# Supporting Information

# For

# Glutathione-Responsive Degradable Amphiphilic Polyester-Based Nanocarrier for Targeted Drug Delivery

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**Materials and Methods:** All reagents were obtained from commercial suppliers and used without further purification unless otherwise mentioned. Enzyme lipase B from Pseudomonas cepacia was purchased from Sigma Aldrich. 4-Bromo-1,8-naphthalic anhydride, Pentafluorophenol, Adipoyl chloride, 2-amino-1,3-propanediol, Diethanolamine, Bis(2-hydroxyethyl) disulfide, Glutathione (reduced form), DL-Dithiothreitol (DTT) and biotin were purchased from TCI Chemicals. 4-Dimethylaminopyridine (DMAP) was purchased from Avra Synthesis Pvt. Ltd. and Piperidine were procured from Spectrochem Chemicals. Doxorubicin.HCl was purchased from BLD Pharmatech Pvt. Ltd. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from HiMedia Laboratories. All the solvents were dried properly following standard procedures before setting up the reactions. $^{1}$H NMR and $^{19}$F NMR spectra were recorded on a Bruker Ascend 500 MHz or 400 MHz spectrometer using deuterated solvents from Eurisotop. Chemical shifts ($\delta$) are reported in a ppm unit with TMS as the internal standard. The coupling constants ($J$) are reported in hertz (Hz). Column chromatography was carried out on silica gel (100-200 mesh). Number average molecular weight ($M_n$) and dispersity ($Đ$) of the polymers were measured by size exclusion chromatography (SEC) mixed *N, N*-Dimethylformamide (DMF) as an eluent at 60 °C or THF as an eluent at 30 °C with a flow rate of 0.5 mL/min, where the GPC instrument contained a Waters 515 HPLC pump, a Waters 2414 refractive index (RI) detector, one PolarGel-M guard column (5037.5 mm) and two Polar Gel-M analytical columns (30037.5 mm). FT-IR spectra were recorded in a PerkinElmer Spectrum 100 FT-IR Spectrometer. UV-vis spectra were recorded in a JASCO V750 spectrophotometer. Fluorescence spectra were recorded in a FluoroMax-3 spectrophotometer, from Horiba Jobin Yvon. Spectroscopic grade solvents were used for UV-Vis and photoluminescence studies. Transmission Electron Microscopy (TEM) studies were captured in a JEOL-2010EX machine operating at an accelerating voltage of 200 kV. Dynamic light scattering (DLS) and Zeta potential measurements were carried out in the Malvern instrument. HRMS were done on XEVO G2-XS Q Tof and Micromass Q-Tof Micro machine. The absorbance of the MTT assay at 570 nm was monitored by the microplate reader (VARIOSKAN, Thermo Fisher). Confocal laser scanning microscopy (CLSM) images were collected in the Leica STELLARIS 8 STED.

**Synthesis and Characterization**

Synthesis of **A1**, **B2**, **B3, P3, P4** and **P5** were reported by us previously.[1–4]

**Synthesis of the polymer P1: A1** (243.1 mg, 0.508 mmol) was dissolved in 100 µL of dry DMF in a polymer vessel. To this solution, DMAP (12.4 mg, 0.101 mmol) and **B1** (80.3 mg, 0.52 mmol) were added with stirring. The whole mixture was then degassed by purging dried argon gas for 20 minutes before stirring it at 100 °C in an oil bath for 24 hours. The crude polymer obtained was dissolved in chloroform and purified by precipitation from cold ether and collected after drying under reduced pressure to yield 122 mg of the pure polymer as a brown sticky mass. $^{1}$H NMR (500 MHz, DMSO-$d_6$): $\delta$ 4.33 (t, 3H), 2.95 (t, 4H), 2.36 (s, 4H), 1.67 (s, 4H). Experimental $M_n$ and $M_w$ from SEC analysis = ~7900 g/mol and 9600 g/mol, respectively ($Đ$ = 1.37) with respect to the polystyrene (PS) standard and THF as eluent, which closely matches with the theoretical molecular weight (10,560 g/mol) calculated from Carothers' equation.

**Synthesis of the polymer P2: A1** (353.6 mg, 0.74 mmol) was dissolved in 100 μL of dry DMF in a polymer vessel. To this solution, DMAP (18.5 mg, 0.14 mmol) and **B1** (11.4 mg, 0.0739 mmol) were added with stirring followed by addition of **B2** (183.3 mg, 0.554 mmol) and **B3** (39.3 mg, 0.111 mmol). The whole mixture was then degassed by purging dried argon gas for 20 minutes before stirring it at 100 °C in an oil bath for 48 hours. The crude polymer obtained was dissolved in methanol and purified by precipitation from 5% cold methanol in ether and collected after drying under reduced pressure to yield 92 mg of the pure polymer as a yellow sticky mass. $^1$H NMR (500 MHz, DMSO-$d_6$): $\delta$ 8.46 – 8.19 (m, 3H), 7.76-7.84 (m, 1H), 6.98 (b, 1H), 6.47 – 6.31 (m, 2H), 4.66 – 4.51 (b, 5H), 4.36-4.27 (m, 5H), 4.14-4.08 (m, 16H), 3.50 (bs, 6H), 3.10 (bs, 2H), 2.95 (bs, 2H), 2.83 (m, 2H), 2.29 (b, 27H), 1.83 (bs, 4H), 1.66 (m, 4H), 1.37 (m, 30H). Experimental $M_n$ from SEC analysis = 18100 g/mol, $M_w$ = 23500 g/mol, $Đ$ = 1.30 with respect to polystyrene (PS) standards and DMF as eluent.

**Experimental Section:**

**Sample preparation for spectroscopic studies:** Stock solutions of **P1** and **P2** were prepared by dissolving measured amounts of each in a fixed volume of HPLC-grade methanol within a clean, dry glass vial. From there, measured quantities were taken out into a vial, and methanol was evaporated by heating the vial to obtain a thin film, which was then dispersed in a measured amount of HPLC-grade water, phosphate buffer solution of pH 7.4 or complete DMEM solution to attain the required concentrations. The dispersion was subjected to heating followed by cooling at room temperature to ensure proper dissolution of the film. These solutions were then used for spectroscopy (UV-vis, PL), DLS and biological studies.

