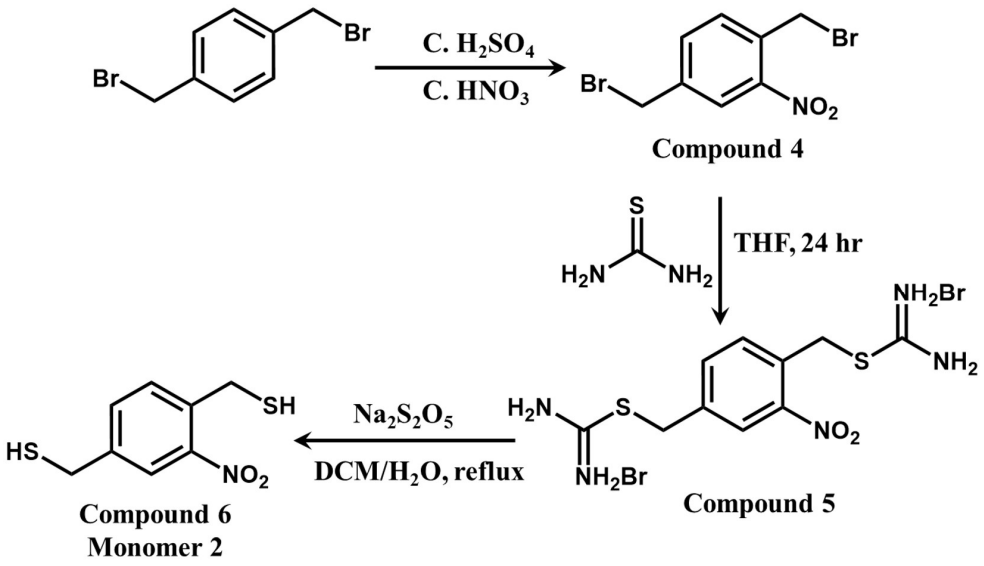
Scheme S_1: Synthetic scheme of Tertiary amine containing di-acrylate (TADA) amphiphilic monomer (M1)

Synthesis of 1,4-bis(methylthio)-2-nitrobenzene (BMTNB) as crosslinker monomer (M2):

Compound 4: In this experiment, a solution of 1,4-bis(bromomethyl)benzene (1 g, 0.0038 mol) was dissolve in concentrated H_2SO_4 (98%, 2 mL) under ice bath with continuous stirring after 30 minutes 1 mL of Nitric acid (69%) was slowly added drop by drop under 0°C. After

allowing the reaction to warm to room temperature and stirring it overnight, the resulting mixture was poured into 50 mL ice-water and filtered. The yellow solid obtained was washed three times with water (3*200 mL) and final product 1,4-bis(bromomethyl)-2-nitrobenzene was obtain as brownish yellow solid.

^{1}H-NMR (400 MHz, Chloroform-d) δ 8.1 (d, 1H), 7.7 (d, 1H), 7.6 (d, 1H), 4.8 (S, 2H), 4.5 (S, 2H).



Scheme S2: Synthetic scheme of 1,4-bis(methylthio)-2-nitrobenzene (BMTNB) as crosslinker monomer (M2)

Compound 5: Compound 4 (0.7 grams, 0.002 mmol) and 2.5 equivalent of thiourea (0.46 grams, 0.006 mmol) were dissolved in 20 mL of dry THF and cooled to 0° C. The mixture was stirred overnight at room temperature, resulting in the formation of white precipitates. After being filtered and washed with ethyl acetate with 3 times, the yellowish white solid was dried under vacuum, as compound 5 (80% yield).

^{1}H NMR (400 MHz, CD_3OD: δ 8.29 (s, 1H), δ 7.86 (d, 1H), δ 7.79 (d, 1H), δ 4.79 (s, 2H), δ 4.60 (s, 2H).

Compound 6: Compound 5 (0.5 g, 0.0011 mol) was suspended in a mixture of dichloromethane (6 mL) and water (4 mL) and stir 30 minutes and then eight equivalents of sodium metabisulfite ($Na_2S_2O_5$) (1.7 g, 0.009 mmol) were added to this heterogeneous mixture. The resulting solution mixture was then refluxed for 4 h under 55°C. After cooling to room temperature, the bilayer solution was washed 3 times with excess dichloromethane (DCM). The combined organic layers were dried over anhydrous Na_2SO_4, filtered, and concentrated, and given as yellowish solid as compound 6 (BMTNB) with a 98% yield which was used as a monomer 2.

^{1}H-NMR (400 MHz, Chloroform-d) δ 8.01 (s, 1H), 7.58 (d, 1H), 7.45 (d, 1H), 4.02 (d, 2H), 3.81 (d, 2H).

NMR Characterization:

^{1}H and ^{13}C NMR spectra were recorded on a Bruker DPX-300 NMR spectrometer at 25 °C using $CDCl_3$ as the solvent (purchased from Sigma-Aldrich). Chemical shifts are reported in parts per million (ppm) and referenced to tetramethylsilane (TMS, δH = 0.00 ppm). The spectra were calibrated using the residual proton signal of chloroform at δH = 7.26 ppm. All spectra were interpreted using first-order analysis. The following abbreviations are used throughout: s (singlet), br s (broad singlet), d (doublet), t (triplet), q (quartet), quin (quintet), dd (doublet of doublets), dt (doublet of triplets), and m (multiplet).

Electrospray Ionization Mass Spectrometry Characterization:

For mass spectrometric analysis, the samples were dissolved in suitable solvents and directly injected into the instrument. All spectra were recorded at room temperature.

Determination of Aqueous self-assembly of the amphiphile:

Calculated amount of TADA (5 mg) was dissolved in 500 µl of acetone. Subsequently, 10 mL of HPLC water was added very slowly, and the heterogeneous solution mixture was kept open for 6 hours to allow the acetone to evaporate. Finally, the resulting solution was used as mother

stock solution (concentration = 1 mM). Next, a series of screw-capped vials were filled with different concentrations taking from stock solution, ranging from 1 mM to 0.06 mM per 1ml of HPLC water. Prior to spectroscopic analysis, exact 20 µL DiI dye solution (stock solution = 1mg/1 ml acetone, 1 mM) was added dropwise to each vial and kept open for 1hr with continuous slow stirring to allow the acetone to evaporate, where concentration of DiI dye in final resulted solution was 2×10^{-5} M. After that, each DiI dye-encapsulated amphiphilic solution sets were used to measure the UV-vis spectra. Finally, the concentration of the DiI dye encapsulated amphiphilic solution was plotted against the absorbance intensity at 562 nm, and the inflection point was identified as the Critical Aggregation Concentration (CAC) of the monomer TADA based amphiphilic compound.

