Supporting Information

for

Mitochondria Targeting Nanostructures from Enzymatically Degradable Fluorescent Amphiphilic Polyesters

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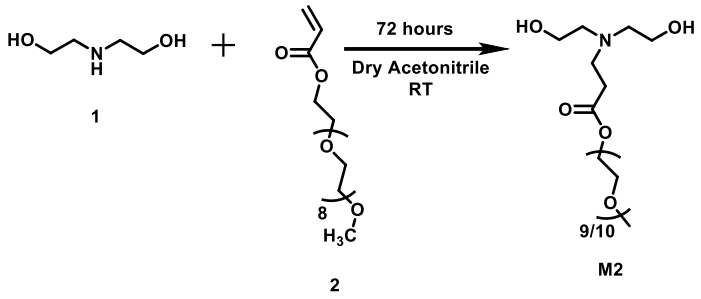
Materials and methods: All reagents were obtained from commercial suppliers and used without further purification unless otherwise mentioned. Diethanolamine was purchased from TCI Chemicals and poly(ethylene glycol) methyl ether acrylate (PEGMEA, average molecular weight = 480 g/mol, contains 100 ppm MEHQ as inhibitor, 100 ppm BHT as inhibitor) was purchased from Sigma Aldrich. 4-Dimethylamino pyridine (DMAP) was purchased from Avra Synthesis Pvt. Ltd. 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 600 MHz or 400 MHz spectrometer using deuterated solvents from Eurisotop. Chemical shifts (δ) are reported in a ppm unit with TMS as the internal standard. The coupling constants (*J*) are reported in hertz (Hz). Number average molecular weight (M_n) and dispersity (Đ) of the polymers were measured by size exclusion chromatography (SEC) with N, N-Dimethylformamide (DMF) as an eluent at 75 °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. Cryo-TEM images were captured in JEOL JEM 2100 PLUS Cryo- TEM with cryogen cooled pole piece. One drop of the aqueous solution of the polymers (C = 0.1mg/mL) was syringed out and mounted on 300 mesh carbon coated Cu grid hung on to GATAN cryoplunger which was then immediately transferred to a cryogen cooled specially designed GATAN sample holder and examined under 120 kV electron beam. Transmission Electron Microscopy (TEM) studies were captured in a JEOL-2010EX machine operating at an accelerating voltage of 120 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 TCS SP8 microscope. Fluorescence-activated cell sorting (FACS) analysis was performed in BD FACSAria III. All the images were analyzed using ImageJ software with the JACoP plugin for the determination of the correlation coefficients.

Synthesis and Characterization

Synthesis of **P1**, **P2, M1** and **A1** were reported by us previously.[1,2]



Scheme S1: Synthetic scheme for the monomer **M2**.

Synthesis of the monomer M2

The synthesis of **M2** was followed from the reported literature[3] with some modifications. 130 μL of diethanol amine (**1**) was measured in a 50 mL round bottom flask, to which 545 mg of polyethylene glycol monomethyl ether acrylate (**2**), dissolved in 25 mL of dry acetonitrile was added dropwise with stirring under room temperature. The stirring was continued for 72 hours. A workup was conducted in DCM-water mixture after the evaporation of dry acetonitrile under reduced pressure. The DCM part was collected and passed through anhydrous Na_2SO_4 for removing the residual water. The collected DCM part was then concentrated to yield a brown liquid as the pure product. Amount obtained = 494 mg; % yield = 74. ^{1}H NMR (600 MHz, Chloroform-*d*) δ 4.26 – 4.24 (m, 2H), 3.76 – 3.70 (m, 4H), 3.65 – 3.62 (m, 45H), 3.60-3.58 (t, 3H), 3.53-3.52 (m, 2H), 2.87-2.84 (m, 2H), 2.64-2.63 (t, 4H), 2.53-2.51 (t, 2H). Theoretical $[M+H]^+$: 588.360, Experimental $[M+H]^+$: 588.407.

Synthesis of the polymer P3

A1 (134.95 mg, 0.282 mmol) was dissolved in 100 μL of dry DMF in a polymer vessel. This solution was kept in a water bath at 50 °C for 5 minutes to ensure complete dissolution. To this solution, DMAP (3.44 mg, 0.028 mmol) and **M1** (82.93 mg, 0.141 mmol) were added with stirring after which **M2** (50 mg, 0.141 mmol) was added. The whole mixture was then degassed by purging dried argon gas for 20 minutes before stirring it at 120 °C in an oil bath for 48 hours. The crude polymer obtained was dissolved in methanol and purified by precipitation from cold ether and collected after drying under reduced pressure to yield 64 mg of the pure polymer as a brown sticky mass. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.44 – 8.29 (m, 3H), 7.78 (b, 1H), 7.03-6.99 (m, 1H), 5.46 (b, 1H), 4.55 (b, 4H), 4.13 – 4.05 (m, 4H), 3.51 (m, 35H), 3.24-3.17 (m, 7H), 2.20-1.36 (m, 21H). Experimental M_n from SEC analysis = ~9000 g/mol, $Đ$ = 1.73 with respect to the polystyrene standard and DMF as eluent.

