Supporting Information for

Reducing Agent Triggered Templated Synthesis of Dynamic Covalent Poly(disulfide)s Nanonetwork: Remarkable Tuning in Noncovalent Encapsulation Stabilities and Cargo Release

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Materials and Methods:

All total starting materials, reagents and solvents were purchased from commercial vendors (such as Alfa Aesar, Sigma-Aldrich, TCI, and Avra) and utilized without performing any additional purification. Each NMR spectroscopic measurement was made at 25°C using a Bruker DPX-300 MHz spectrometer with $CDCl_3$ solvent which was bought from Sigma-Aldrich. All chemical shifts, including the ^{13}C and proton (^1H) signals, were measured in parts per million (δ) units. Using the Q-tof micro-YA-263 mass spectrometer and an ESI method, high resolution mass spectroscopic data were obtained. For column chromatography, silica gel with a mesh size of 60–120 was used as stationary phase. Through the use of gel permeation chromatography (GPC), the molecular weight (Mn) and polydispersity index (PDI) value were determined. The Waters GPC system was outfitted with a Waters 515 HPLC pump and a Waters 2414 RI (refractive index) detector using DMF solvent, and it was calibrated using poly(methyl methacrylate) (PMMA) standards. The XEVO G2-XS Q Tof and Micromass Q-Tof micro machines were used for HRMS studies. Labtronics spectrometer model number LT-

291 was used to record UV-vis spectrometric data and Horiba Jobin Yvon FluoroMax-4 spectrophotometer was used to measure the fluorescence spectra. The Malvern Nano-zetasizer instrument was used to measure the DLS data. Using a 200 kv transmission electron microscope, JEOL-JEM 2100HR with EELS was used to capture the TEM pictures. Using an Olympas fluorescence microscope, an optical fluorescence microscope image was obtained. The MicroCal Origin software program (version 7.0) was used to analyze the ITC data.

Synthesis of lipoic acid based amphiphilic compound:

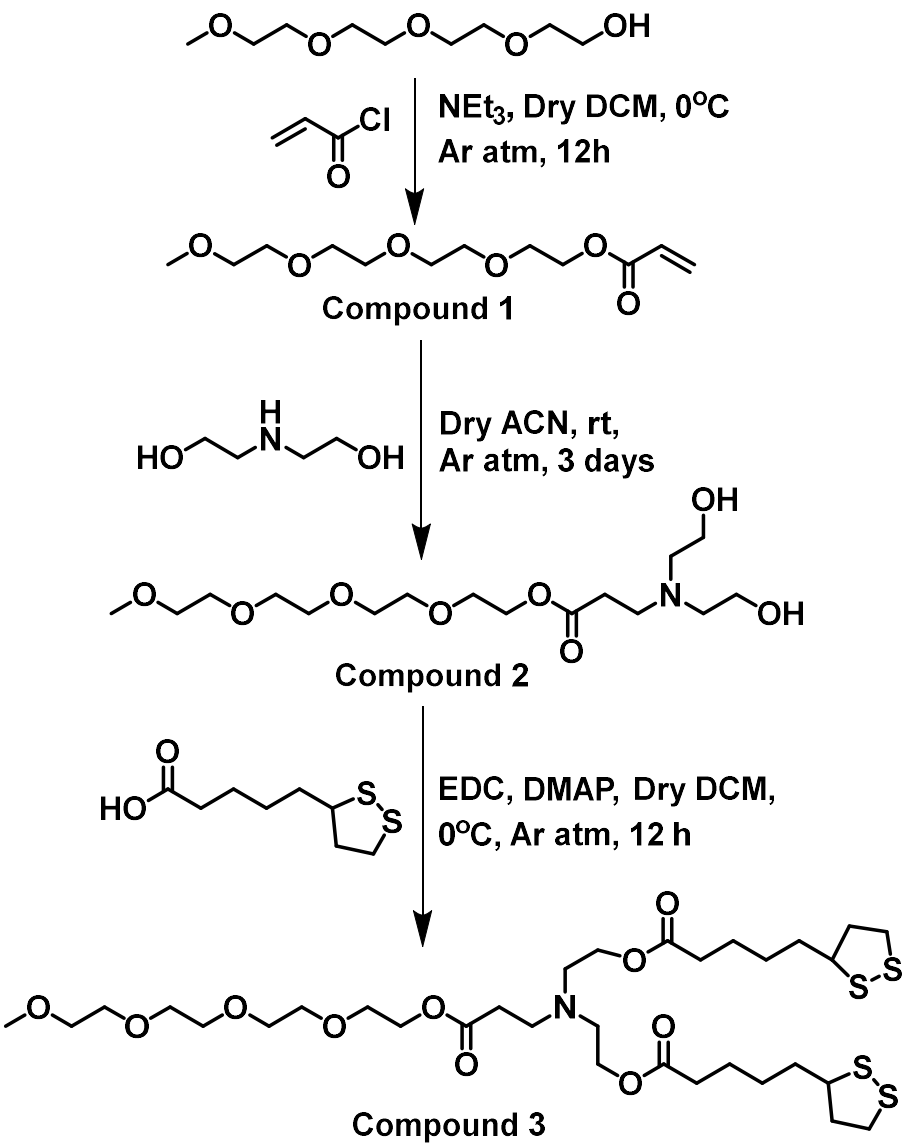
Synthesis of compound **1** and compound **2** is described in the literature[1]. Synthesis of final amphiphilic compound **3** is explained below.

Compound 3: Compound 2 (0.3 grams, 0.82 mmol) and 1,2 dithiotic acid (0.41 grams, 1.9 mmol) were dissolved in 20 mL of dry dichloromethane (DCM) and cooled to 0°C. Next, an ice-cold solution containing N-(3-Dimethylaminopropyl) -N'-ethylcarbodiimide hydrochloride (EDC.HCl) (0.39g, 2.04 mmol) and 4-(dimethylamino) pyridine (DMAP) (0.25g, 2.04 mmol) in 10ml of dry DCM was slowly added drop by drop to the previous cold solution mixture using a dropping funnel. The mixture was then stirred at room temperature for 12 hours under argon atmosphere. Subsequently, the reaction was halted and the solution was workup with brine solution (3×30 ml). The organic phase was dried using anhydrous sodium sulphate (Na_2SO_4) and evaporated under reduced pressure to obtain the crude product in the form of a yellowish oil. The crude product was then further purified using column chromatography, using silica gel as the stationary phase and 20% EAA in hexane solution as the eluent, the pure product was obtained as a yellowish liquid with a yield of 87%.

^{1}H-NMR (400 MHz, Chloroform-d) δ 4.23 (t, 4H), 4.11 (t, 2H), 3.70 (t, 2H), 3.67 (12H), 3.59-3.53 (m, 6H), 3.39 (s, 3H), 3.23 – 3.08 (m, 4H), 2.78 (t, 2H), 2.51-2.44 (m, 2H), 2.35 (t, 4H), 1.96-1.89 (m, 4H), 1.73-1.63 (m, 8H), 1.53 – 1.43 (m, 4H).

^{13}C NMR (101 MHz, CDCl3) δ 173.49, 172.24, 71.93, 70.60, 69.17, 63.48, 62.43, 59.05, 56.34, 52.67, 40.22, 38.49, 34.60, 33.94, 28.74, 24.61.

ESI-MS: m/z calculated for $C_{32}H_{57}O_{10}S_4$ $(M+H)^+$ = 744.29, observed = 744.2893



Scheme S1: Synthetic scheme of lipoic acid based amphiphilic compound.