Isothermal Titration Calorimetric (ITC) Experiments:

In the ITC experiment, the sample cell was filled with an aqueous nanoaggregate solution (1mg/100µl HPLC water, 2 mM) using an injection volume of 1µL and a total of 40 injections. The reference cell, on the other hand, was filled with deionized water. The sample was injected with a time interval of 120 seconds between each addition, while continuously stirring at a speed of 400 rpm, and maintaining a temperature of 25°C. The calculation of the free energy (ΔG) of micellization can be determined using equation-1 provided below. In this equation, CAC represents the critical aggregation concentration, T represents the temperature, and R represents the universal gas constant. The entropy (ΔS) of micellization can be determined using Gibb's-Hemholtz equation (eq.2).

$\Delta G = RT \ln CAC$ ……. **eq.1**

$\Delta G = \Delta H - T\Delta S$ ...……...**eq.2**

Fabrication of Nanogel:

In order to generate a nanoaggregate solution, a weighed quantity of compound TADA (0.5 mg, 1 mM) was dissolved in 100 µl of acetone. Subsequently, 1 mL of HPLC water was added

dropwise to the previous mixture and the mixture was then left open for 1 hour to allow the acetone to evaporate. Next, dithiol based BMTNB (0.24 mg, 1.1 mM in 0.1 ml acetone) and hexylamine (0.14µl, 1.1 mM in 0.1 ml acetone) were simultaneously added to the nano assembled solution to fabricate the nanogel. Then the solution was stirred for 24 hours and after that it was filtered with a 0.45 µm syringe filter.

^{1}H NMR Analysis of Crosslinked Nanogel:

The nanogel was prepared via in situ thiol–acrylate Michael addition within the aqueous nanoassembled solution, as described previous fabrication of nanogel section. Briefly, TADA (10 mg) was dissolved in acetone (1.5 mL) and added dropwise into HPLC-grade water (10 mL) to form nanoassemblies, followed by evaporation of acetone under ambient conditions. Subsequently, BMTNB (5 mg) and hexyl amine (catalytic amount) dissolved in acetone (1 mL) were added to the nanoassembled solution and stirred for 24 h at room temperature to facilitate core crosslinking. The resulting crosslinked nanogel dispersion was then lyophilized to obtain a solid sample. The ^{1}H NMR of lyophilized solid material was recorded in CDCl (Figure S1).

Gel Permeable Chromatography (GPC) Study:

At first, a weighed quantity (2mg) of the monomer (TADA) and cross-linked nanogel were dissolved in 1ml of DMF. Then the solutions were sonicated and vortexed for 3 minutes. The homogeneous solutions were produced by allowing them to settle at room temperature for few hours and then the solutions were filtered using a membrane filter with 0.45 µm pore size. Finally, the solutions were injected into the GPC column one by one to estimate the molecular weight of the monomer and nanogel, where the flow rate and the column temperature were kept at 0.8ml /min and 25°C, respectively.

Transmission Electron Microscope (TEM) Study:

To investigate the TEM images, 1.0 mM solutions of nanoassembly and nanogel were drop-casted on a carbon coated copper grid (300-mesh) and then the samples were then left to dry in the air for 24 hours before the pictures were captured.

Dynamic Light Scattering (DLS) Study:

The above same solutions i.e, 1.0 mM of nanoassembly and nanogel were filtered using a membrane with a 0.22 μm pore size before the measurements were taken for the DLS analysis. The measurements were performed at room temperature.

Dilution stability test:

The Size modifications studies were measured using DLS instruments after diluting by water and an organic solvent DMF for both the nanoaggregate and nanogel solution.

DiI dye Encapsulation and pH and light responsive release Studies:

A measured volume (15 μL) of pre-prepared DiI dye solution (1 mg mL^{-1} in acetone, 1 mM) was added dropwise to the pre-formed nanoaggregate solution. The nanogel was subsequently fabricated by the addition of dithiol-based BMTNB and hexylamine, followed by slow stirring at room temperature for 5 h to obtain a homogeneous dispersion. Prior to further experiments, the DiI-loaded nanoaggregate and nanogel solutions were passed through a hydrophilic membrane filter (0.45 μm pore size) to remove any unencapsulated dye.

The resulting dye-encapsulated systems were then subjected to UV–visible absorbance measurements to evaluate the extent of DiI encapsulation. Upon confirmation of dye loading, light- and pH-responsive guest release studies were carried out. Time-dependent UV–visible spectra were recorded to monitor the release of DiI. The percentage of dye release was calculated from the absorbance intensity of DiI at 548 nm using the following equation: $[(I_o-I_t)/I_o]\times100$ where Io is the initial intensity of absorbance and it is the absorbance intensity at any time ‘t’.

Calculation of photon flux:

The photon flux of the light source was calculated from the measured optical power assuming monochromatic irradiation. The energy of a single photon was determined using $E=hc/\lambda$, where h is Planck's constant, c is the speed of light, and λ is the irradiation wavelength. The photon flux [Φ (phi)] was then obtained by dividing the incident optical power by the energy per photon. i.e, $\Phi=P\lambda/(hc)$. Reflections and scattering losses were not considered in this estimation, where P = light power (W = J/s), λ = 365 nm, h = 6.626×10−34 J·s and c = 3.00×108 m/s.

Fluorescence Microscopy Study:

Optical polarization microscopy (OPM) was carried out for both DiI-encapsulated nanoaggregate and nanogel samples. For this purpose, 50 µL of each dye-loaded solution was drop-cast onto clean glass slides, followed by placement of a cover slip. The samples were imaged at 40× magnification using an Olympus BX-51 fluorescence microscope. Although the spatial resolution of optical microscopy is insufficient to precisely identify the location of individual dye molecules, the observation of red-emitting spherical features under fluorescence OPM clearly indicates the successful incorporation of DiI within the assembled structures.

Calculation of Dye Encapsulation Efficiency and Encapsulation Capacity:

The dye encapsulation efficiency (EE) & dye encapsulation capacity (EC) of crosslinked nanogel solution was calculated by absorption spectroscopy using following equations:

EE (%) = [weight of dye in micelle/weight of dye in feed] ×100%

EC (%) = [weight of dye in micelle/weight of dye loaded micelle] × 100%

Ellman'S Test:

During the conversion of nanoaggregates into a nanogel via a Michael addition reaction, TADA (0.5 mg in 1 mL $CHCl_3$, 1 mM) was cross-linked using a dithiol linker, BMTNB (0.24 mg, 1.1 mM). At predetermined time intervals, 10 µL aliquots were withdrawn from the reaction mixture and immediately added to a pre-prepared basic solution of Ellman's reagent (0.2 mg

mL^{-1} in chloroform). The resulting solutions were analyzed by UV–visible spectroscopy to monitor the consumption of free thiol groups. The progress of the cross-linking reaction was confirmed by tracking changes in the characteristic absorption bands of Ellman's reagent at 490 nm.

