Efficient approach to prepare multi-anticancer drug Conjugated nanocarrier

Vijayakameswara Rao N[1], Himadri Dinda[1], Mutyala Naidu Ganivada[1], Jayasri Das Sarma[2]* and Raja Shunmugam[1]*

[1]Polymer Research Centre, Department of Chemical Sciences,
[2]Department of Biological Sciences,
Indian Institute of Science Education and Research Kolkata.
E-mail: sraja@iiserkol.ac.in

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Characterization:

Gel Permeation Chromatography (GPC). Molecular weights and PDIs were measured by Waters gel permeation chromatography in THF relative to PMMA and PS standards on systems equipped with Waters Model 515 HPLC pump and Waters Model 2414 Refractive Index Detector at 35 ^{0}C with a flow rate of 1 mL/min. HRMS analysis was performed with Q-TOF YA263 high resolution (Waters Corporation) instruments by +ve mode electrospray ionization.

Fluorometry. Fluorescence emission spectra were recorded on a Fluorescence spectrometer (Horiba Jobin Yvon, Fluromax-3, Xe-150 W, 250-900 nm).

Nuclear Magnetic Resonance (NMR). The ^{1}H NMR spectroscopy was carried out on a Bruker 500 MHz spectrometer using $CDCl_3$ as a solvent. ^{1}H NMR spectra of solutions in $CDCl_3$ were calibrated to tetramethylsilane as internal standard (δ_H 0.00).

Fourier Transform Infra Red (FT-IR). FT-IR spectra were obtained on FT-IR Perkin-Elmer spectrometer at a nominal resolution of 2 cm^{-1}.

Ultra Violet (UV) Spectroscopy. UV-visible absorption measurements were carried out on U-4100 spectrophotometer HITACHI UV-vis spectrometer, with a scan rate of 500 nm/min.

Dynamic Light Scattering (DLS). Particle size was measured by dynamic light scattering (DLS), using a Malvern Zetasizer Nano equipped with a 4.0 mW He-Ne laser operating at λ = 633 nm. All samples were measured in aqueous as well as methanol at room temperature and a scattering angle of 173°.

Transmission Electron Microscopy (TEM). Low resolution transmission electron microscopy (TEM) was performed on a JEOL 200 CX microscope. TEM grids were

purchased from Ted Pella, Inc. and consisted of 3-4 nm amorphous carbon film supported on a 400-mesh copper grid.

Confocal Laser Scanning Microscopy (CLSM). Confocal Microscope images were taken in LSM 710 with microscope axio observer Z.1, Carl Zeiss.

Experimental Section

Materials: 5-norborene-2-carboxylic acid (mixture of endo and exo isomers), doxorubicin hydrochloride, indomethacin, chlorambucil, cis-5-norbornene-exo-anhydride, 2-aminoethanol, triethylamine, *N*, *N*- Diisopropylethylamine, *N*-(9-Fluorenylmethoxycarbonyloxy) succinimide (FmocOSu), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), folic acid, dicyclohexylcarbodiimide (DCC), poly(ethylene glycol) [**HO-PEG–OH**; M_n = 619; L_n = 14 by mass spectroscopy MS (ESI), (**Figure** S10)], ethyl vinyl ether, 4-aminobenzoic acid, acetic anhydride, sodium acetate, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride, N- hydroxysuccinimide, 1-hydroxybenzotriazole, 4-dimethylamino-pyridine, 2-aminoethanol, tertiary butyl carbazate,trimethyl silyl bromide, diethyl ether, dimethyl sulphoxide, second generation Grubbs' catalyst, $CDCl_3$ and DMSO-D_6 were purchased from Sigma Aldrich. Dichloromethane (DCM), acetone, toluene were dried and used for the reactions.

For Cell Studies: Dulbecco's modified Eagle's medium (DMEM), minimal essential medium (MEM), penicillin, streptomycin and fetal bovine serum (FBS) were purchased from Invitrogen. 3-(4, 5 dimethyl-2-thiazolyl)-2, 5-diphenyl-2H- tetrazolium bromide (MTT) were purchased from USB (Cleveland, OH). Vectashield mounting medium with DAPI (Vector Laboratories).