Experimental Section

Sample preparation for spectroscopic studies: A measured amount of **P1**, **P2**, and **P3** was taken in a clean and dry glass vial, and to it a fixed volume of HPLC-grade methanol (for **P1** and **P2**) or chloroform (for **P3**) was added to make the stock solutions. From there, measured quantities were taken out into a vial, and methanol was evaporated by heating the vial at 70 °C 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 at 100 °C, 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 zeta potential measurements.

FT-IR Study: For analyzing the spectral signature of pristine **P3** polymer, a sample was prepared by directly making a dry KBr pellet of the solid polymer **P3** and 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. For the degradation study, an aqueous solution of **P3** (*C* = 1 mg/mL), was added with a 500 μL of enzymatic solution of Lipase B from *Pseudomonas cepacia* (6.6 mg/mL in phosphate buffer solution of pH = 7.4) and incubated at 37 °C for 18 hours at 200 rpm before lyophilizing and recording their FT-IR spectra. The same sample preparation method was followed for monitoring the enzymatic degradation of **P3** by size exclusion chromatography.[1,2,4,5] For the degradation by Esterase enzyme from porcine liver, 2 mg/mL of polymer solutions in phosphate buffer solution of pH = 7.4 was made, to which 87.18mg (20U) of the Esterase solution was added and incubated at 37 °C at 200 rpm.[6] These solutions were directly used to measure the FT-IR spectra at 4 h, 18h and 24 h time intervals. In a similar manner, solutions were made in the complete DMEM solution used for cell culture for recording the spectra after 24 hours.

Critical Aggregation Concentration (CAC) Determination: A stock solution of pyrene (*C* =1 $x10^{-4}$ M) was prepared in $CHCl_3$ and measured amount of it was taken to adjust the final concentration to 10^{-6} (M) in the total volume of 500 μL in all the vials. Measured amount of the polymer stock solutions in their respective good solvents were also taken in different vials as signed for different concentrations of the polymers and the solvents were evaporated by drying after which 500 μL of water was added. The individual solutions were then subjected to heating at 100 °C for 2 minutes and cooled at room temperature and equilibrated for 50 minutes before recording their emission at an excitation wavelength of 336 nm.[7,8]

Cell culture condition: Human cervical cancer (HeLa) and normal kidney epithelial (NKE) cells were used for cellular uptake studies described. Both the cells were seeded in a high glucose Dulbecco's Modi fied Eagle Medium (DMEM), which was supplemented with 10% fetal bovine serum (FBS) and 1% L-Penicillin-Glutamine-Streptomycin (PSG), which forms the complete media. Cells were

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.[9]

Cell viability study by MTT assay: The cell viability of the HeLa cells was checked in the presence of all the polymers under study by 3-(4,5-dimethylthia zol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as say. Approximately, 104 cells per well 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 (50, 100, 200 and 400 μg mL^{-1}) and incubated for another 24 h. After 24 h, the media containing polymers was again 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 un treated cells were considered as the positive control.[9]

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]

Fluorescence-activated cell sorting (FACS) analysis: Approximately 105 cells (HeLa and NKE 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 100 μg/mL of each of the polymers and cells were further incubated at 37 °C or 4 °C for predetermined time (1 h) period. To study the effect of inhibitors on the cellular internalization, the adherent cells were first incubated with commercially available inhibitors known to inhibit endocytosis (Genistein, chlorpromazine hydrochloride or 5-(N, N-dimethyl) amiloride hydrochloride) for 1 h at 37 °C. Subsequently after the completion of the incubation, the cell culture media was replaced with fresh complete media containing same concentration of inhibitors and 0.1 mg/mL of the polymers followed by incubation for another 1 h. After this, the media was removed, and cells were treated with trypsin EDTA, and cell pellets were collected after centrifugation. After the removal of super natant, the cells were re-suspended 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.[9]

Confocal laser scanning microscopy (CLSM) imaging: 105 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 polymers (C = 100 μg/mL) followed by incubation for the desired time. For co-localization experiment, cells which were pre-incubated with the polymer solution of (C = 100 μg/mL) at 37 °C for 1 h, were again incubated with Hoechst 33342 and MitoTracker Red for 10

min each. After this, the cells were washed thrice with fresh media and imaged. Finally, live cell microscopy imaging was performed in presence of complete media. The red channel was excited at 543 nm, whereas for green channel and blue channel, 488 nm and 405 nm respectively, was used for excitation. Images were captured using a confocal laser-scanning Eclipse Ti-E microscope equipped with a plan apochromatic VC 60×/1.4 oil objective and Digital Sight DS-Qi1MC monochromatic camera with NIS AR software (Nikon, Tokyo, Japan). During the time dependent experiment, the cells were incubated for 15 minutes with **P1** and **P2** polymers followed by washing and incubating again with MitoTracker Red and LysoTracker Green for 10 min each. A similar set was incubated with the polymers for 15 minutes and was washed with complete media, followed by incubation for 4 h to eliminate the effect of residual nano particles. They were then washed thrice with fresh complete media and imaged at the earliest.[9]