Forster Resonance Energy Transfer (FRET) Studies:

Stock solutions were prepared by dissolving 1mg of each FRET acceptor DiI dye (1,10-di-octadecyl-3,3,30,30-tetramethylindocarbo cyanine perchlorate) and donor DiO dye (3,30 -di-octadecyloxacarbocyanine perchlorate) independently in 1mL of acetone. Twenty microliters each of DiI and DiO solutions were thoroughly mixed, taken from their respective stock solutions and added dropwise to series of pre-prepared nanoaggragate solutions (1 mM) drop by prop with continuous very slowly stirring for 2 h. After that, the solution was cross-linked by using dithiol based BMTNB to fabricate the nanogel followed by final resulting nanogel solution was then filtered using a hydrophilic membrane (pore size: 0.45 μm) before FRET studies. These final crosslinked nanogel solutions was subjected to FRET investigations with the donor excitation at 470 nm and the slit width kept at 2/2, with a scan speed of 5 nm/s. Time dependent photoluminescence (PL) spectra were measured, and the Förster resonance energy transfer (FRET) ratio Ia/(Ia+Id) was calculated at various time intervals, where Ia represents the emission intensity of the acceptor dye at 584 nm, while Id represents the emission intensity of the donor dye at 508 nm.

Additional Figure:

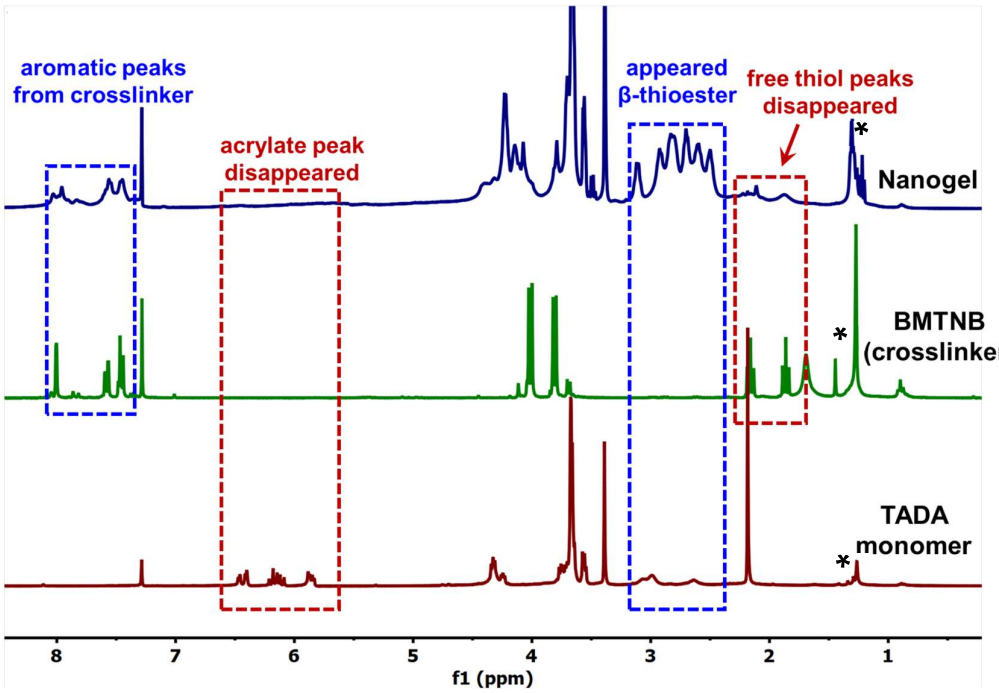


Figure S1: ^{1}H-NMR stack plot of TADA monomer, BMTNB monomer (as dithiol crosslinker) and crosslinked nanogel (*=denotes residual solvents peak)

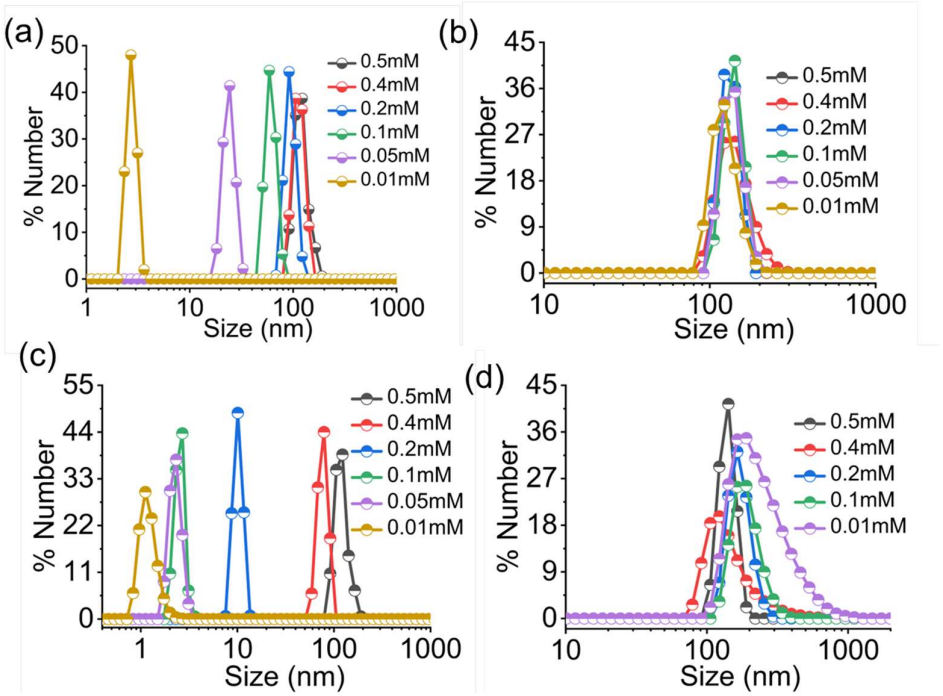


Figure S2: DLS profile of (a), (c) nanoaggregate and (b), (d) nanogel solution upon dilution with water and an organic solvent (DMF).