NMR Characterization:

All ^{1}H and ^{13}C NMR spectroscopic data were collected using a Bruker DPX-300 NMR spectrometer at a temperature of 25°C and used $CDCl_3$ as solvent, which was bought from Sigma-Aldrich. The chemical shifts were measured in parts per million (ppm) and referenced to tetramethylsilane (TMS) with a singlet at δH= 0.00. The calibration process involved utilizing the residual solvent signal of chloroform at a proton chemical shift of δ H = 7.26. The analysis was conducted using first-order and the following acronyms were utilized consistently in the text: s = singlet, br. s = broad singlet, d = doublet, t = triplet, q = quartet, quin = quintet, dd = doublet of doublets, dt = doublet of triplets, m = multiplet.

Electrospray Ionization Mass Spectrometry Characterization:

The samples were dissolved in appropriate solvents and then injected in the column for recording mass spectra at room temperature.

Determination of Aqueous self-assembly of the amphiphile:

A certain amount of compound-3 (5mg) was dissolved in 500 µl of acetone. Subsequently, 5 mL of HPLC water was added very slowly, and the heterogeneous solution mixture was kept open for 5 hours to allow the acetone to evaporate. Finally, the resulting solution was used as mother stock solution (concentration = 0.67mM). Next, a series of screw-capped vials were filled with different concentrations taking from stock solution, ranging from 0.67mM to 0.03mM per 1ml of HPLC water. Prior to spectroscopic analysis, exact 20 µL Nile red dye solution (stock solution = 1mg/1 ml acetone, 3mM) was added dropwise to each vial and kept open for 1hr with continuous slow stirring to allow the acetone to evaporate, where concentration of Nile red dye in final resulted solution was 6×10^{-6} M. After that, each Nile red dye-encapsulated amphiphilic solution sets were used to measure the UV-vis spectra. Finally, the concentration of the Nile red dye encapsulated amphiphilic solution was plotted against the

absorbance intensity at 537 nm, and the inflection point was identified as the Critical Aggregation Concentration (CAC) of the lipoic acid based amphiphilic compound.

Isothermal Titration Calorimetric (ITC) Experiments:

In the ITC experiment, the sample cell was filled with an aqueous nanoaggregate solution (1.2mg/100µl HPLC water, 1.6mM) 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**

Gel Permeable Chromatography (GPC) Study:

Initially, a weighed amount (2mg) of the compound 3 dissolved in 1 mL of HPLC grade DMF was placed in several screw-capped vials. Each vial was then subjected to vortexing and sonication for 5 minutes. Subsequently, different percentage of DTT (1%, 2%, 6%, 10%, 20%, 40%, 50%) were added to the solution vials, while one vial was left without DTT as a control. To ensure a uniform and standardized polymeric solution of compound 3, all solutions were allowed to settle at room temperature for 6 hours. The polymeric solution was then filtered using a membrane filter with a pore size of 0.45 µm. Following this, each solution was individually injected into the GPC column to determine the polymer's molecular weight. The flow rate was maintained at 0.8 ml/min, and the column temperature was kept at 25°C.

Fabrication of Nano-network (NN):

In order to generate a nanoaggregate solution, a weighed quantity of compound 3 (0.3mg, 0.4mM) was dissolved in 150 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. According to the GPC results, in order to get better fabricated Nano-network solution either 1% or 2% DTT was added to the previous nanoaggregate solution in comparison to other percentages of DTT (such as 6%, 10%, 20%, 40%). After 6 hours of slowly stirring, the resulting nanonetwork solution was filtered using a 0.45 mm syringe filter.

Transmission Electron Microscope (TEM) Study:

For TEM experiments, 0.4mM of nanoaggregate solution, the most efficient nano-network solutions (0.4mM, using 1% DTT), and nanoaggregate (0.4mM) solution treated with 50% DTT were applied onto a 300-mesh carbon coated copper grid by drop-casting. 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, nanoaggregate and various fabricated nanonetwork solutions (using 1%, 2%,6%, 10%, 20%, 40%, 50% DTT) 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.

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 the effective nano-network (2% DTT) solution.

Calculation % crosslinking density:

A series of each nanoaggregate (0.4mM /ml HPLC water) solutions were subjected to treated different % of DTT to get various % crosslinked nanonetwork solution. Then this final

nanonetwork solutions were used for absorbance measurement to check the % of crosslinking density by UV/vis instrument. The % crosslinking can be quantified from the absorbance spectrum using absorption intensity of each nanonetwork solution at 282 nm the following equation: $[\{(IN_1-IN_a)-(IN_x-IN_a)\}/IN_1]\times100$ where IN_x= absorbance intensity of nanonetwork (where x=1, 2, 6, 10, 20, 40 i.e, used for nanonetwork fabrication using 1%, 2%, 6%, 10%, 20%, 40% DTT respectively). The NN1, NN2, NN3, NN4, NN5 and NN6 used for nanonetwork fabricated using 1%, 2%, 6%, 10%, 20%, 40% DTT respectively.

IN_a= Absorption intensity of nanoaggregate = 0.541 at 282 nm.

IN1 = Absorption intensity of NN1 (1% DTT) = 1.095 at 282 nm.

Nanonetwork	Absorbance intensity (INx) value from figure 3e	Output result from equation	Amount of % Decrosslinking	Amount of % Crosslinking
NN1	1.095	0	0	100
NN2	1.017	14.08	14	86
NN3	0.950	26.17	26	74
NN4	0.818	50	50	50
NN5	0.718	68.05	68	32
NN6	0.612	87.18	87	13

Nile red dye Encapsulation and release Studies:

Fifteen microliter of pre-prepared Nile red dye (stock solution = 1mg/mL acetone, 3 mM) was added very slowly to series of pre-prepared nanoaggregate solution, then doing nano-network by using 2 % DTT with slowly stirred for 5 hours to get homogeneous solutions at room temperature. Before any experiments were conducted, the Nile red encapsulated nanoaggregate and nano-network solutions were filtered through a (0.45 μm pore size) hydrophilic membrane to remove unencapsulated Nile red.

Next, this dye-encapsulated solutions were used for absorbance measurement to check the extent of dye encapsulation. Redox responsive guest release was examined as Nile red encapsulation was confirmed. Redox responsive guest release was examined as by adding 10 mM GSH to Nile red encapsulated nano-network solution. Then time dependent UV−vis spectra were recorded to examine the Nile red release. The release % can be quantified from the absorbance spectrum using absorption intensity of Nile red at 548 nm 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'.

Fluorescence Microscopy Study:

The Optical polarization microscopy (OPM) was also performed by using each Nile red dye encapsulated nanoaggregate and nanonetwork solution, briefly 50 µl of each Nile red encapsulated solutions were drop-casted on series of cleaned glass slide, and a cover glass was placed on it. Images were captured at a magnification of 20 times using an OLIMPUS BX-51 fluorescent microscope. Although the resolution of optical microscopy does not allow us to determine the precise location of the dye molecules, the red emitting spherical particles under the fluorescence optical polarization microscope (OPM) confirmed its presence within the assembly.