Additional Figures

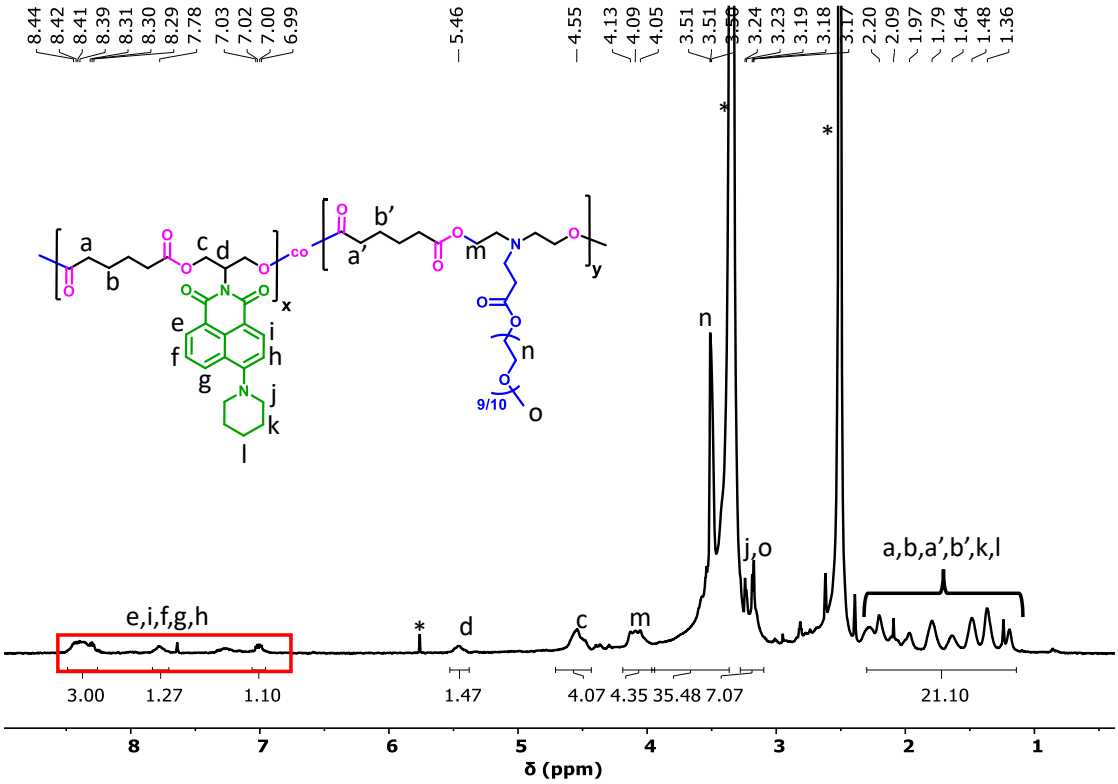


Figure S1: ^{1}H NMR of **P3** polymer in DMSO-d_6 solvent. "*" denotes solvent peaks.

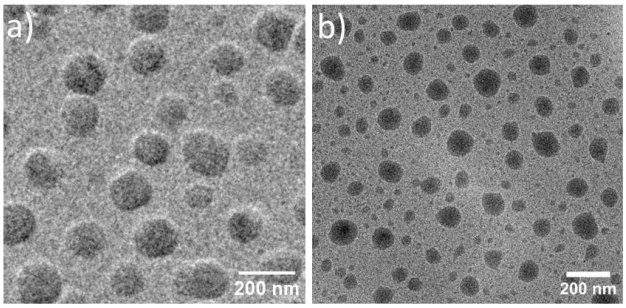


Figure S2: Cryo TEM images of (a) **P1**, and (b) **P2** in water**;** *C* = 0.1 mg/mL.