**FT-IR Study:** For analyzing the spectral signature of the crude and purified **P1** and **P2** polymers, spectral measurements were carried out with scan range = 4000−1000 cm$^{-1}$, resolution = 1.0 cm$^{-1}$, number of scans = 64, and T = 25 °C.

**Critical aggregation concentration (CAC) determination:** The critical aggregation concentration (CAC) of the **P2** polymer was determined from fluorescence spectroscopy using Nile Red or pyrene as hydrophobic probes. 10 μL of Nile Red or pyrene solution ($c$ = 10$^{-4}$ M) was added followed by addition of appropriate volume of polymer solution ($c$ = 4 mg/mL) in methanol to individual vials to create a series of solutions containing varying polymer concentrations (ranging from 0.002 to 0.25 mg/mL) while maintaining fixed dye concentrations. The solvents were then evaporated using heat to make thin films and 1 mL of water was then added to each vial. The solutions were further heated to dissolve the thin films which were then cooled to room temperature and allowed to stand for an additional 12 h. Fluorescence spectra corresponding to Nile Red were then recorded ($\lambda_{ex}$ = 565 nm) for individual vials at 655 nm. For pyrene ($\lambda_{ex}$ = 336 nm) the ratio of emission intensities at 383 nm and 372 nm were plotted againstvarying concentrations of **P2**. The inflection point in such a plot was considered as CAC.

**Degradation study with GSH and Lipase B:** For the degradation study, measured amount of **P2** from the stock solution ($c$ = 4 mg/mL in methanol) was taken in a glass vial and the solvent was evaporated by gentle heating to make a thin film. To this, measured amount of phosphate buffer

(pH 7.4) solution was added to make the final concentration of **P2** = 0.5 mg/mL. To this, GSH was then added (final concentration of GSH 10 mM) and the whole solution was then allowed to stir at room temperature for 24 hours. For degradation with Lipase B, 500 µL of enzymatic solution of Lipase B from *Pseudomonas cepacia* (6.6 mg/mL in phosphate buffer solution of pH = 7.4) was added to the polymer solution (0.5 mg/mL in water). Time-dependent DLS analysis was then performed to check the size of the aggregates in the presence of GSH and Lipase B, separately, followed by morphological characterization by Transmission Electron Microscopy (TEM). After that, the samples are lyophilized and dissolved in DMF for the SEC experiment.

**Time- and GSH-concentration dependent release kinetics of Nile Red:** For the Nile Red (NR) release study, measured volume of NR stock solution in $CHCl_3$ and the polymer stock solution in methanol were mixed in a clean glass vial followed by heating to make a thin film. To this thin film, measured volume of phosphate buffer solution was added to make the final concentration of NR = $10^{-6}$ M and **P2** = 0.1 mg/mL. After addition of the GSH (10 mM) or Lipase B, time-dependent emission ($\lambda_{ex}$ = 565 nm) corresponding to Nile Red was measured at 655 nm. For the GSH-concentration-dependent studies for Nile Red release of **P2** and **P5** polymer, NR-encapsulated polymer nanoaggregates of **P2** and **P5** were prepared following the same procedure as previously discussed, keeping the polymer concentration fixed at 0.1 mg / mL. A stock solution of GSH was prepared in water (concentration of GSH = 0.1 M). Furthermore, different volumes of GSH from the stock solution were added sequentially to both these polymer solutions, and the emission ($\lambda_{ex}$ = 565 nm) corresponding to Nile Red was recorded at $\lambda_{em}$ = 655 nm. For each GSH addition, samples were equilibrated for 15 minutes before recording the spectra. For calculating the Nile Red release %, the following equation was used:

$$\text{NR Release \%} = [(I_0 - I_t) / I_0] \times 100$$

where $I_0$ is the emission ($\lambda_{em}$ = 655 nm) corresponding to the NR emission immediately after adding GSH or Lipase B, and $I_t$ is the emission at any given time t.[5]

**DOX encapsulation and release method:** Commercially available DOX.HCl was neutralized using excess triethyl amine solution and the neutralized hydrophobic DOX was collected from the DCM after workup in DCM/water medium. A measured quantity (0.5 mg or 0.9 µM) of hydrophobic DOX in HPLC grade $CHCl_3$ was added to a solution of the polymer **P2** in $CHCl_3$ (*c* = 4 mg/mL). A thin film was then prepared by removing the organic solvent by gentle heating, which was followed by the dissolution of the film in water. Then that DOX-loaded polymer solution was subjected to dialysis in water for 48 hours in the dialysis bag of 3500 $M_w$ cutoff. The resulting solution was kept for further usage (final concentration of DOX 0.15 mg/mL in water). Before any experiment, this solution was again filtered through a 0.45 µm syringe filter (nylon) and diluted to prepared the desired concentration.[6] The release kinetcis for DOX was probed by fluorescence spectroscopy studies. A solution of DOX loaded **P2** was taken inside a dialysis bag of 3500 $M_w$ cutoff and was dialyzed against GSH solution (10.0 mM) in PBS buffer of pH 7.4. With increasing dialysis time, DOX fluorescence was detected from the solution outside the

dialysis bag ($\lambda_{ex}$ = 485 nm). % of released DOX was calculated from the emission spectra using the following equation:

% release = $\{(I_t - I_0)/I_0\}$ x 100, where $I_t$ and $I_0$ represent emission intensity (@555 nm) at t = 0 (just after GSH addition) and at a particular time t, respectively.[7]

**Cell culture experiment:** Human cervical cancer (HeLa) and non-cancerous NIH 3T3 cells were used for cellular uptake studies in this work. Both the cells were seeded in a high glucose Dulbecco's Modified Eagle Medium (DMEM), which was supplemented with 10% fetal bovine serum (FBS) and 1% L-Penicillin-Glutamine-Streptomycin (PSG), which forms the complete media. Approximately 1 x $10^4$ cells were seeded and maintained by passaging them at ~ 80% confluency at 37 °C in the presence of 5% $CO_2$ in an incubator provided with a humidified environment.[6]