Calculation of Dye Encapsulation Efficiency and Encapsulation Capacity:

The dye encapsulation efficiency (EE) and dye encapsulation capacity (EC) of different crosslinked nanonetwork solutions were calculated by absorption spectroscopy using the 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:

A series of each nanoaggregate (0.3mg, 0.4mM /ml HPLC water) solutions were subjected to treated different % of DTT to get various % crosslinked nanonetwork solution. Then each polymeric nanonetwork solutions were added to pre-prepared basic solution of Ellman's reagent (1mg/ml chloroform) and to get better homogeneous solution, all polymeric reaction mixture solutions were allowed to settle at room temperature for 30 minutes. The UV/Vis spectra of the solution were examined both before and after Ellman's treatment in order to confirm the presence of free thiol functionality through monitoring both the absorption band at 330 nm and 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 solution were thoroughly mixed, taken from their respective stock solutions and added dropwise to series of pre-prepared nanoaggragate solutions (0.5mM) drop by prop with continuous very slowly stirring for 1h. After that, different % of DTT (1%, 2%, 6%, 10%, 20%, 40%) were added to the dye encapsulated resulting nanoaggregate solution. All solutions were allowed to settle at room temperature for 6 hours and the each final resulting nanonetwork solution was then filtered using a hydrophilic membrane (pore size: 0.45 μm) before FRET studies. These final varied crosslinked nanonetwork solutions w 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 Figures:

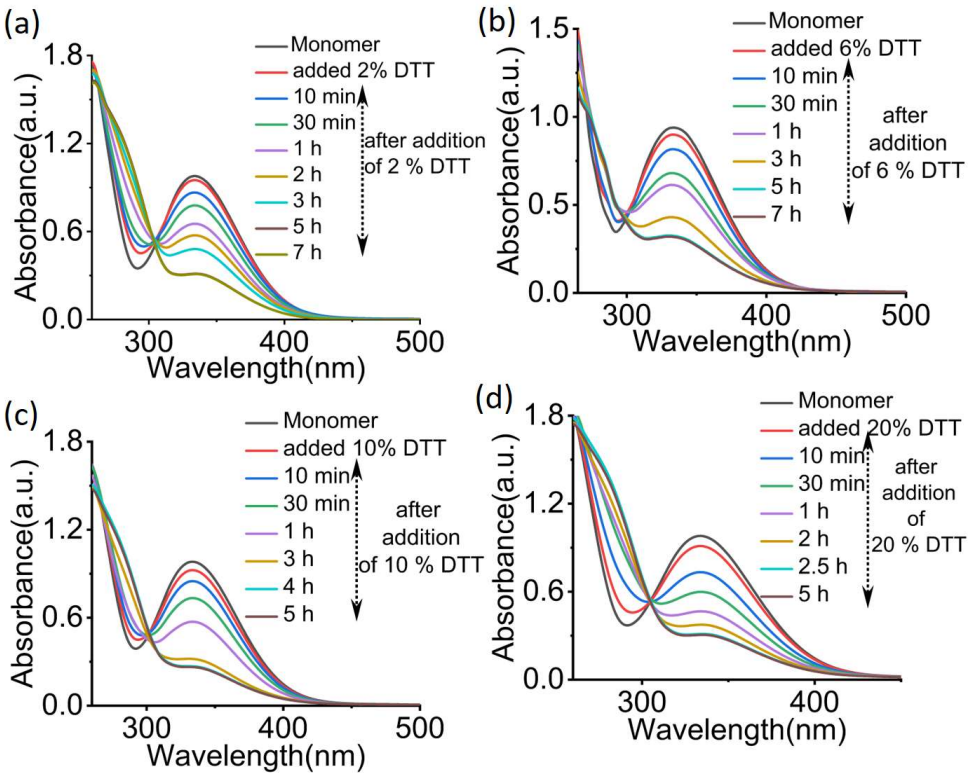


Figure S1: Monitoring polymerization by time dependent UV/Vis spectroscopy by using (a) 2% DTT (b) 6% DTT (c) 10% DTT (d) 20 % DTT as the monomer has a characteristic absorption band at 325 nm.

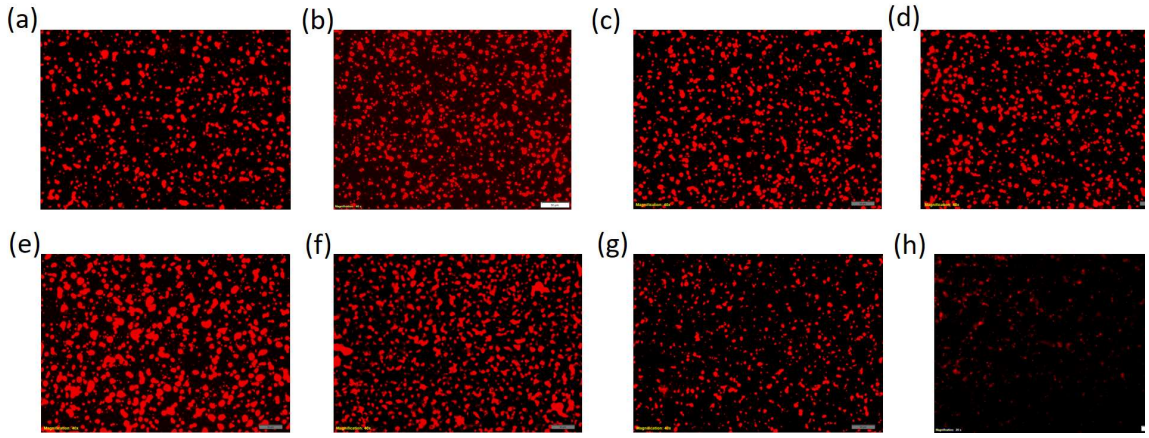


Figure S2: Optical polarizing microscopy (OPM) image of Nile red encapsulated (a) nanoaggregates and various nano-network solution by using (b) 1% DTT (c) 2% DTT (d) 6% DTT (e) 10 % DTT (f) 20% DTT (g) 40% DTT (h) 50% DTT. Scale bar = 50 μm and magnification= 40x in the OPM image.

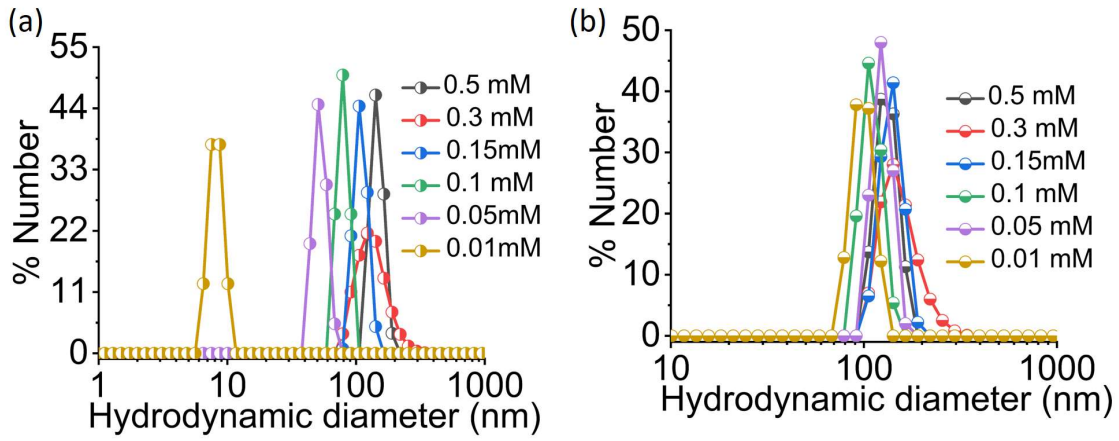


Figure S3: DLS profile of (a) nanoaggregate and (b) nano-network (by using 2% DTT) solution upon dilution with water.