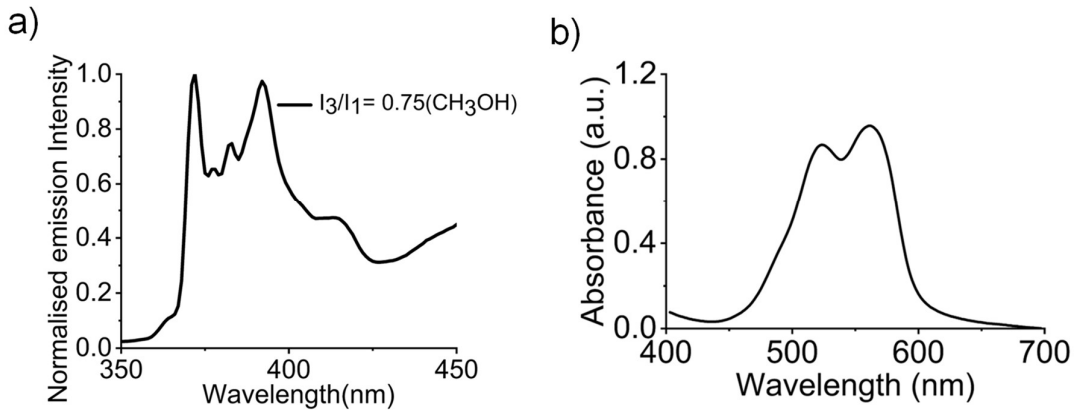


Figure S3: Plots for calculation of encapsulation efficiency (EE) and encapsulation capacity (EC) (a) Emission spectrum of pyrene encapsulated micelle; $I_3/I_1 = 0.75$ (CH_3OH) (b) Absorption spectrum of DiI encapsulated micelles.

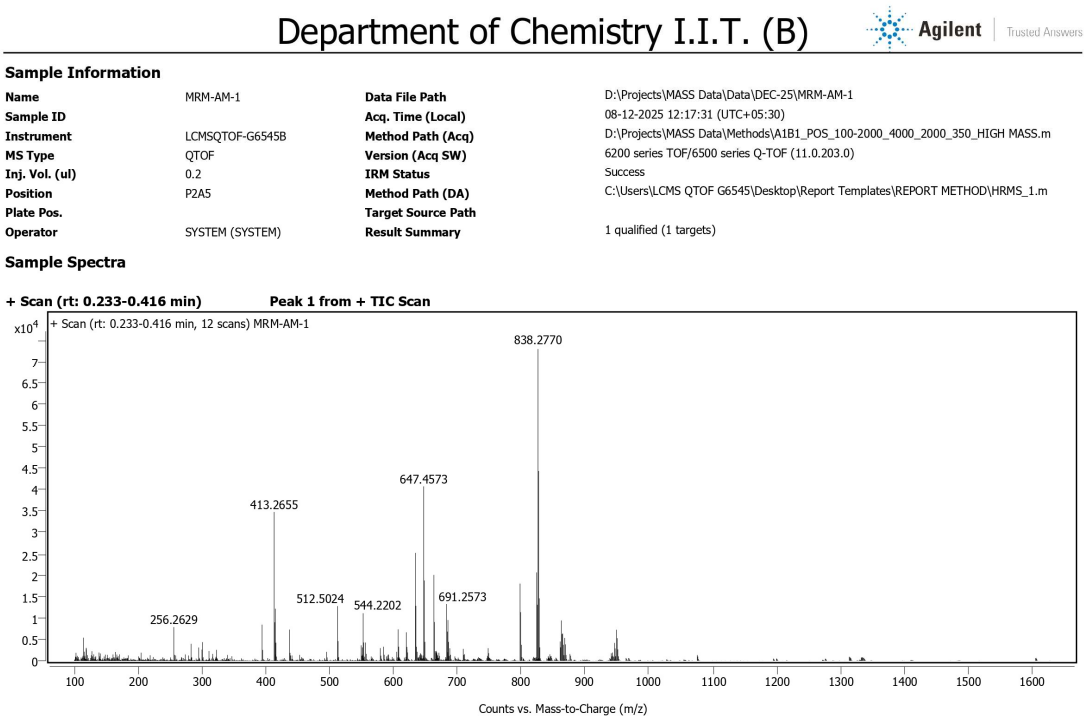


Figure S4: Mass profile of the photolytic degradation of the nanogel under ambient conditions (25ºC) upon exposure to UV light (λ = 365 nm, 200 W) (m/z values = 544.2202, 691.2573 and 838.2770.)

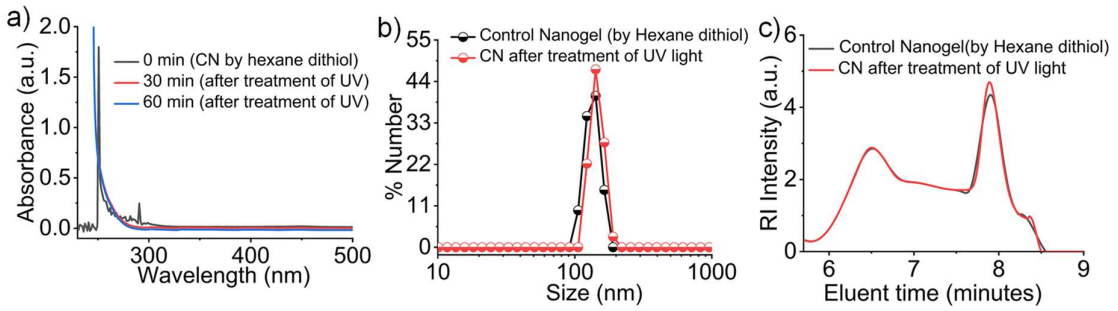


Figure S5: (a) UV–vis absorption profiles of the control crosslinked nanogel (CN) under UV irradiation at different time intervals. Comparison of (b) DLS profile and (c) size exclusion chromatography (SEC) profile of that control nanogel(CN) before and after UV light exposure.