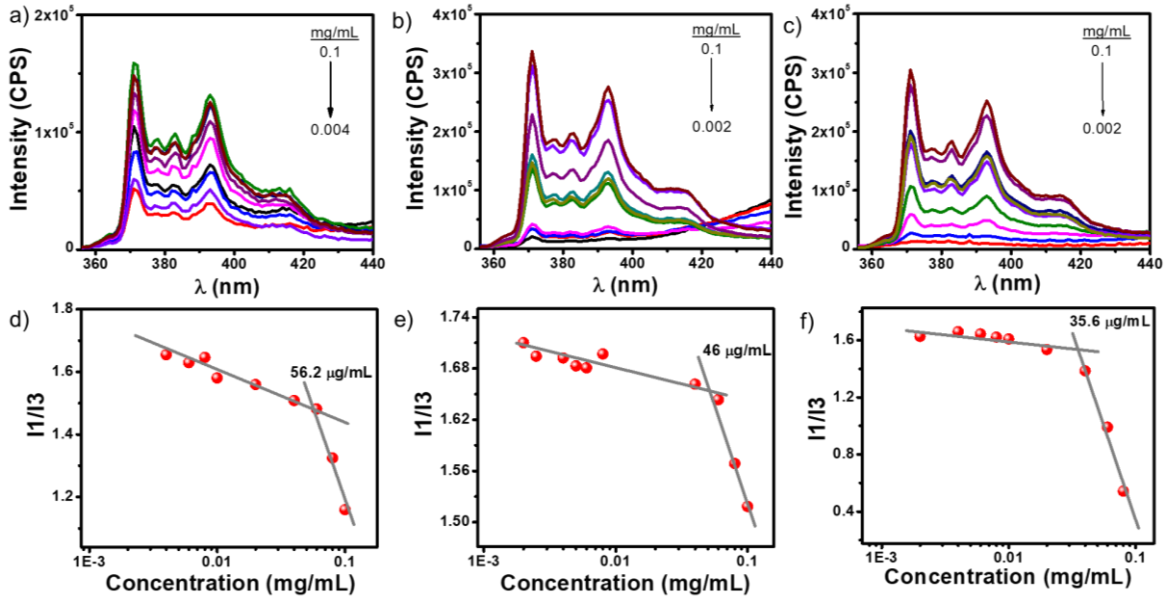


Figure S3: Critical aggregation concentration (CAC) determination[6] of **P1** (a,d), **P2** (b,e) and **P3** (c,f) in water using pyrene as a fluorescence probe. Emission intensity of encapsulated pyrene at different concentrations of the polymers (a-c); Pyrene fluorescence intensity ratios (I_{371}/I_{383}) versus logarithm of polymer concentration (d-g); CAC was determined from the from the inflection point observed in the plot. $\lambda_{exc.}$= 336 nm, Slit = 2_2, path length = 10 mm; Conc. of pyrene = 2 x10^{-6} (M).

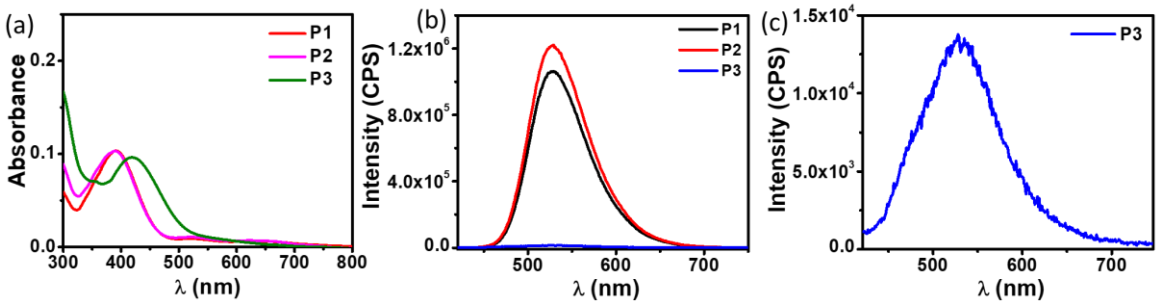


Figure S4: (a) UV-vis plot and (b) fluorescence emission plot of **P1**, **P2**, and **P3** in water at the concentrations of 0.1 mg/mL for **P1** and **P2** and 0.2 mg/mL for **P3**; (c) zoomed fluorescence emission plot of **P3** in water; [$\lambda_{exc.}$= 400 nm, Slit=1_1].

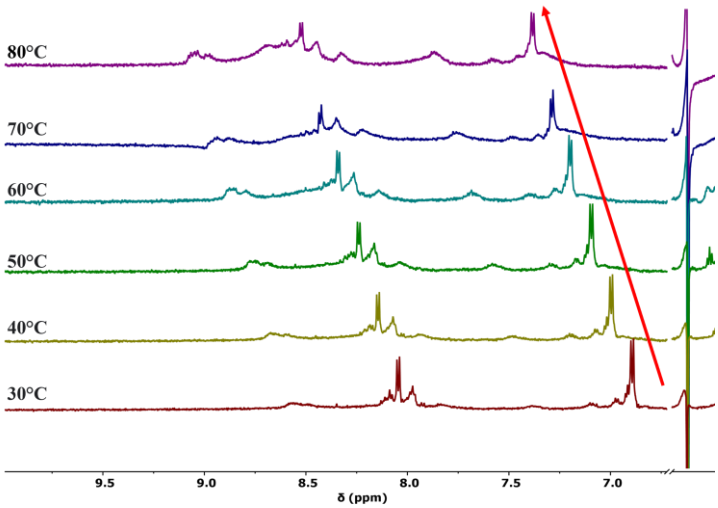


Figure S5: Variable temperature ^{1}H NMR plot of **P3** polymer in D_2O in water suppression mode. *C* = 0.1 mg/mL

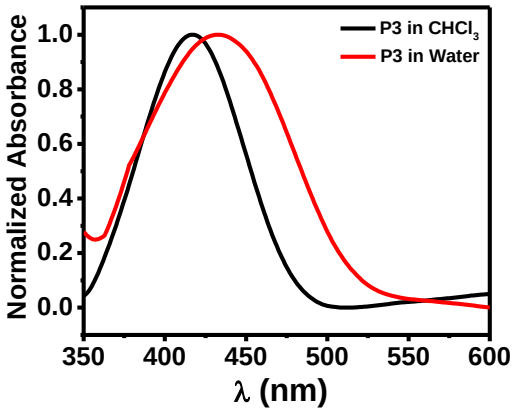


Figure S6: UV-vis spectra of **P3** polymer in $CHCl_3$ and aqueous solvent (*C* = 0.2 mg/mL).