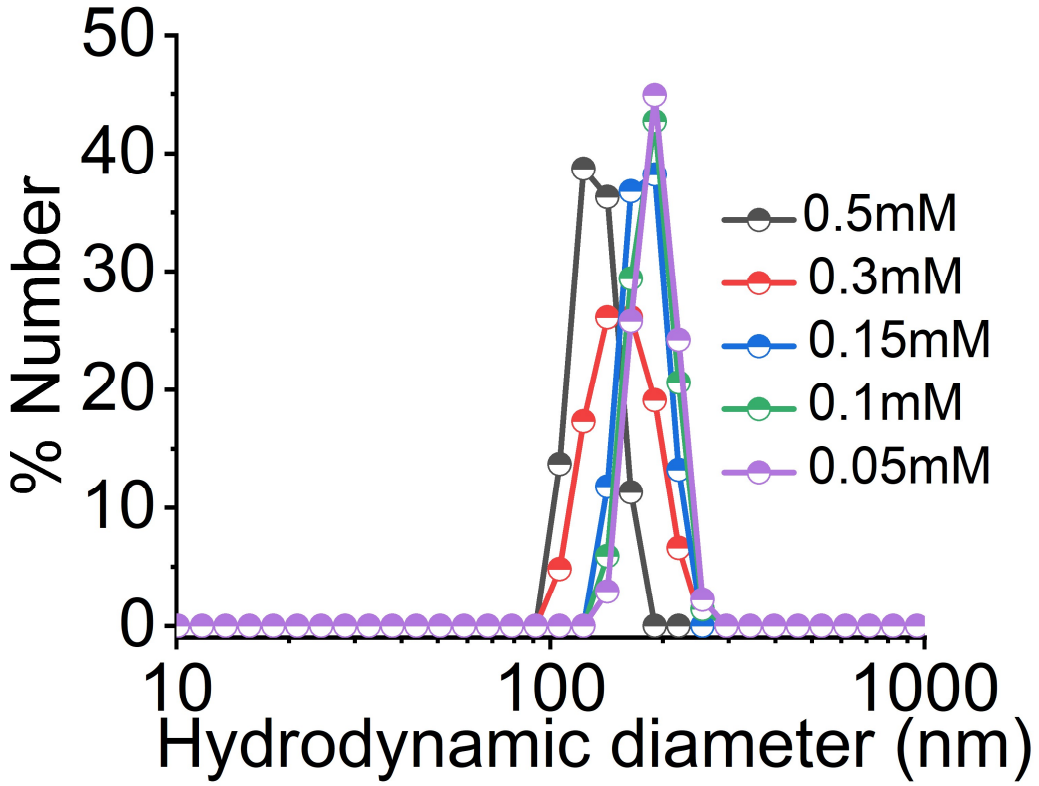


Figure S4: Dilution of nano-network (by using 2% DTT) solution with an organic solvent DMF and monitoring of size by DLS measurements.

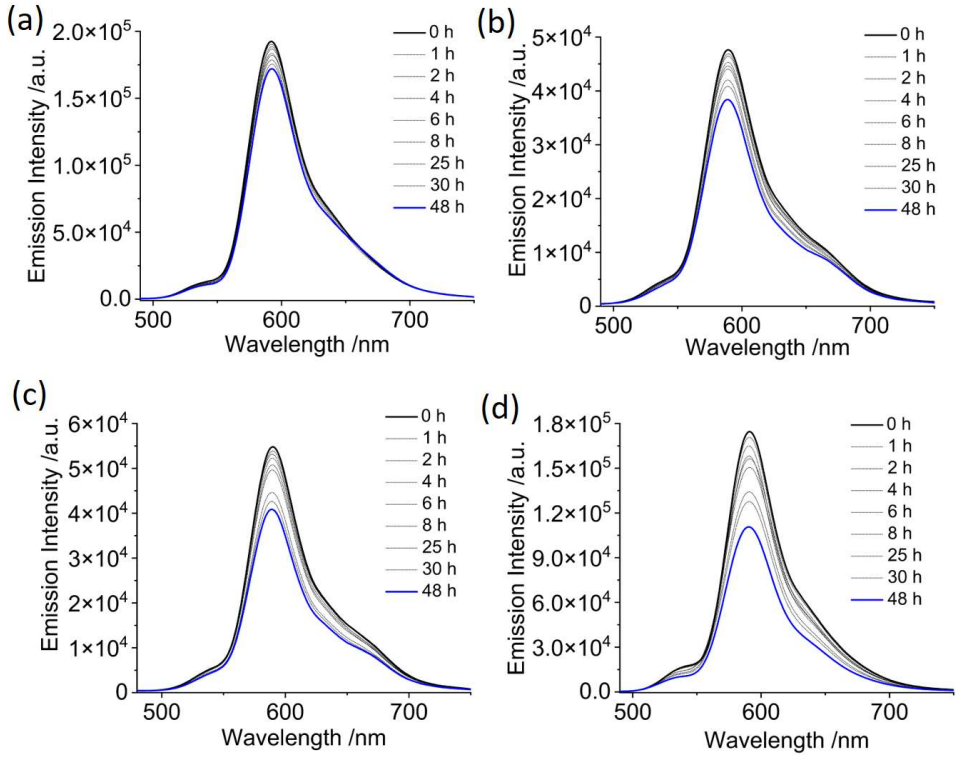


Figure S5: Monitoring of FRET over 48 h by emission spectroscopy of DiI and DiO loaded various nanonetwork solution by using (a) 2% DTT, (b) 6% DTT, (c) 10% DTT, (d) 20 % DTT.

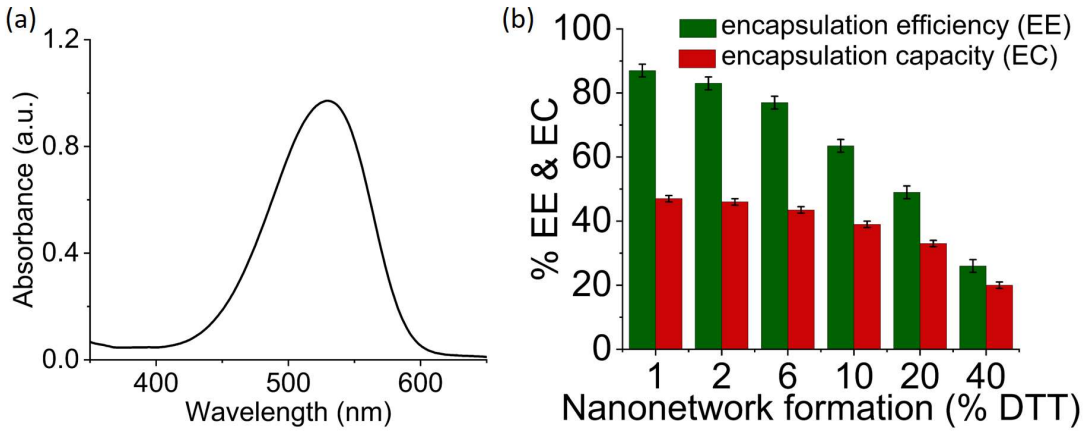


Figure S6: Plots for calculation of encapsulation efficiency (EE) and encapsulation capacity (EC). (a) Absorption spectrum of the known concentration of nile red solution in organic solvent to determine extinction coefficient, (b) comparison diagram for % EE & EC of various crosslinked nanonetwork.