**Cell viability study by MTT assay:** The cell viability of the HeLa cells was checked in the presence of **P2** polymer by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay. Approximately 1 x $10^4$ cells were seeded in a 96-well plate in complete media and left overnight for the cells to adhere. The next day, the spent media was replaced with fresh complete DMEM containing polymers at various concentrations (100, 200, 300, 400 and 500 μg/mL) and incubated for another 24 h or 48 h. For the cell viability test with free DOX, a stock solution ($c$ = 1 mg/mL) of free DOX was prepared in water then diluted with DMEM media to obtain the required concentrations. After that, the media containing polymers was removed and 100 μL of fresh media was added followed by 50 μL of 5 mg $mL^{-1}$ MTT salt per well and incubated for another 4 h at 37 °C. After 4 h, the media was carefully removed without disturbing the formazan crystals formed at the base of each well. Dimethyl sulfoxide (DMSO) (100 μL per well) was added to dissolve the purple formazan crystals and incubated for another 30 min before the absorbance of these treated cells was recorded at 570 nm using a plate reader (VARIOSKAN, ThermoFisher). MTT added to the untreated cells were considered as the positive control. The cell viability (% percentage of cell death) was calculated as: [O.D. of the polymer treated cells / O.D of the untreated cells] X 100, where O.D. stands for optical density].[8]

**Fluorescence-activated cell sorting (FACS) analysis:** Cells (HeLa and NIH 3T3 cell) were seeded in 35 x 10 mm cell culture plates in complete media and incubated overnight for the cells to adhere. The next day the spent media was removed, and fresh complete media was added containing 200 μg/mL or 400 μg/mL of polymer and cells were further incubated at 37 °C for 48 h. Subsequently after the completion of the incubation, the cell culture media was replaced with fresh complete media. After this, the media was removed, and cells were treated with trypsin EDTA, and cell pellets were collected after centrifugation. After the removal of supernatant, the cells were resuspended in complete media and transferred to FACS tube (12 x 75 mm polystyrene round bottom style). Data for 10000 events of live cells were collected and analyzed in BD FACSAriaTM III.[6] For the biotin inhibition experiment, cells were pretreated with biotin and incubated for 24 hours prior to the addition of polymer solution.

**Confocal laser scanning microscopy (CLSM) imaging:** Cells were seeded in confocal imaging dishes (dimension 35 X 10 mm purchased from Genetix, Biotech Asia Pvt. Ltd) and were incubated over night for the cells to get adhered to the dish. The next day the media was removed and replaced with fresh complete media containing **P2** ($c$ = 400 µg/mL) followed by incubation for the desired time. Further cells which were pre-incubated with the polymer solution $c$ = 400 µg/mL) at 37 ºC for 48 h, were again incubated with Hoechst 33342 for 10 min for staining the cell nuclei. After this, the cells were washed thrice with fresh media and were used for imaging. Similar procedure was followed for the cell imaging with DOX-loaded self-assembled **P2**, where the polymer concentration was maintained at 0.3 mg/mL and DOX concentration at 0.02 µg/mL. Finally, live cell microscopy imaging was performed in the presence of complete media. The red channel was excited at 543 nm, whereas for the green channel and blue channel, 488 nm and 405 nm, respectively, was used for excitation. Treated cells were then washed thrice with fresh complete media and used subsequently for imaging.

**Additional Figures:**

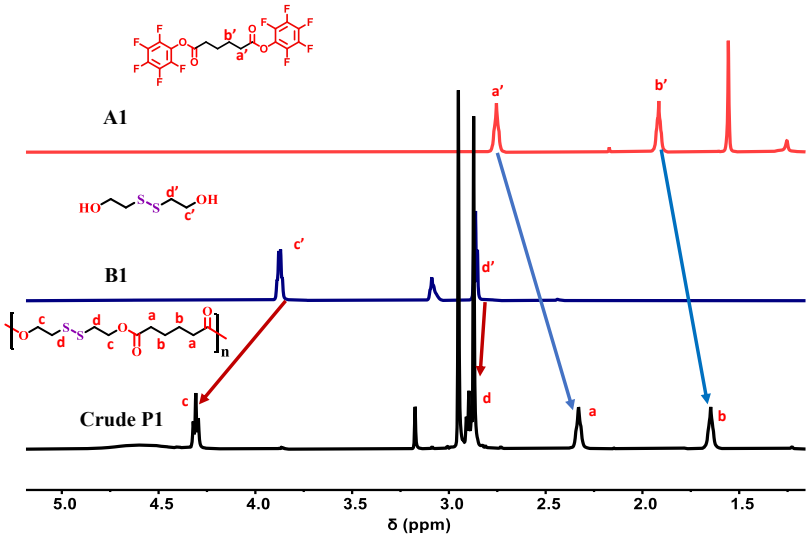



**Figure S1**: Stacked $^1$H NMR spectra of **A1**, **B1** and crude **P1** in $CDCl_3$ showing complete **A1** conversion.