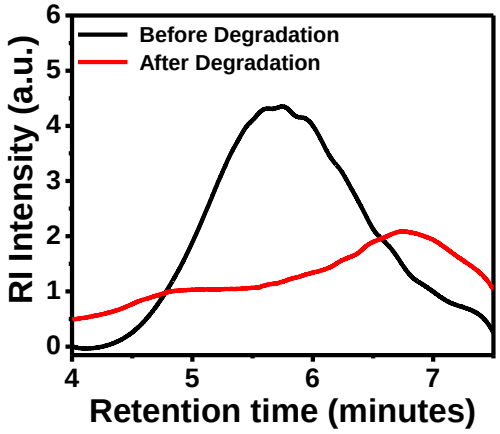


Figure S7: SEC plot of the **P3** polymer upon degradation with Lipase B enzyme from *Pseudomonas cepacia* with DMF as the eluent.

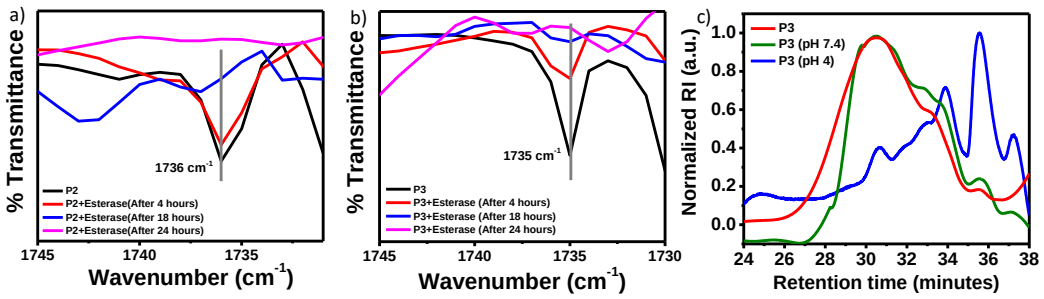


Figure S8: FT-IR plot of the (a) **P2** and (b) **P3** polymers in phosphate buffer solution of pH 7.4 showing backbone degradation with Esterase enzyme from porcine liver (showing selected ester region); (c) SEC traces of **P3** polymer at variable pH with DMF as the eluent.