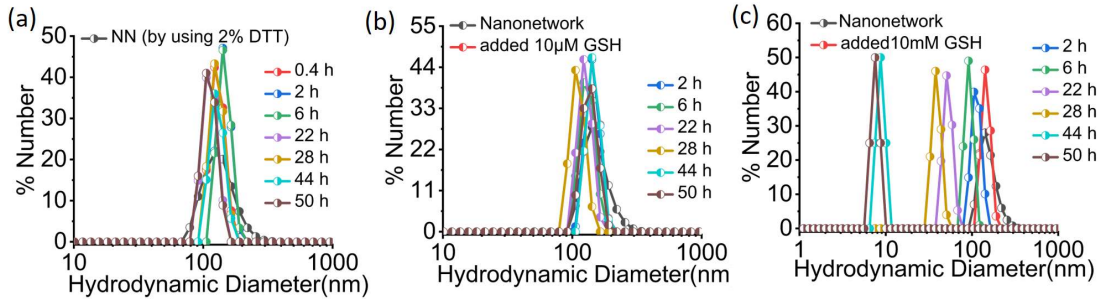


Figure S7: Monitoring the size of redox responsive degradation of the poly(disulfide)s nanonetwork (by using 2% DTT) by DLS measurements (a) in absence of GSH (b) in presence of 10μM GSH (c) in presence of 10mM GSH

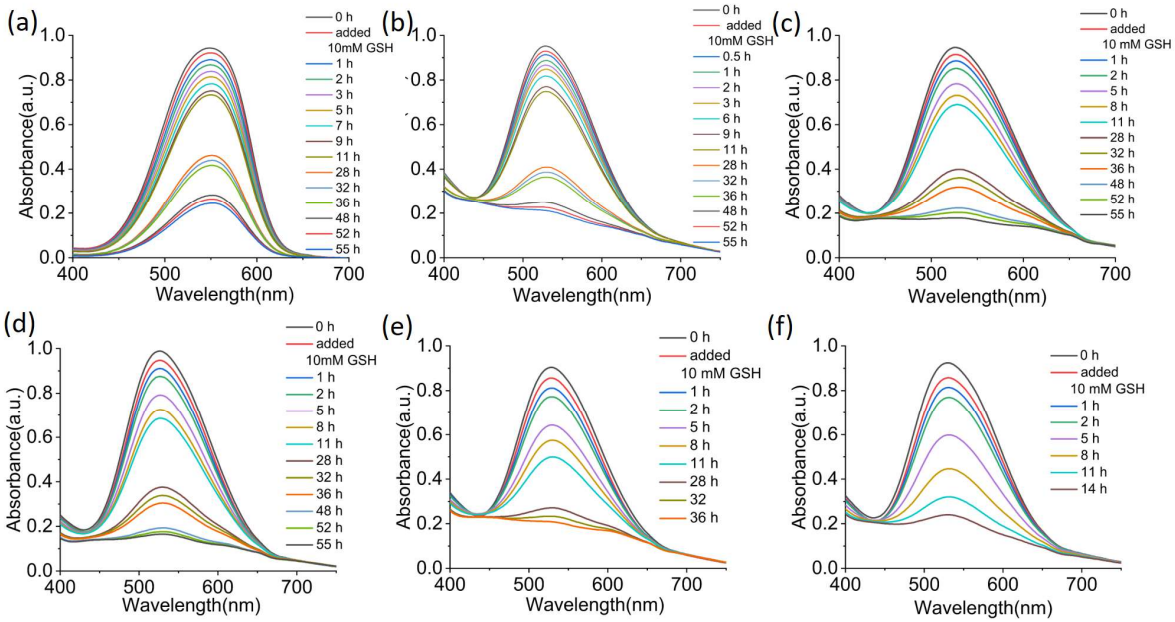


Figure S8: In presence of 10 mM GSH stimuli the guest release profile of Nile red encapsulated various nano-network solution by using (a) 1% DTT (b) 2% DTT (c) 6% DTT (d) 10 % DTT (e) 20% DTT (f) 40% DTT.

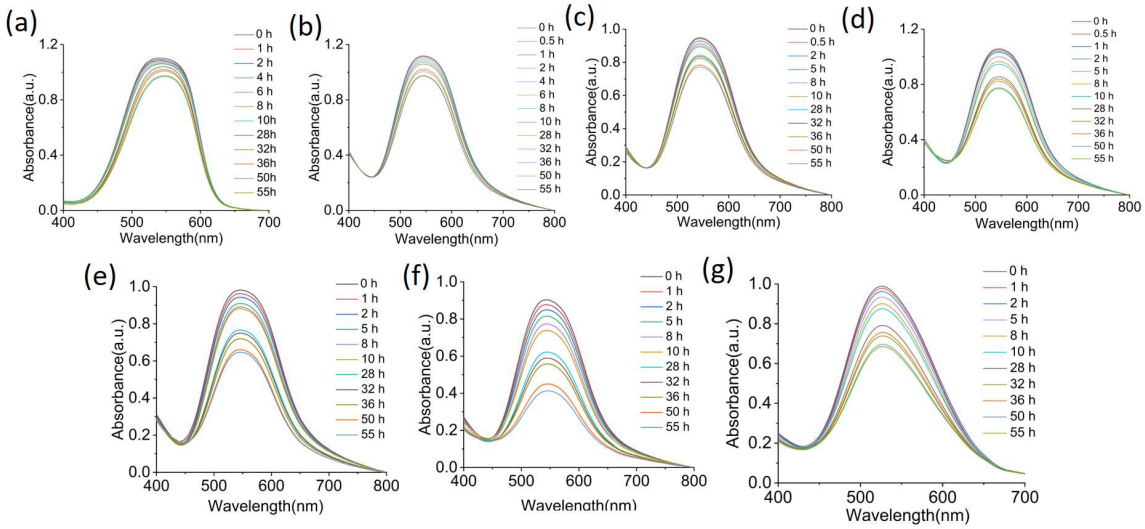


Figure S9: In absence of any stimuli the guest release profile of Nile red encapsulated various nano-network solution by using (a) 1% DTT (b) 2% DTT (c) 6% DTT (d) 10 % DTT (e) 20% DTT (f) 40% DTT.