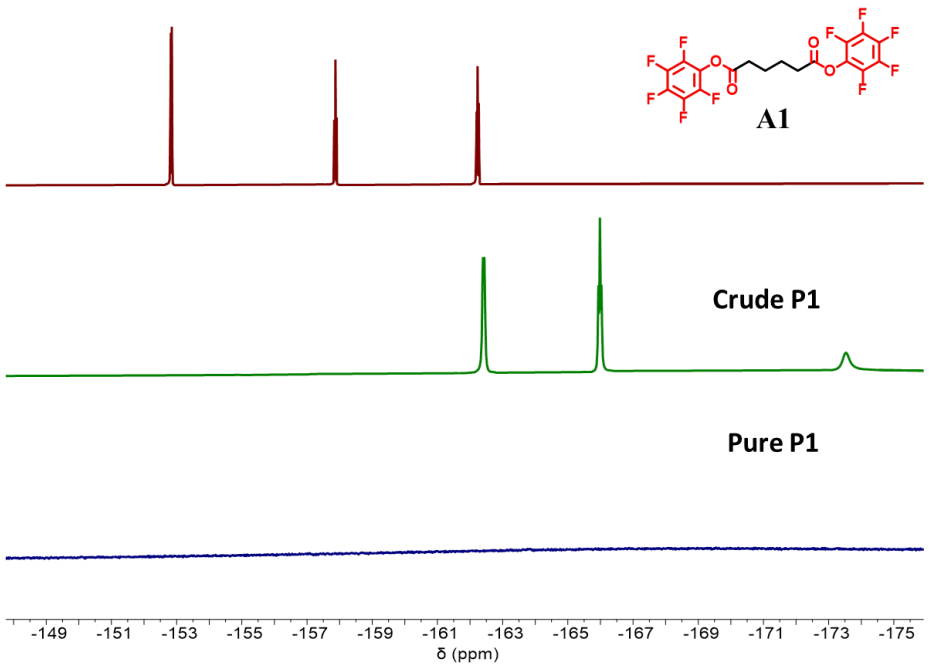



**Figure S2**: Stacked $^{19}F$ NMR spectra of **A1**, crude **P1** and purified **P1** in $CDCl_3$ showing complete **A1** consumption.