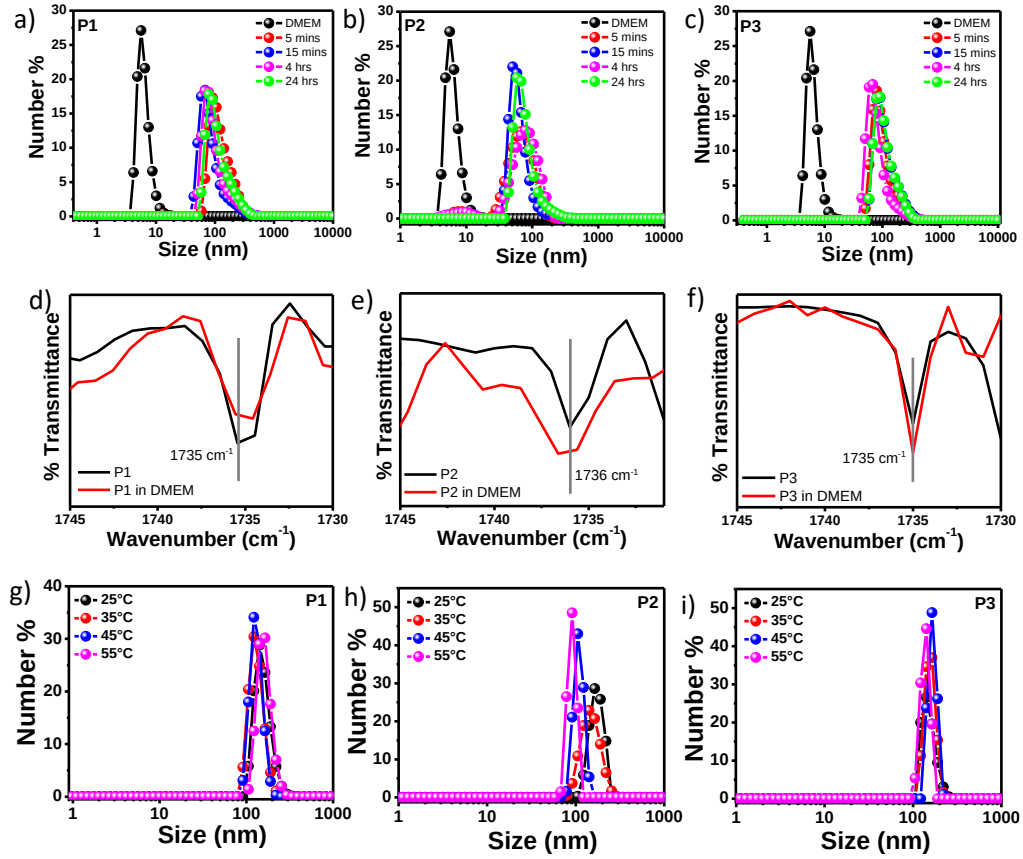


Figure S9: DLS plot of (a) **P1**, (b) **P2** and (c) **P3** polymers in complete DMEM solution monitored at different intervals of time; FT-IR plot of (d) **P1**, (b) **P2** and (c) **P3** in complete DMEM solution after 24 hours (showing selected ester region); DLS plot of (g) **P1**, (h) **P2** and (i) **P3** polymers in buffer of pH 7.4.

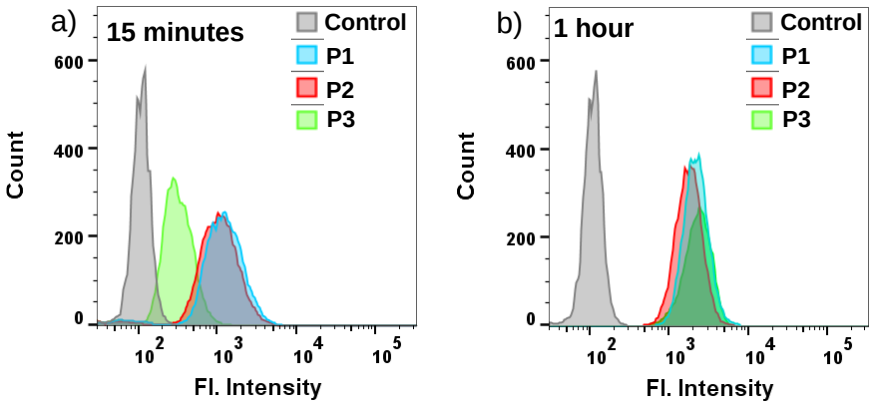


Figure S10: FACS analysis showing relative fluorescence intensity variation of polymers **P1**, **P2** (*C* = 100 µg/mL) and **P3** (*C* = 200 µg/mL) at (a) 15 minutes and (b) 1 hour of incubation in HeLa cells.

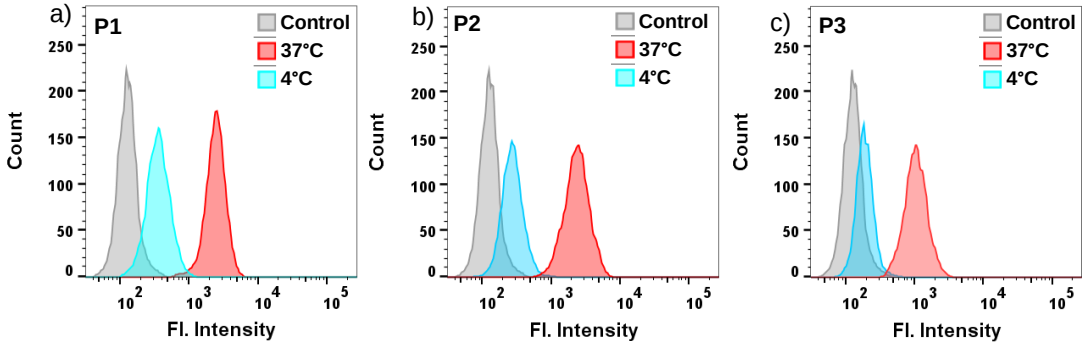


Figure S11: FACS analysis showing relative fluorescence intensity variation of polymers **P1**, **P2** (*C* = 100 µg/mL) and **P3** (*C* = 200 µg/mL) at 37°C and 4°C in HeLa Cell line.