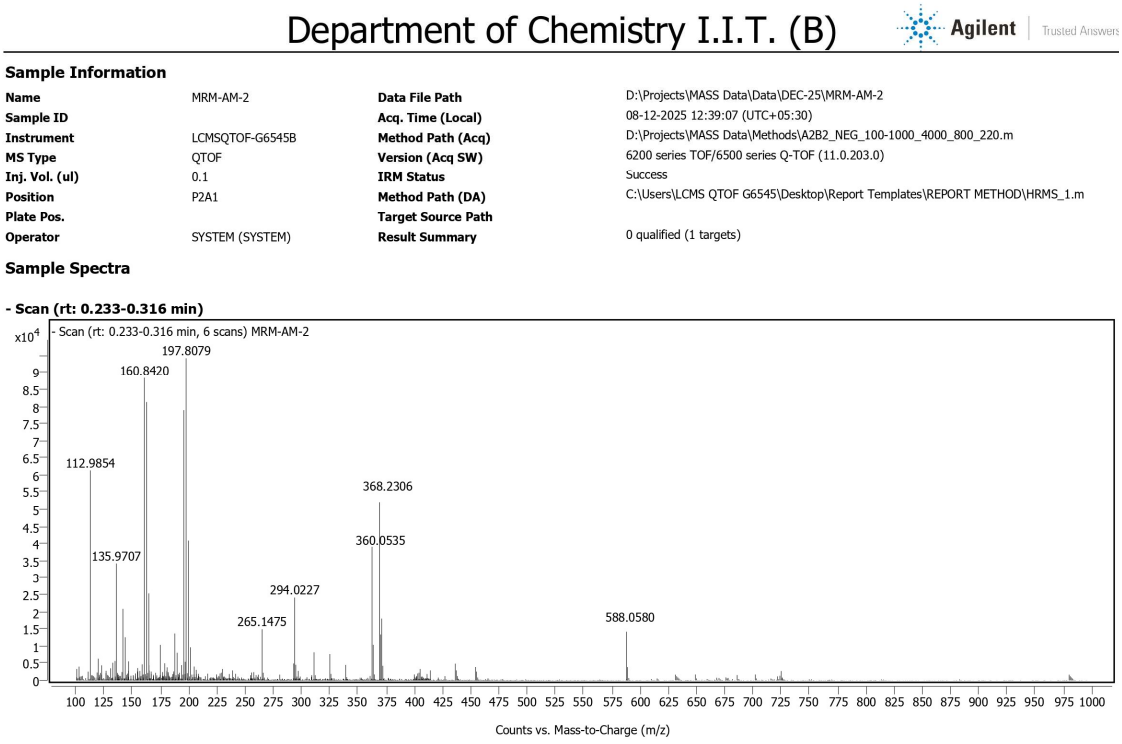


Figure S6: Mass profile of the pH responsive degradation of the nanogel after incubation at pH 5.3 for 55 h). (m/z values = 360.0535 and 368.2306.)

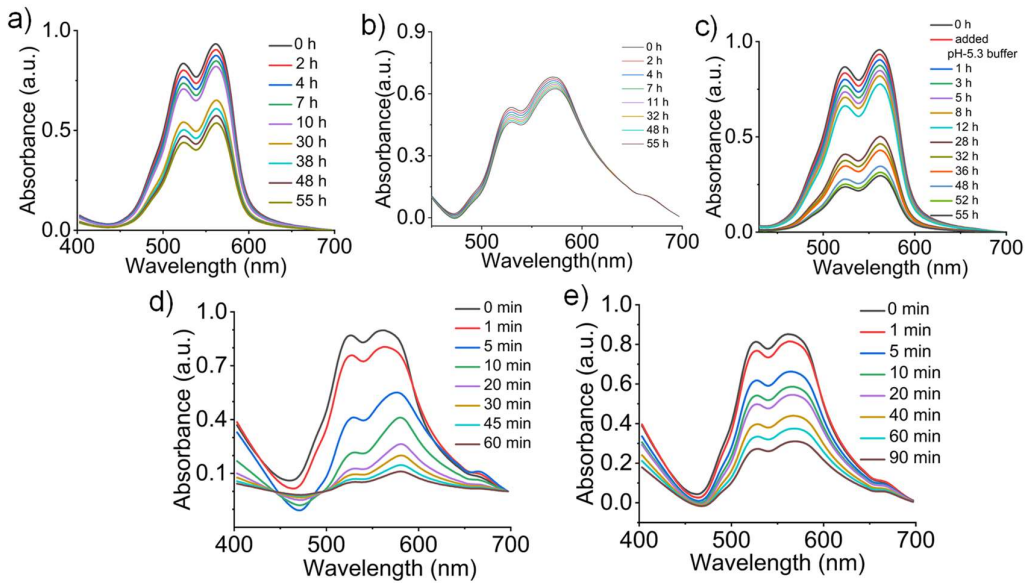


Figure S7: Guest release profile of DiI encapsulated (a) nanoaggregates, (b) nanogel in absence of stimuli and (c) nanogel in presence of acidic medium, pH=5.3. (d) Nanogel in presence of UV light (200 watt) (e) Nanogel in presence of UV light (100 watt).

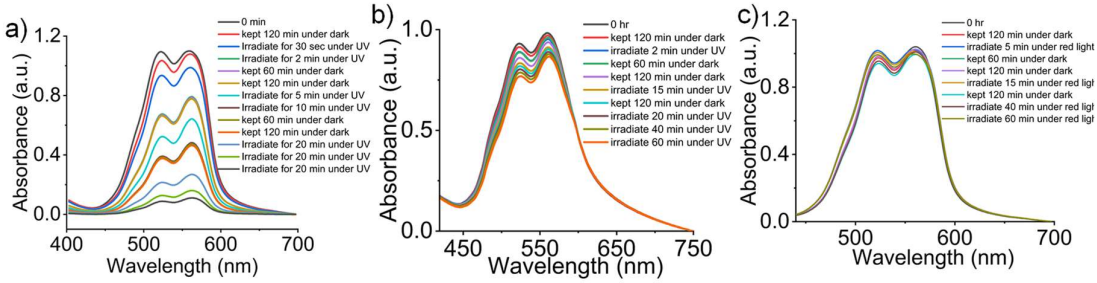


Figure S8: Guest release profile of DiI encapsulated (a) nanogel in the alternating presence of UV light (365nm, 200 watt) and dark, (b) control nanogel in the alternating presence of UV light (365nm, 200 watt) and dark, (c) nanogel in the alternating presence of red light (650 nm, 100 watt) and dark.

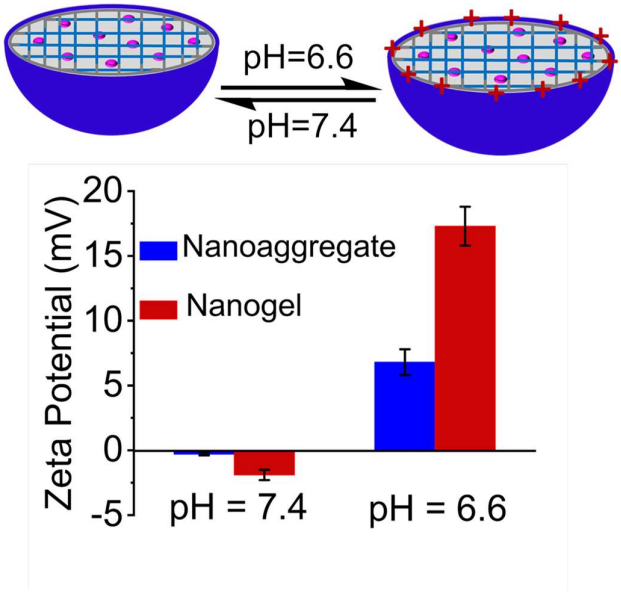


Figure S9: Surface charge of nanoassembled and nanogel solution at tumor-relevant pH (~6.6) and neutral pH (~7.4) measured by zeta potential.