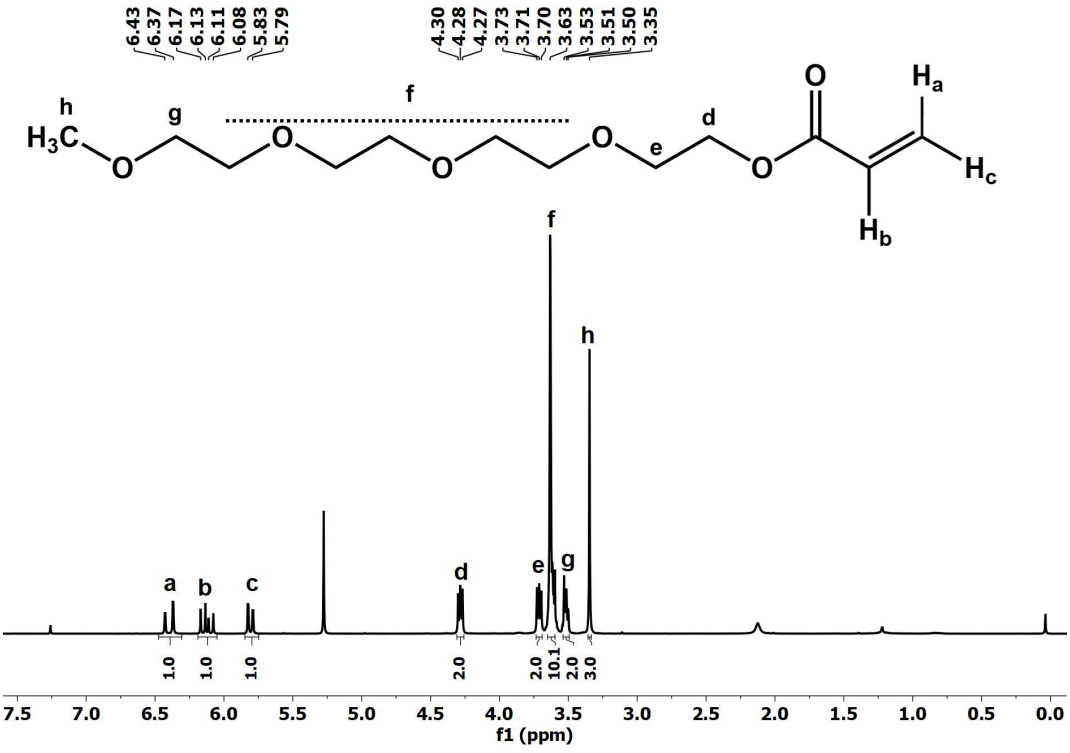


Figure S10: ^{1}H NMR spectrum of compound-1, Solvent= $CDCl_3$

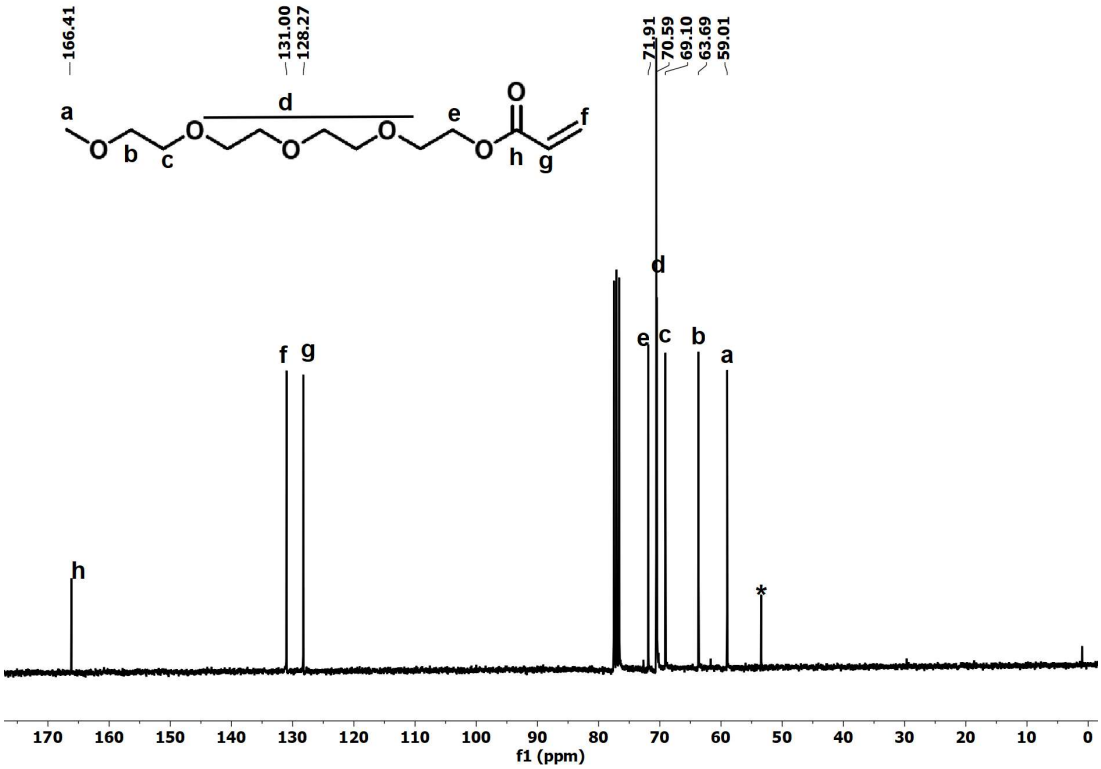


Figure S11: ^{13}C NMR spectrum of compound-1, Solvent= $CDCl_3$

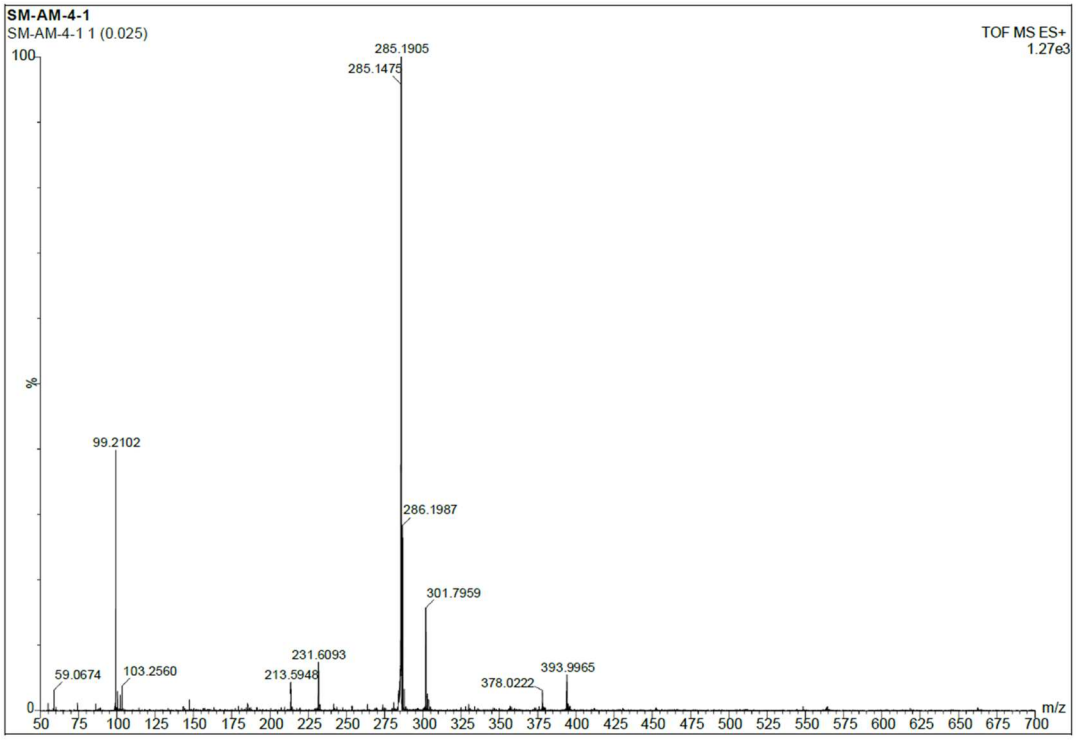


Figure S12: Mass spectrum of Compound **1** [calculated $(C_{12}H_{22}O_6+Na)^+$= 285.14, observed = 285.1905]