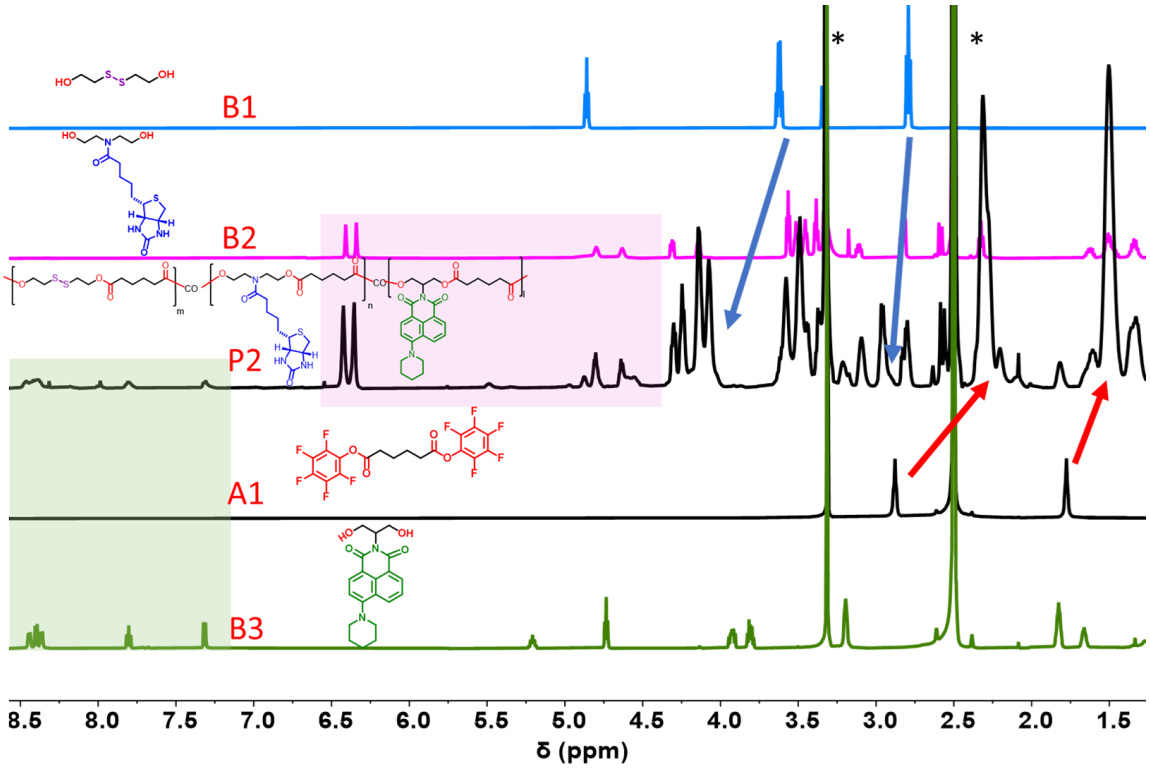



**Figure S3:** Stacked $^{1}H$ NMR spectra of purified **P2** with monomers **A1, B1, B2** and **B3** in DMSO-$d_6$.

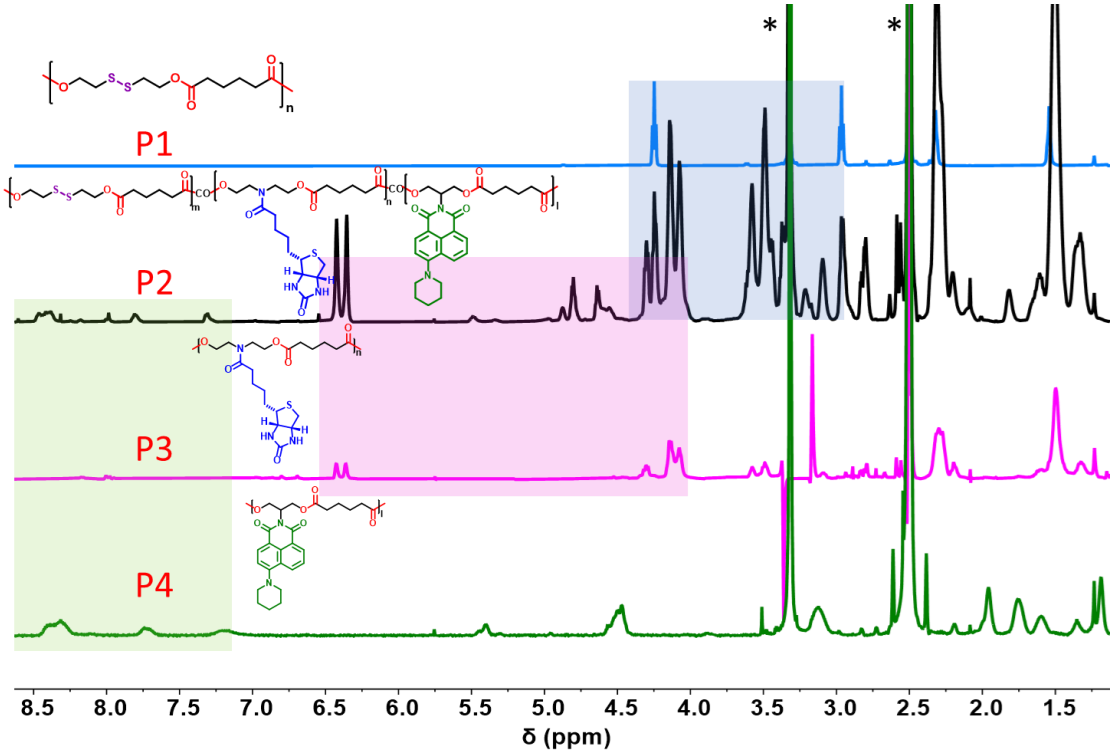



**Figure S4:** Stacked $^{1}$H NMR spectra of purified **P2** with homopolymers **P1**, **P3** and **P4** in DMSO-$d_6$.

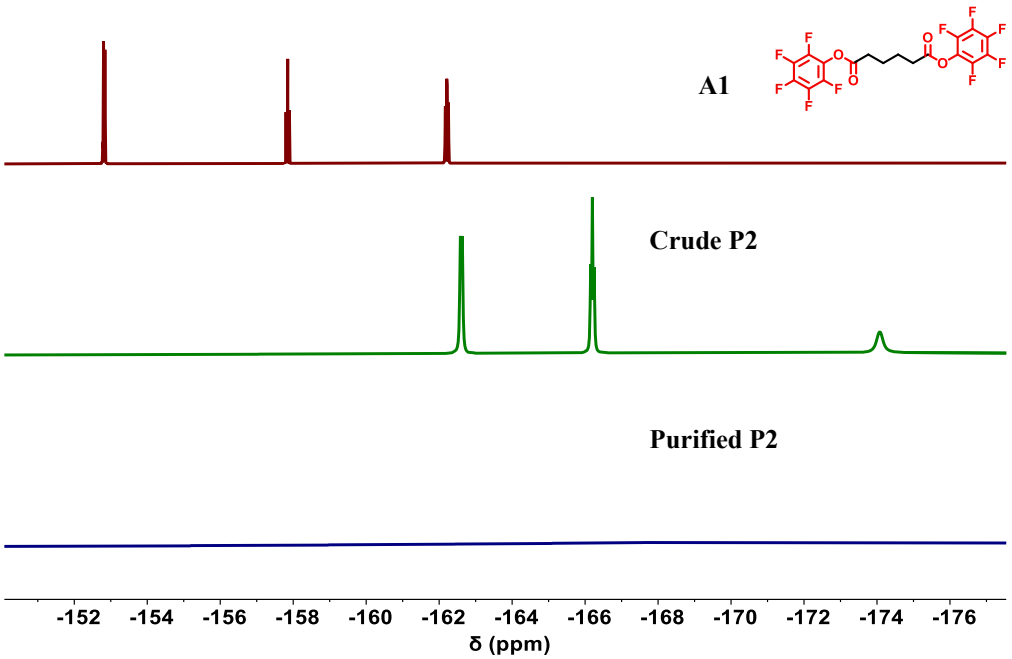



**Figure S5**: Stacked $^{19}$F NMR spectra of **A1**, crude and purified **P2** in DMSO-$d_6$ showing complete **A1** consumption.

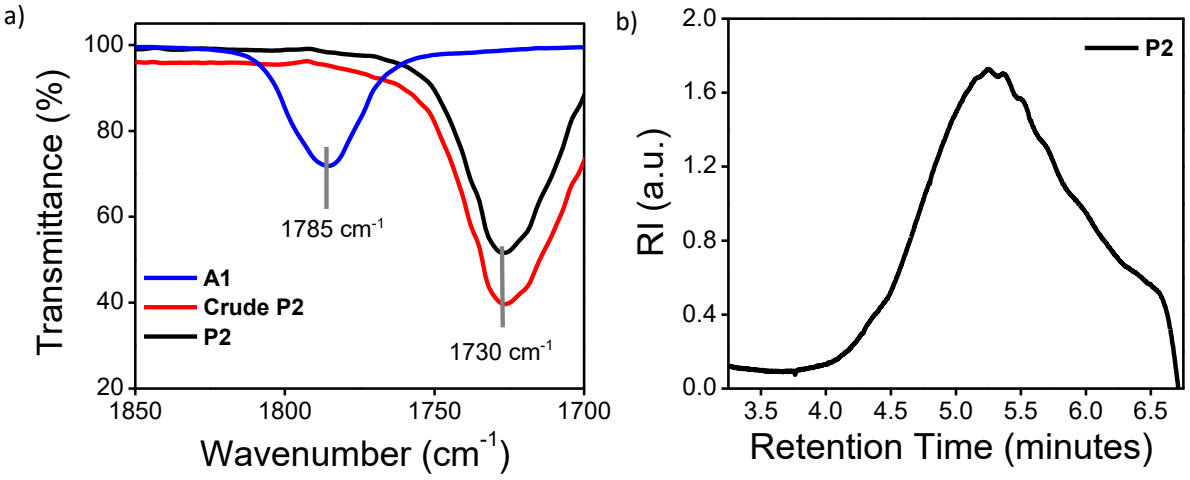



**Figure S6:** a) FTIR spectra of **A1**, crude and purified **P2** (showing selected carbonyl region); b) Size exclusion chromatogram (SEC) of **P2** using DMF as an eluent.