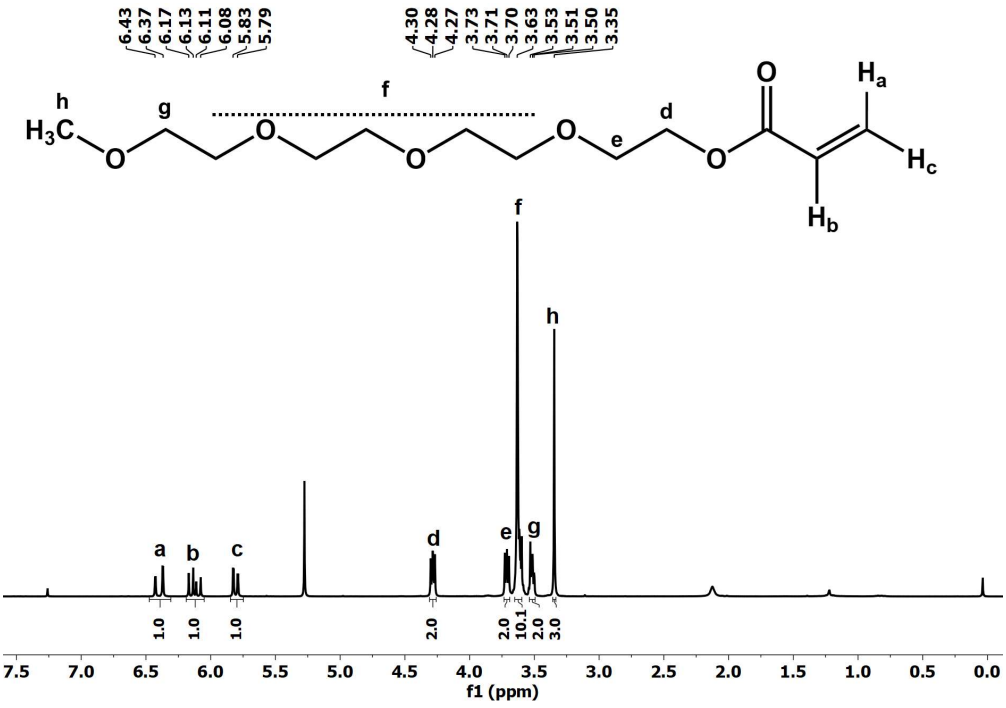


Figure S10: ^{1}H NMR spectrum of compound-1, Solvent= $CDCl_3$

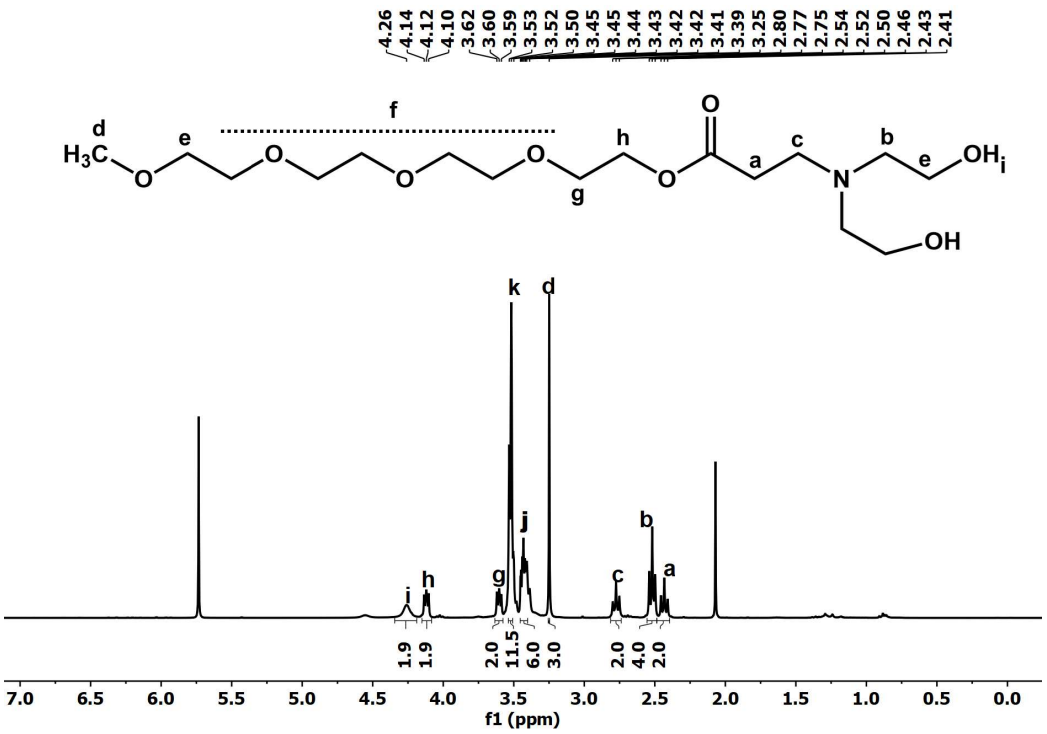


Figure S11: ^{1}H NMR spectrum of compound-2, Solvent= $CDCl_3$

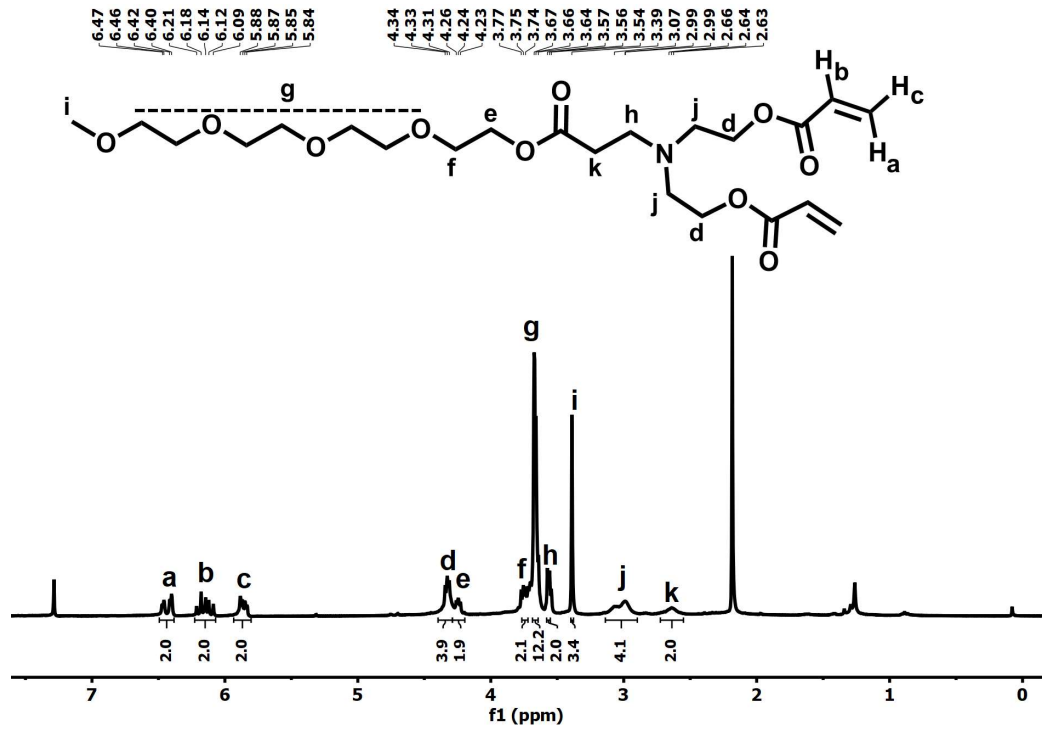


Figure S12: ^{1}H NMR spectrum of compound-3 (M1) (TADA), Solvent= $CDCl_3$.