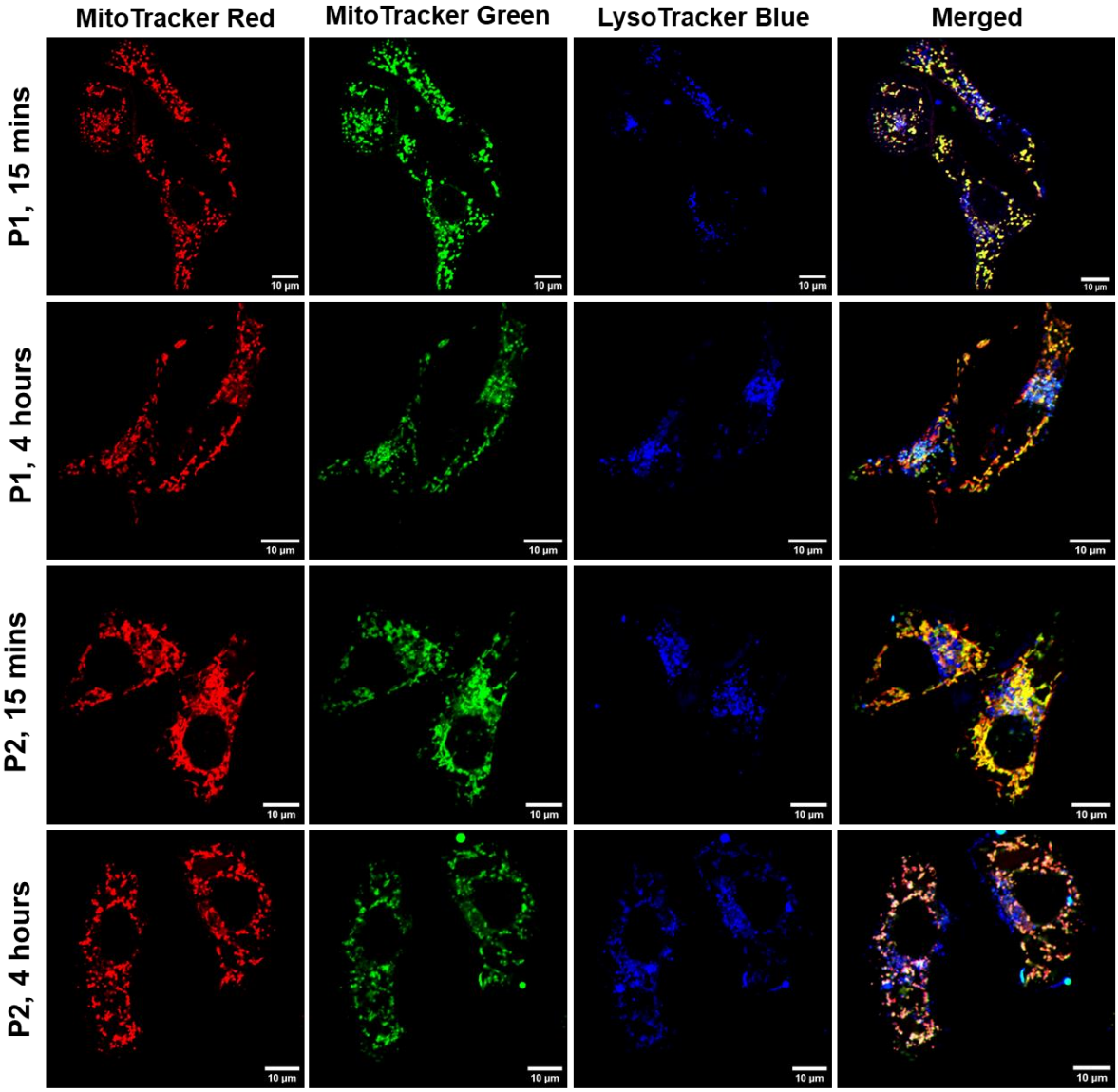


Figure S12: CLSM images of HeLa cells incubated with **P1** and **P2** for 15 minutes and 4 hours (*C* = 100 μg/mL). Images from left to right are arranged as follows: red, green and blue channel emissions due to mitochondria staining MitoTracker Red, intracellular polymer emission, and LysoTracker Blue, respectively, and an overlay of these three images; Scale bar = 10 μm.

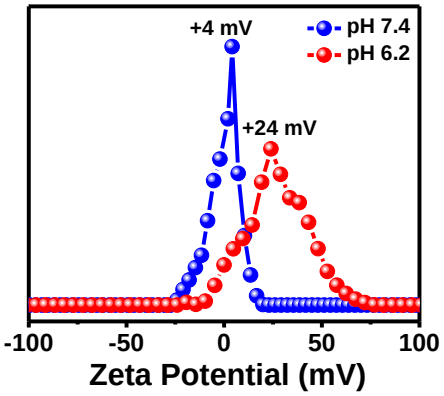


Figure S13: Zeta potential plot of polymer **P3** at pH 6.2 and 7.4 (*C* = 0.1 mg/mL).

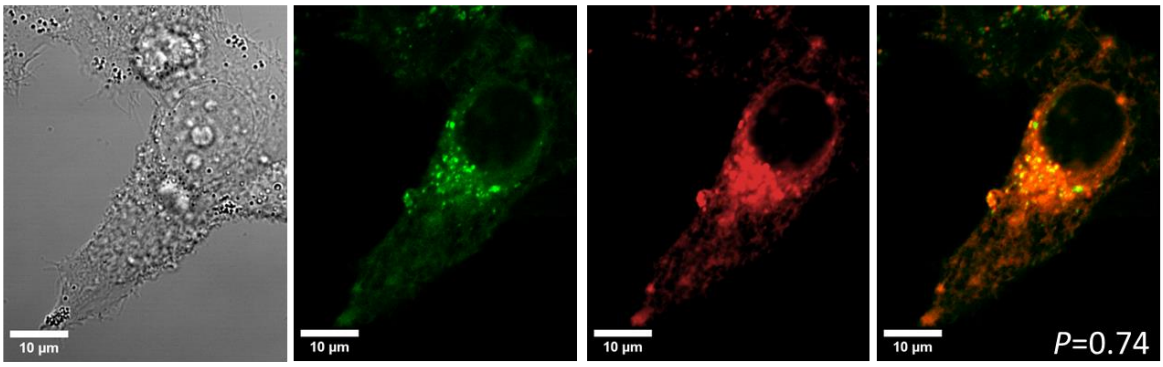


Figure S14: CLSM images of NKE cells incubated with **P2** (*C* = 100 µg/mL) for 1 hour. From left to right: Images from green and red channel emissions are due to **P2** polymer and MitoTracker Red, respectively, and their overlay. Scale bar = 10 µm.