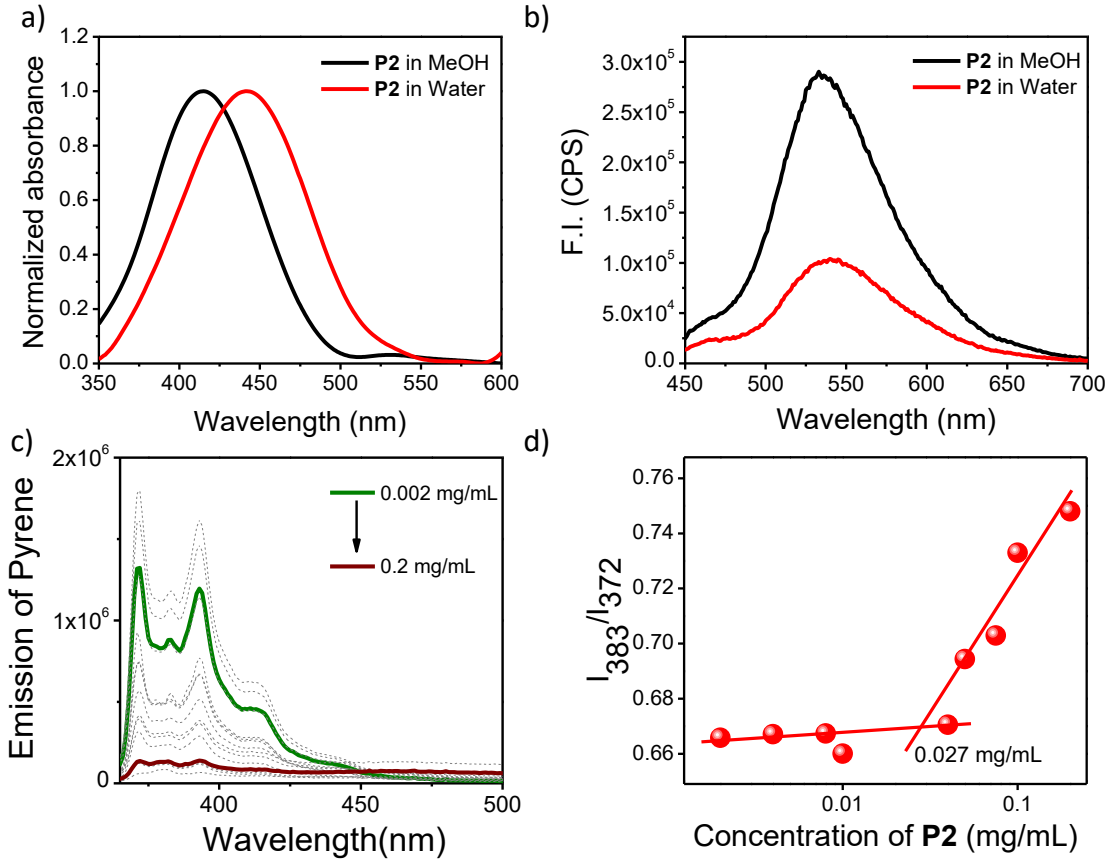



**Figure S7:** a) UV-vis and b) PL spectra of **P2** in MeOH and Water ($c$ = 0.1 mg/mL) ($\lambda_{ex}$ = 420 nm); c) Changes in the pyrene emission intensity with varying concentrations of **P2** ($\lambda_{ex}$ = 336 nm) and d) determination of the critical aggregation concentration (CAC) of **P2** from pyrene emission ($\lambda_{ex}$ = 336 nm) versus varying concentrations of **P2** derived from Figure S7c.

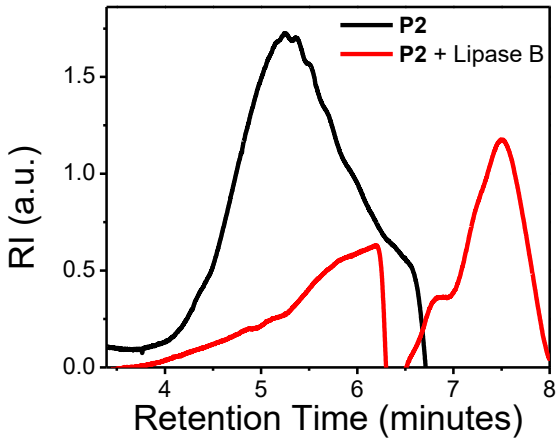
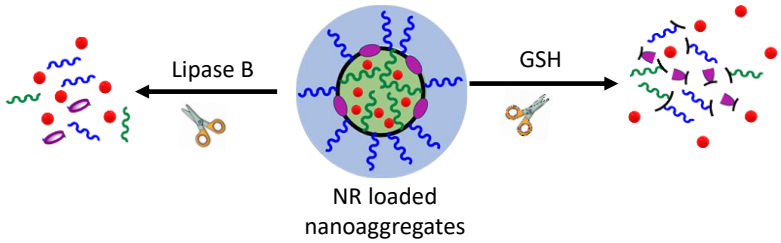



**Figure S8:** SEC profile of **P2** before and after treatment with Lipase B using DMF as an eluent.




**Scheme S1:** Schematic representation for release of encapsulated Nile Red from **P2** nanoaggregates in the presence of GSH and Lipase B due to the polymer backbone degradation.

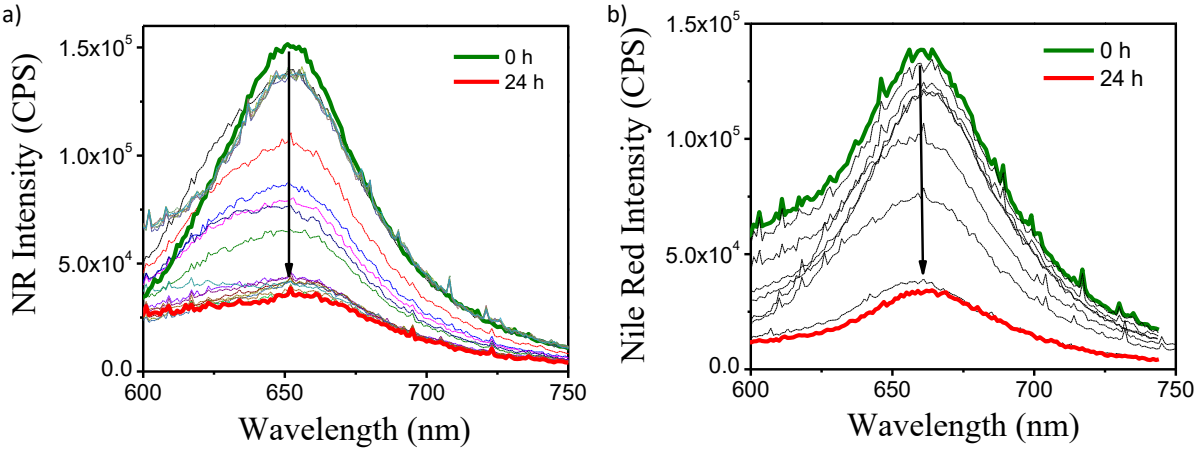



**Figure S9:** Photoluminescence spectra showing time-dependent release of Nile Red from the hydrophobic core of **P2** nanoaggregates ($c$ = 0.1 mg/mL) upon treatment with a) GSH and b) Lipase B from *Pseudomonas cepacia* ($\lambda_{ex}$ = 556 nm).