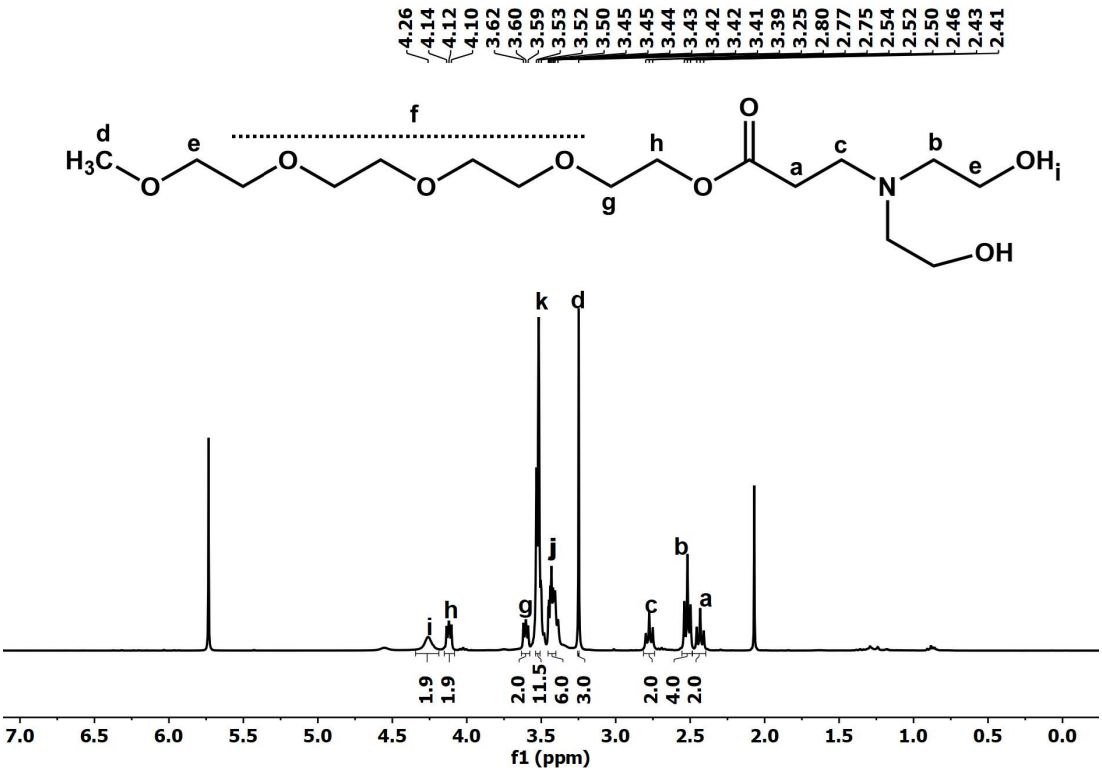


Figure S13: ^{1}H NMR spectrum of compound-2, Solvent= $CDCl_3$

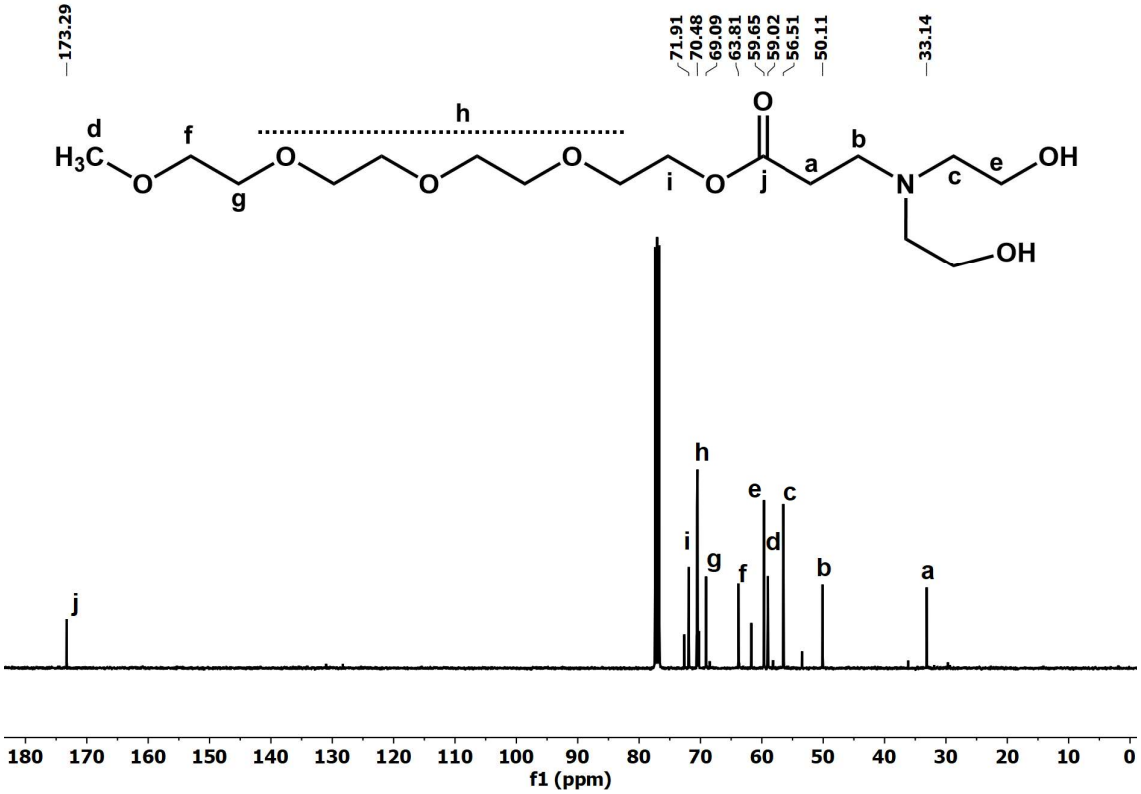


Figure S14: ^{13}C NMR spectrum of compound-2, Solvent= $CDCl_3$

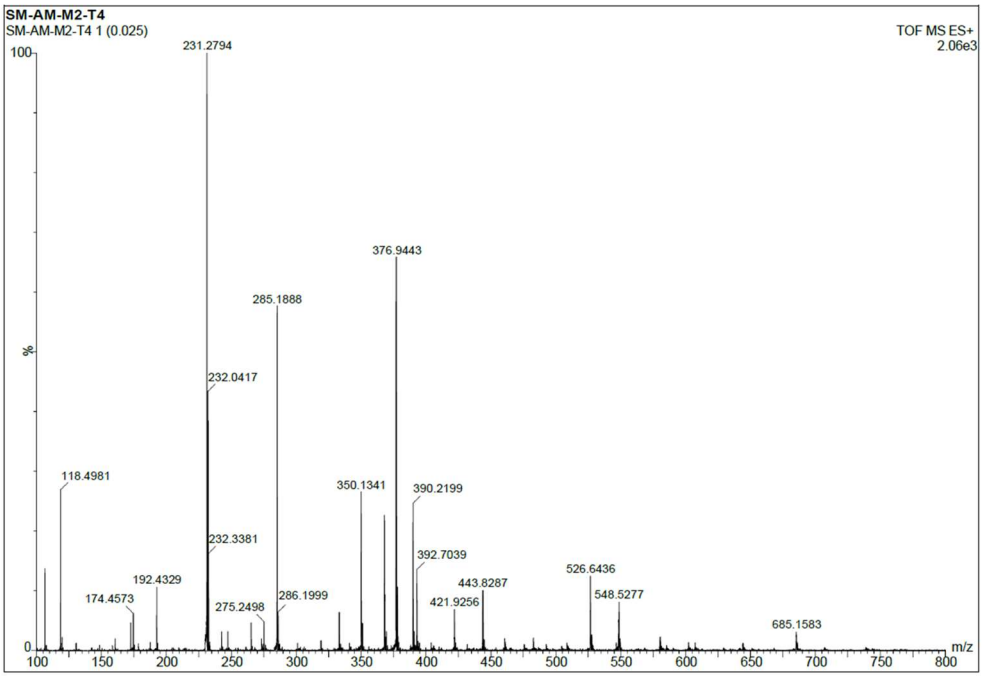


Figure S15: Mass spectrum of Compound **2** [calculated $(C_{16}H_{33}NO_8+Na)^+$= 390.22, observed = 390.2199]