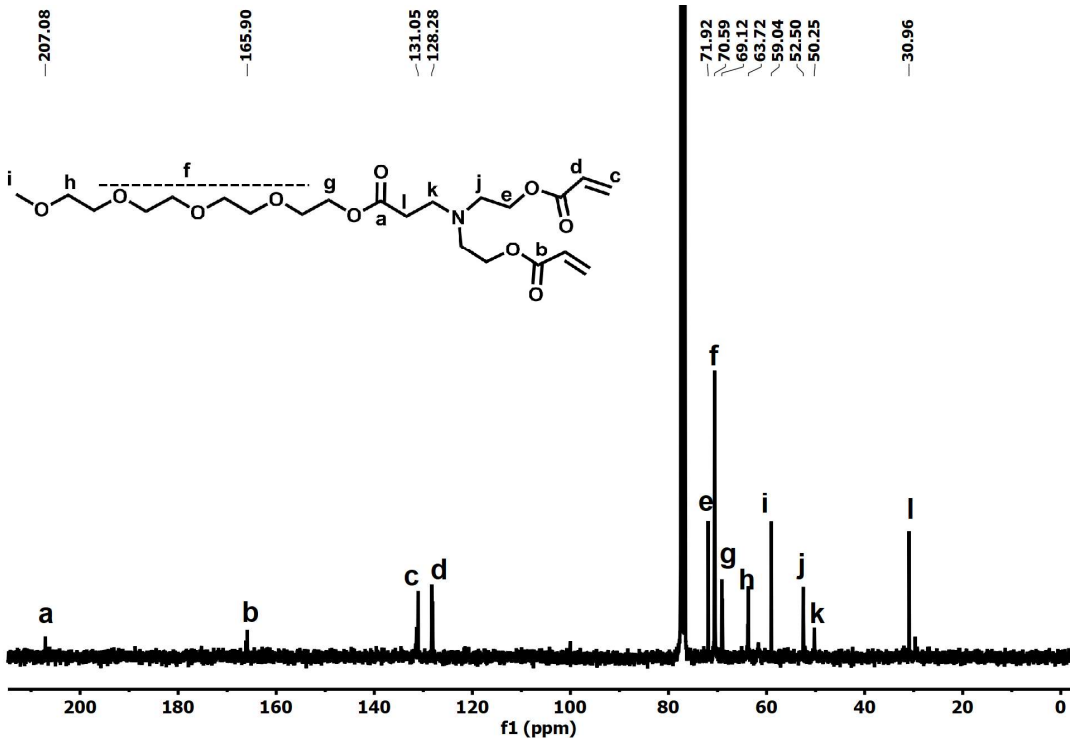


Figure S13: ^{13}C NMR spectrum of compound-3 (M1) (TADA), Solvent= $CDCl_3$

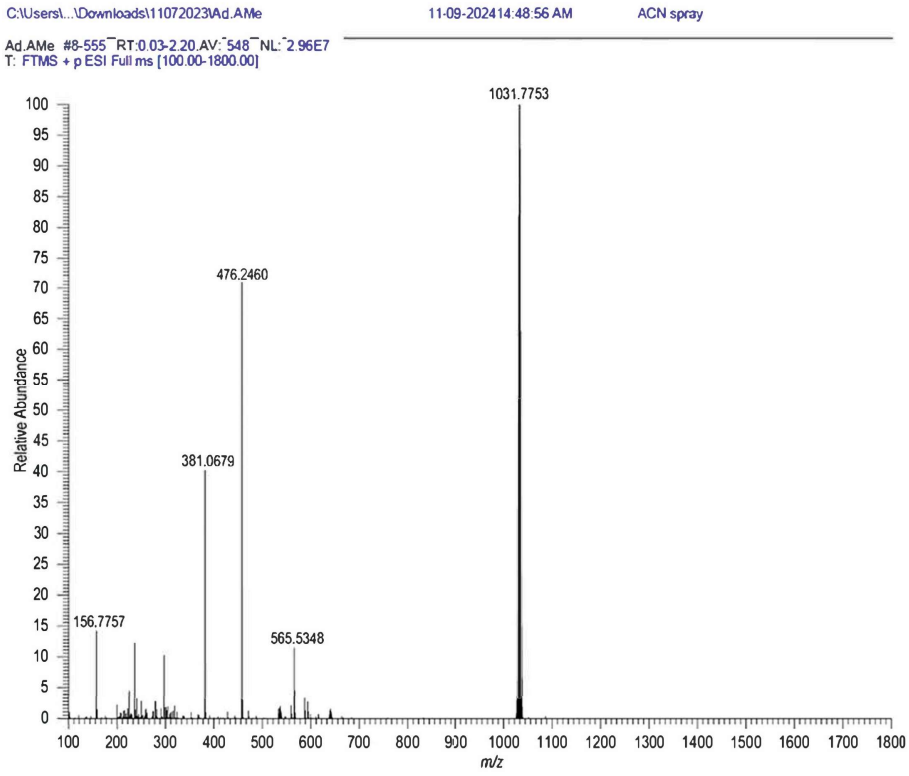


Figure S14: Mass spectrum of Compound **3** [calculated $(C_{22}H_{37}NO_{10}+H)^+$= 476.2417, observed = 476.2460]