Synthesis Procedure:

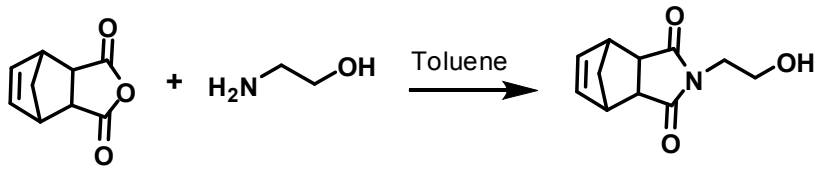
N-Fmoc Doxorubicin: Doxorubicin (200 mg, 0.345 mmol) was dissolved in 2 ml of dry *N*, *N*-dimethylformamide under nitrogen atmosphere. *N*, *N*- Diisopropyl ethylamine, (DIPEA) (150 µl, 0.86 mmol) was added to a solution, followed by *N*-(9-Fluorenylmethoxycarbonyloxy) succinimide (FmocOSu) (230 mg, 0.69 mmol). The mixture was stirred at room temperature for 3 h and concentrated. The resulting residue was triturated

with 0.1% TFA in H_2O (3 x 10 ml) and washed with Et_2O (3 x 10 ml). The resulting red solid was collected by centrifuge and dried under vacuum for 24 h to give as a red powder 190 mg, 70% yield. ^{1}H NMR ($CDCl_3$, 400 MHz) (**Figure** S1), δ (ppm) 7.96 (m, 1H), 7.72 (m, 2H), 7.56 (m, 1H), 7.36 (m, 2H), 7.29 (m, 2H), 7.06 (m, 2H), 5.45 (m, 1H), 5.24 (m, 2H), 4.76 (m, 2H), 4.35 (m, 1H), 4.1 (m, 2H), 4.06 (s, 3H), 3.8 (m, 2H), 3.62 (m, 1H), 3.23 (d, 1H, J = 17.7 Hz), 2.35 (m, 1H), 2.16 (m, 1H), 1.8 (m,1H), 1.31 (d, J = 6.2 Hz, 3H). ^{13}C NMR ($CDCl_3$, 400 MHz), δ (ppm) 215.0, 187.53, 187.04, 163.16, 161.58, 156.48, 156.15,155.85, 144.41, 141.85, 136.46, 135.95, 135.60, 133.95, 128.27, 127.61, 125.58, 121.11, 120.50, 120.38, 119.02, 112.08, 111.76, 77.0, 69.85, 67.18, 66.14, 62.64, 57.25, 47.48, 37.11, 36.15, 34.41,32.07, 17.36.

Isolation of 5-Norbornene-2-exo-carboxylic acid: 25 g of Exo-5-norbornene-2-carboxylic acid was separated from the commercially available mixture of endo and exo 5-norbornene-2-carboxylic acid by the iodolactonization method of Ver Nooy and Rondestvedt.[1] (5 g, 20% yield) ^{1}H NMR (DMSO-D_6, 500 MHZ) (**Figure** S2): δ 1.13 - 1.17 (m, 2H), 1.28 - 1.29 (d, J = 8.5 Hz, 1H), 1.66 - 1.71 (m, 1H), 1.97 - 2.05 (dt, J = 12.7Hz, 1H), 2.76 (s, 1H), 2.9 (s, 1H), 6.03-6.05 (m, 2H), 12.00 (br, 1H). ^{13}C NMR ($CDCl_3$, 500 MHZ) (**Figure** S3): 182.7, 138.1, 135.7, 46.7, 46.4, 43.2, 41.7, 30.3. IR (KBr, cm^{-1}): 2919, 2852, 1700, 1421, 1218, 909, 766. MS (ESI) calculated for $C_8H_{10}O_2Na$ $[M + H]^+$, 138.07; observed 138.09.