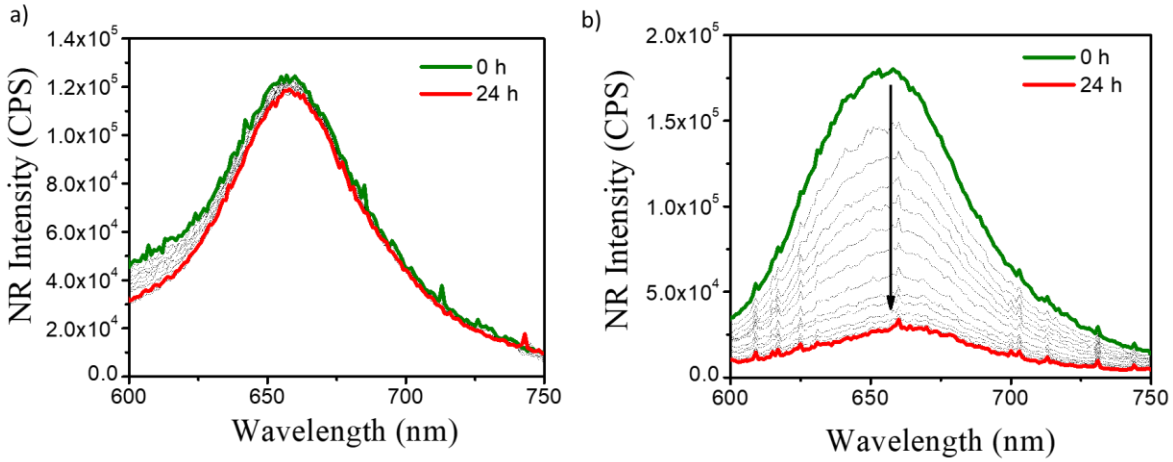
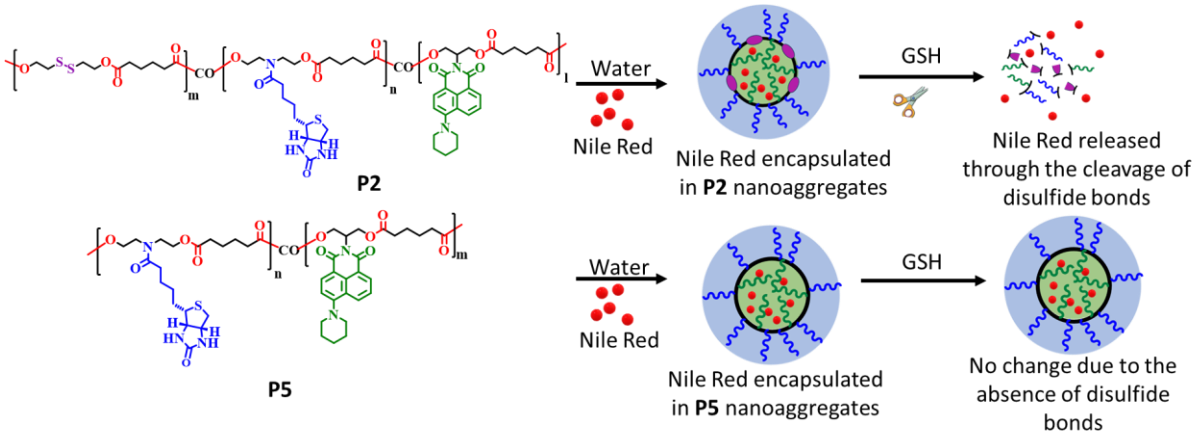



**Figure S10:** Photoluminescence spectra showing time-dependent release of Nile Red from the hydrophobic core of self-assembled **P2** nanoaggregates in a) the absence and b) the presence of both GSH and Lipase B from *Pseudomonas cepacia* ($\lambda_{ex}$ = 556 nm).




**Scheme S2:** Schematic representation of the release of encapsulated Nile Red from the hydrophobic core of self-assembled **P2** nanoaggregates in the presence of GSH, while no release was observed for **P5** nanoaggregates in the absence of the backbone disulfide linkages.

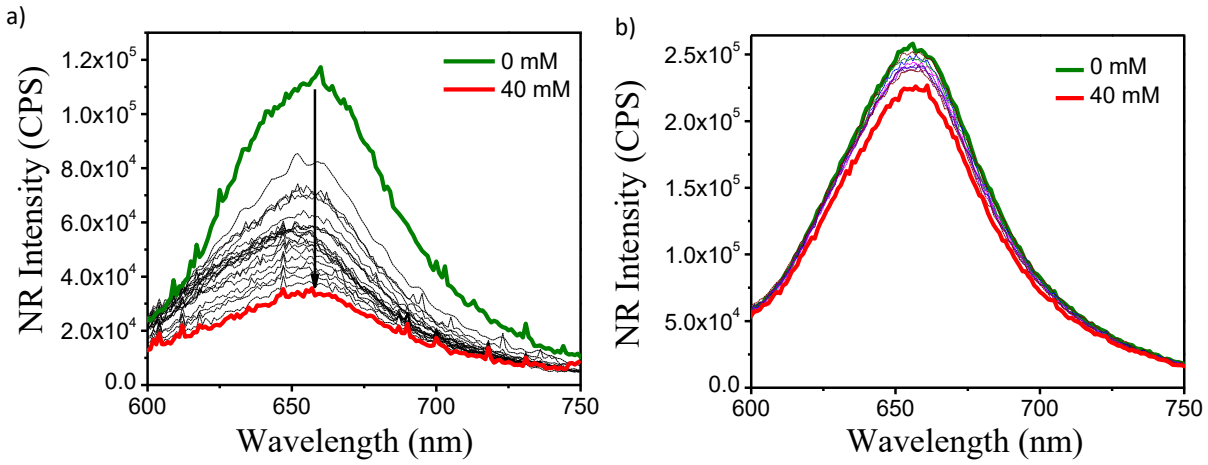



**Figure S11:** Photoluminescence spectra showing GSH concentration-dependent release of Nile Red from the hydrophobic core of self-assembled a) **P2** and b) **P5** as control ($\lambda_{ex}$ = 556 nm).