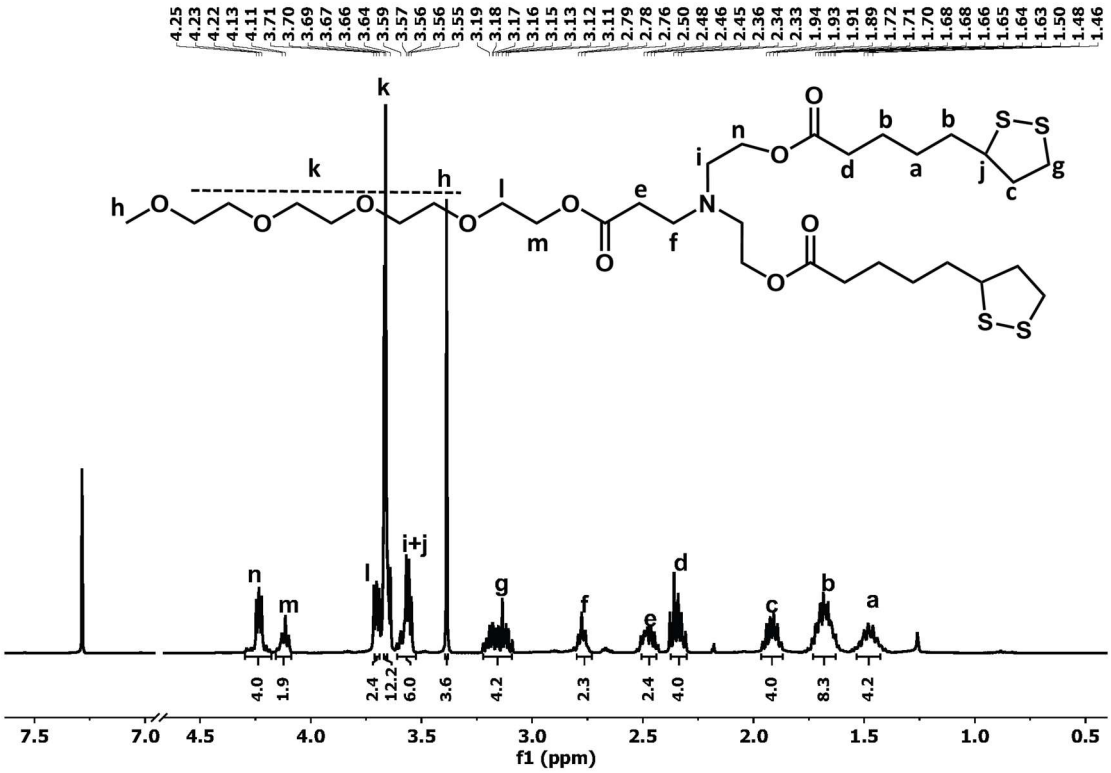


Figure S16: ^{1}H NMR spectrum of compound-3 (GS), Solvent= $CDCl_3$.

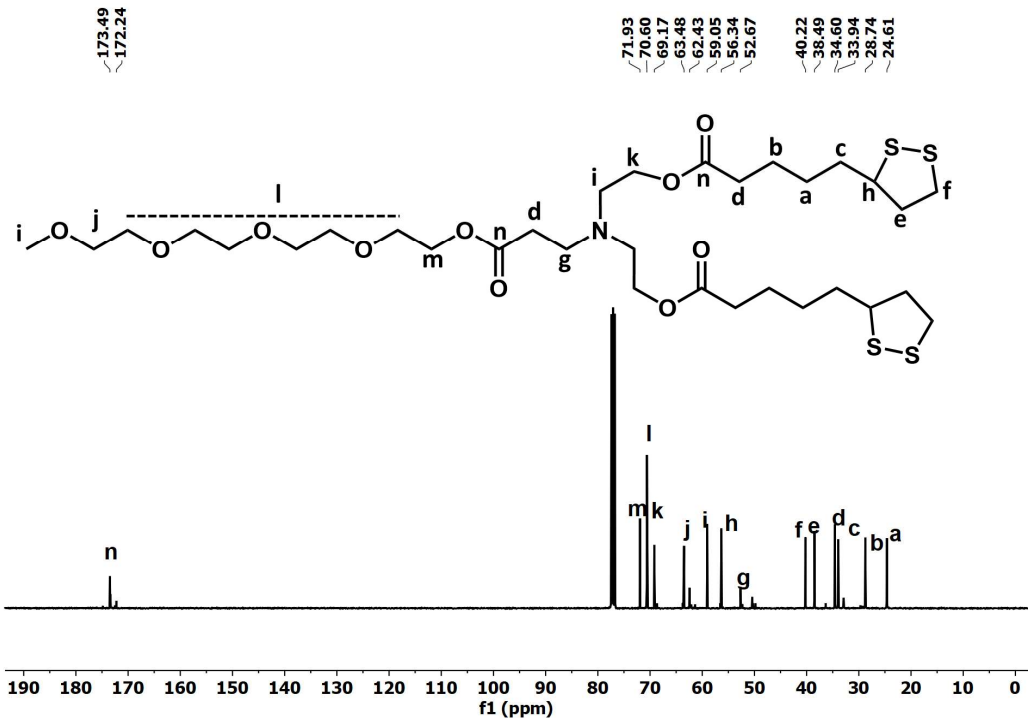


Figure S17: ^{13}C NMR spectrum of compound 3, Solvent= $CDCl_3$

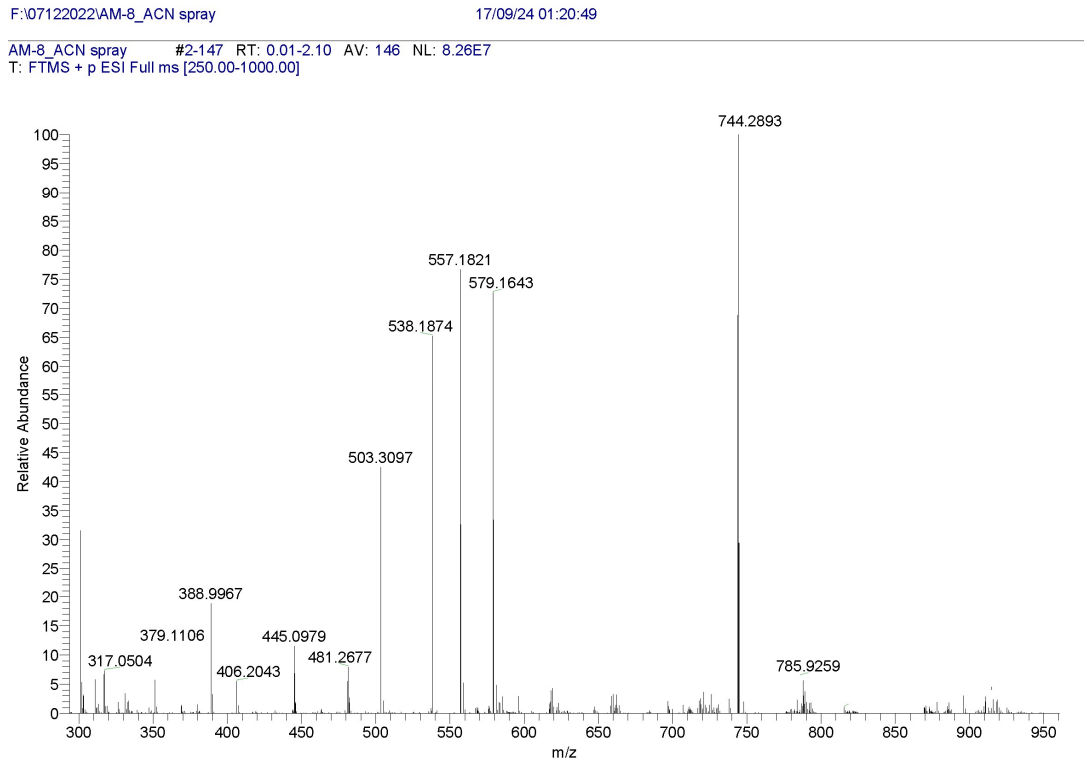


Figure S18: Mass spectrum of compound-3 [calculated $(C_{32}H_{57}NO_{10}S_4+H)^+$= 744.2865, observed = 744.2893]

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.