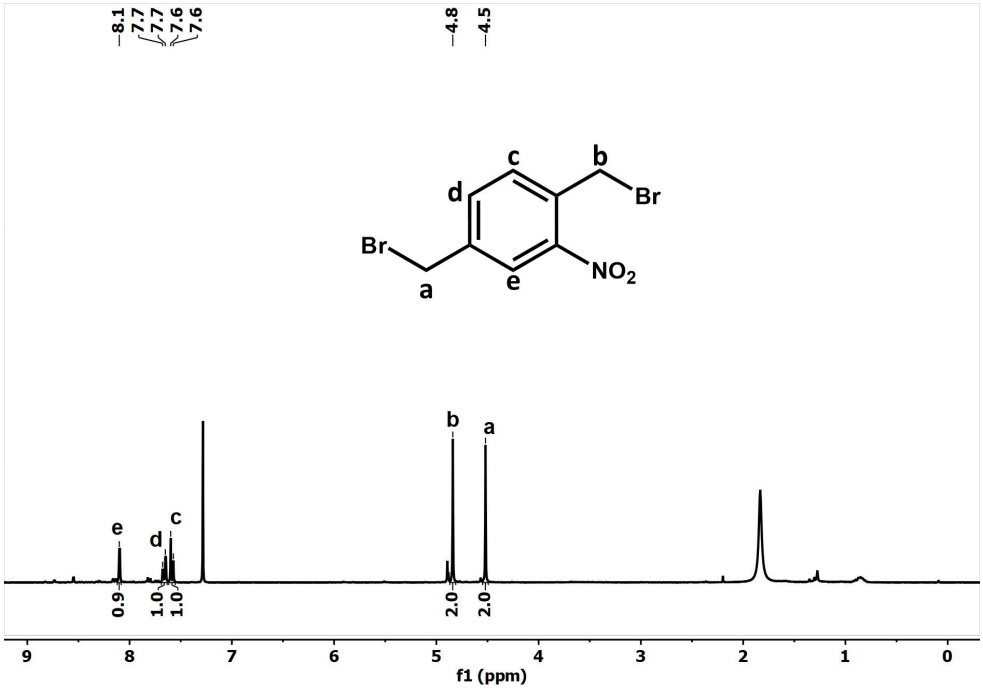


Figure S15: ^{1}H NMR spectrum of compound 4, Solvent= $CDCl_3$

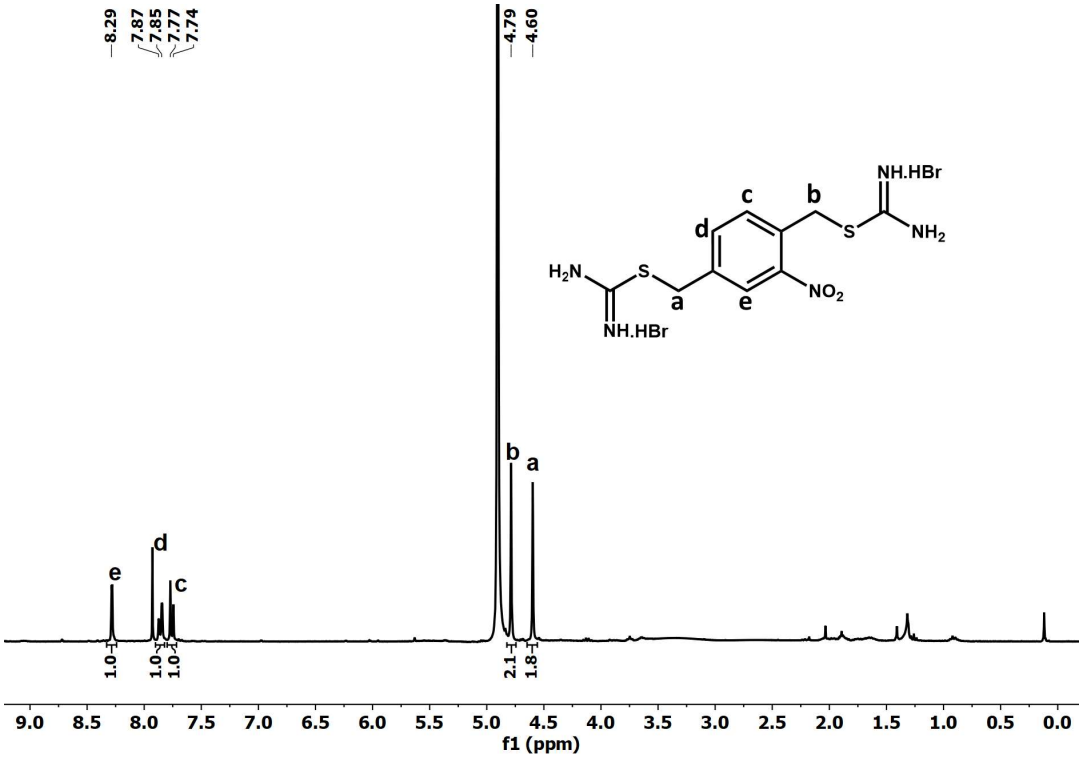


Figure S16: ^{1}H NMR spectrum of compound 5, Solvent= CD_3OD

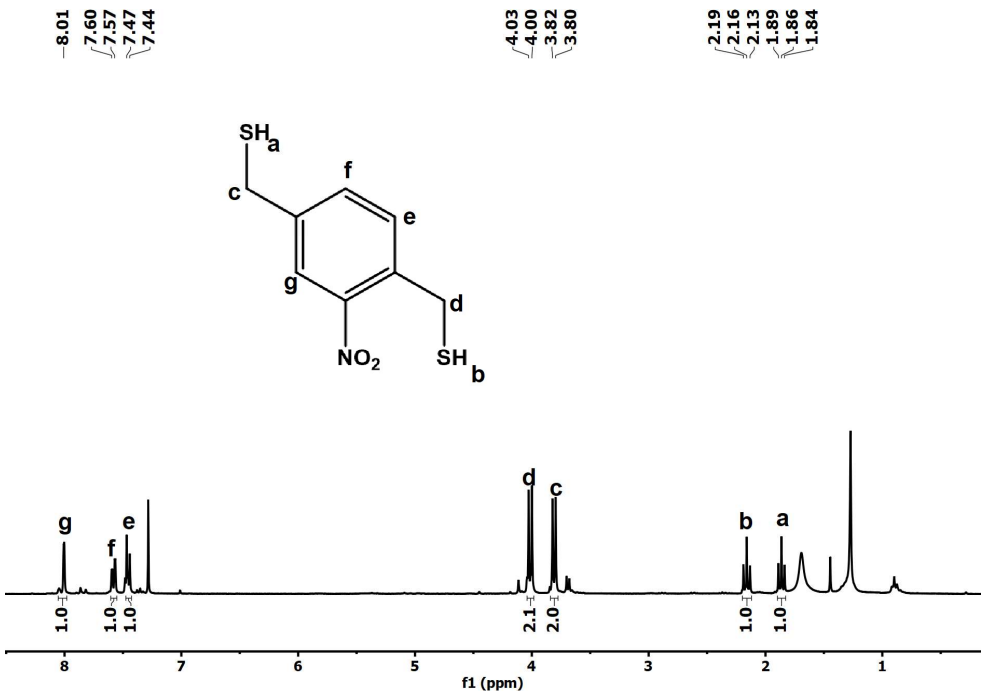


Figure S17: ^{1}H NMR spectrum of compound 6 (M2), Solvent= $CDCl_3$

Reference:

1. S. Kolay, A. Mondal, S. M. Ali, S. Santra and M. R. Molla, *Journal of Macromolecular Science, Part A*, 2022, **59**, 838-848.