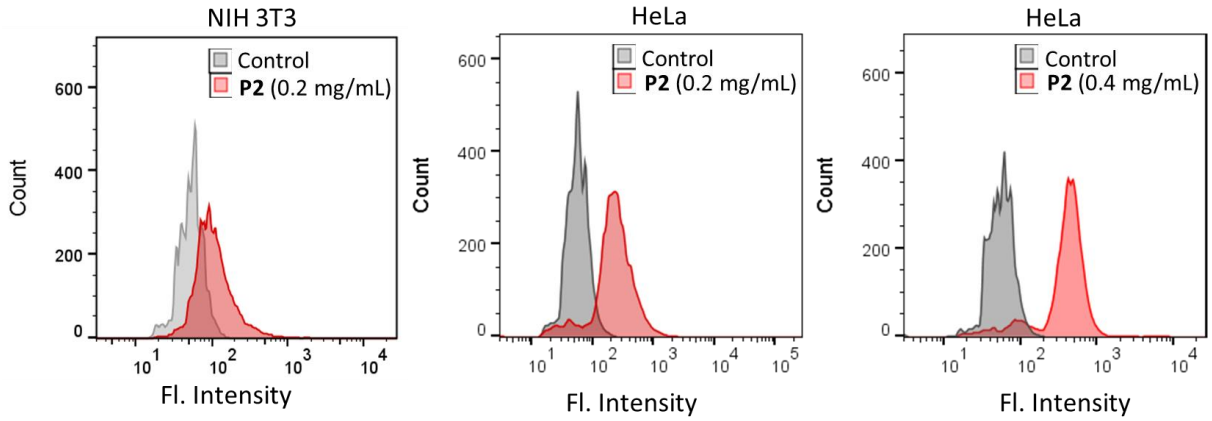



**Figure S12**: FACS analysis of polymer **P2** (*c* = 0.2 mg/mL, incubation time 24 h) in a) non-cancerous NIH 3T3 cells and b) cancerous HeLa cells; c) FACS analysis in HeLa cells upon increasing the incubation time (from 24 to 48 h) and **P2** polymer concentration (from 0.2 to 0.4 mg/mL).

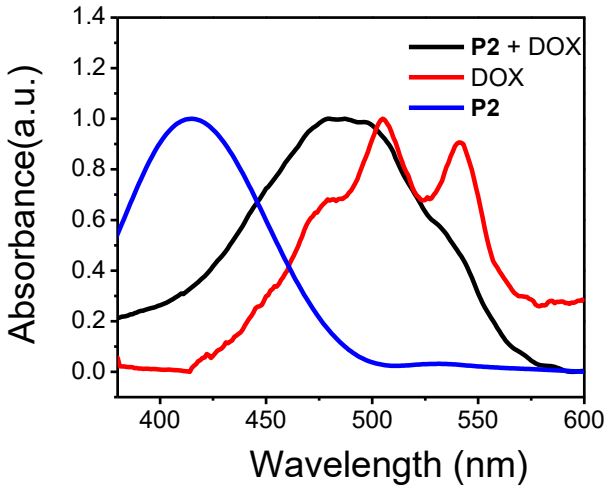



**Figure S13**: UV-vis absorbance spectrum of **P2** + DOX shows red shift as compared to only **P2**, suggesting the encapsulation of DOX in **P2** nanoaggregates (Concentration of **P2** = 1 mg/mL; Concentration of loaded DOX = 0.06 mg/mL).

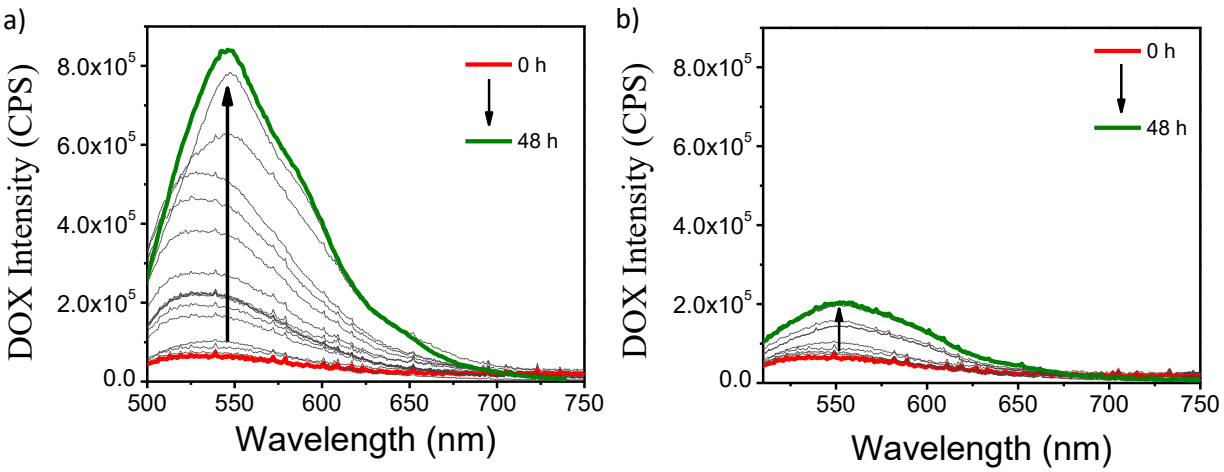



**Figure S14:** Photoluminescence spectra showing time-dependent release of DOX from the **P2** nanoaggregates a) in the presence and b) in the absence of GSH ($\lambda_{ex}$ = 480 nm).

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