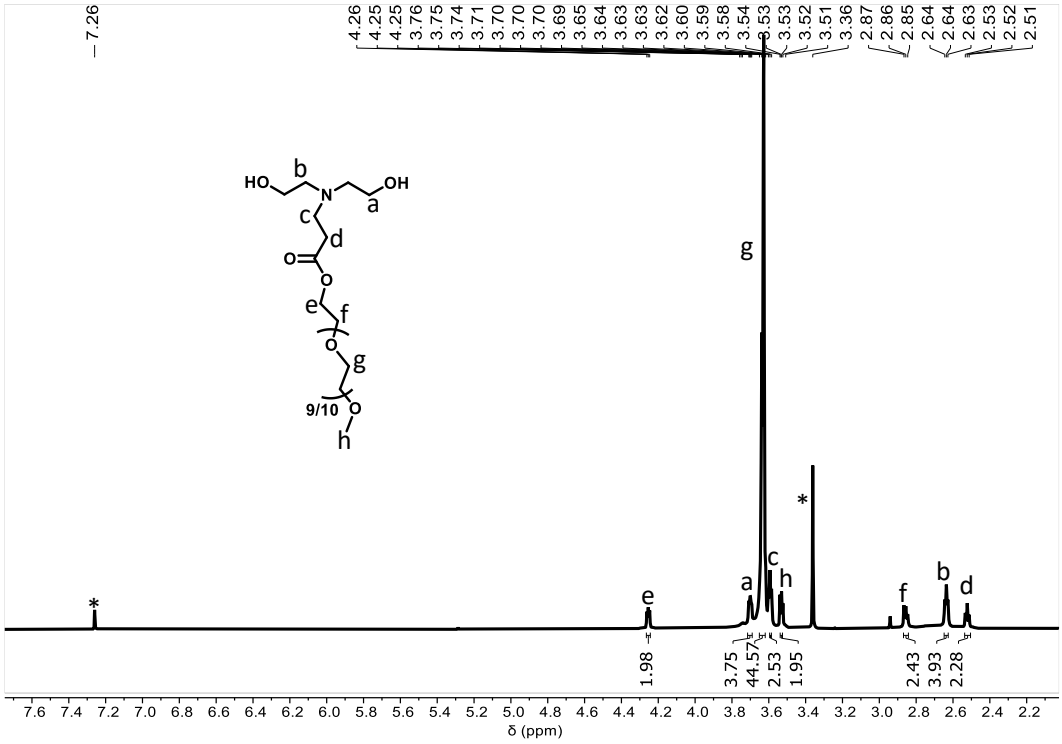


Figure S15: ^{1}H NMR of **M2** monomer in $CDCl_3$ solvent.

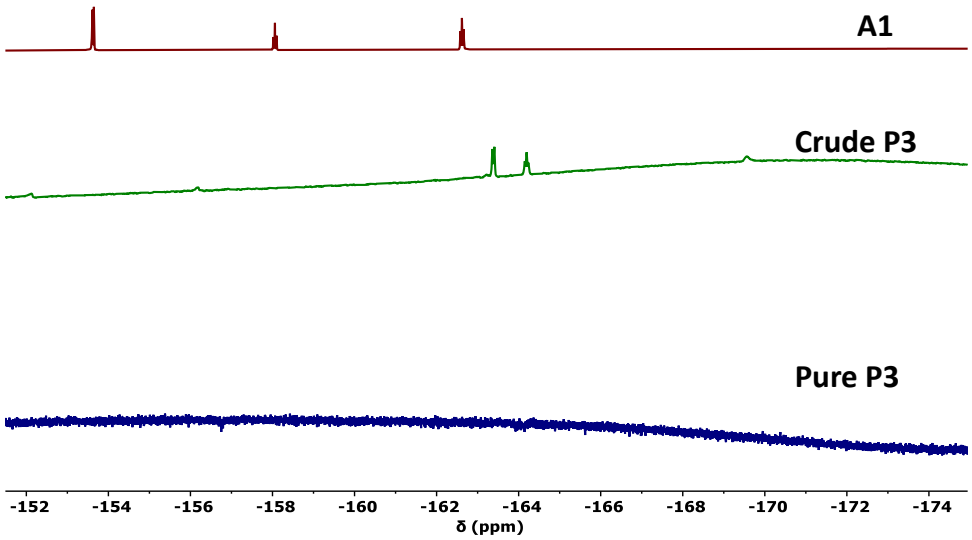


Figure S16: Compared ^{19}F NMR spectra of crude and pure **P3** polymer with **A1** monomer in $CDCl_3$ solvent.

References

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