FMOC DOX-NOR (mono 1): 5-Norbornene-2-exo-carboxylic acid (100 mg, 0.732 mmol) and (647 mg, 0.86 mmol) of Fmoc-Dox, were dissolved in 5 ml dry DCM followed by the addition of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride, EDC, and catalytic amount of (10 mg, 0.072 mmol) dimethyl amino pyridine were added to the mixture at room temperature. Reaction mixture was allowed to stir for 15 h under nitrogen condition. The reaction mixture was diluted with DCM (20 ml). The organic layer was washed with 5% aqueous HCl solution and brine twice, and then concentrated under vacuum to obtain a red colour solid. ^{1}H NMR (DMSO-D_6, 500 MHZ) (**Figure** S4): δ 14.1 (s, 1H), 13.3 (s, 1H), 7.90 - 7.93 (m, 2H), 7.8 - 7.88 (m, 2H), 7.64 - 7.70 (m, 2H), 7.36 - 7.42(m, 2H), 7.28 - 7.30 (m, 2H), 6.30 - 6.40 (s, 2H), 5.44 - 5.48 (s, 1H), 5.20 - 5.26(s, 1H), 4.93 - 4.96 (m, 1H), 4.83 - 4.88 (m, 1H), 4.70 - 4.74 (m, 1H), 4.50 - 4.58 (m, 1H), 3.98 - 4.0 (s, 3H), 3.33 (s, 2H), 2.10 - 2.20 (m, 2H), 1.85-1.89 (m, 1H), 1.46-1.49 (m, 4H), 1.10-1.13 (s, 3H). MS (ESI) calculated for $C_{50}H_{47}NO_{14}$ $[M + H]^+$: 885.3 observed 889.0.

N-(hydroxyethyl)-cis-5-norbornene-exo-2, 3-dicarboximide: Cis-5-norbornene-exo-anhydride (1 g, 0.006 mmol) was added to 15 ml toluene, followed by 2-aminoethanol (800 µl, 0.006 mmol) and triethylamine (200 µl, 0.06 mmol). Reaction mixture was allowed to stir at room temperature. A Dean-Stark trap was attached to the flask, and the reaction mixture was heated at reflux for 12 h. The reaction was monitored by TLC. After the completion of the reaction, the reaction mixture was concentrated to yield residue. This residue was dissolved in 40 ml dichloromethane and washed with 10 ml of 0.1N HCl and 10 ml of brine solution. The organic layer was dried over $MgSO_4$ and concentrated to yield **2** as a white solid in 90% yield. ^{1}H-NMR (DMSO-D_6, 500 MHz) (**Figure** S5): 1.23 - 1.34 (m, 2H), 2.66 (s, 1H), 3.08 (s, 2H), 3.4 - 3.9 (m, 4H), 4.73 - 4.75 (t, 1H), 6.30 (t, J = 5 Hz, 2H). ^{13}C NMR ($CDCl_3$, 400 MHZ) (**Figure** S6): 178, 137, 60, 47, 45, 42, 41.0 IR (KBr, cm^{-1}): 3507, 3605, 2989, 2963, 2885, 1755, 1684, 1454, 1427, 1401, 1351, 1326, 1249, 1239, 1149, 1098, 1065, 991, 951, 935, 881, 854, 806, 771, 734, 693, 647, 607. MS (ESI) calculated for $C_{11}H_{13}NO_3$ $[M + H]^+$: 208.09 observed 207.20 (**Figure** S7)



Scheme S1. Synthesis of compound **1**.

Synthesis of norborene indomethacin (mono 2): Indomethacin (858 mg, 2.40 mmol) and N-(hydroxyethyl)-cis-5-norbornene-exo-2,3-dicarboximide (500 mg, 2.40 mmol) were dissolved in 5 ml of dry dichloromethane. N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride, EDC, (742 mg, 3.60 mmol) and a catalytic amount (1.84 mg, 10 mol %), of dimethyl amino pyridine were added to the mixture at room temperature. The reaction mixture was allowed to stir for 15 h under nitrogen condition. Solution was transferred to a 500-mL separating funnel with water (100 ml). The mixture was washed with 10% aqueous sodium bicarbonate (2 x 100 ml), and saturated brine (2 x 100 ml). The organic layer was collected and dried over Na_2SO_4 and filtered. The solvent was removed on the rotary evaporator, and the residue was purified by column chromatography (5% CH_2Cl_2/MeOH) to give a light yellow colour solid (800 mg, 97%). ^{1}H NMR ($CDCl_3$, 400 MHZ) (**Figure** S8) : δ 7.6 - 7.7(m, 2H), 7.486 - 7.48 (m, 2 H), 6.90 - 6.95 (m, 2H), 6.80 - 6.87 (m, 2H), 6.01 - 6.1

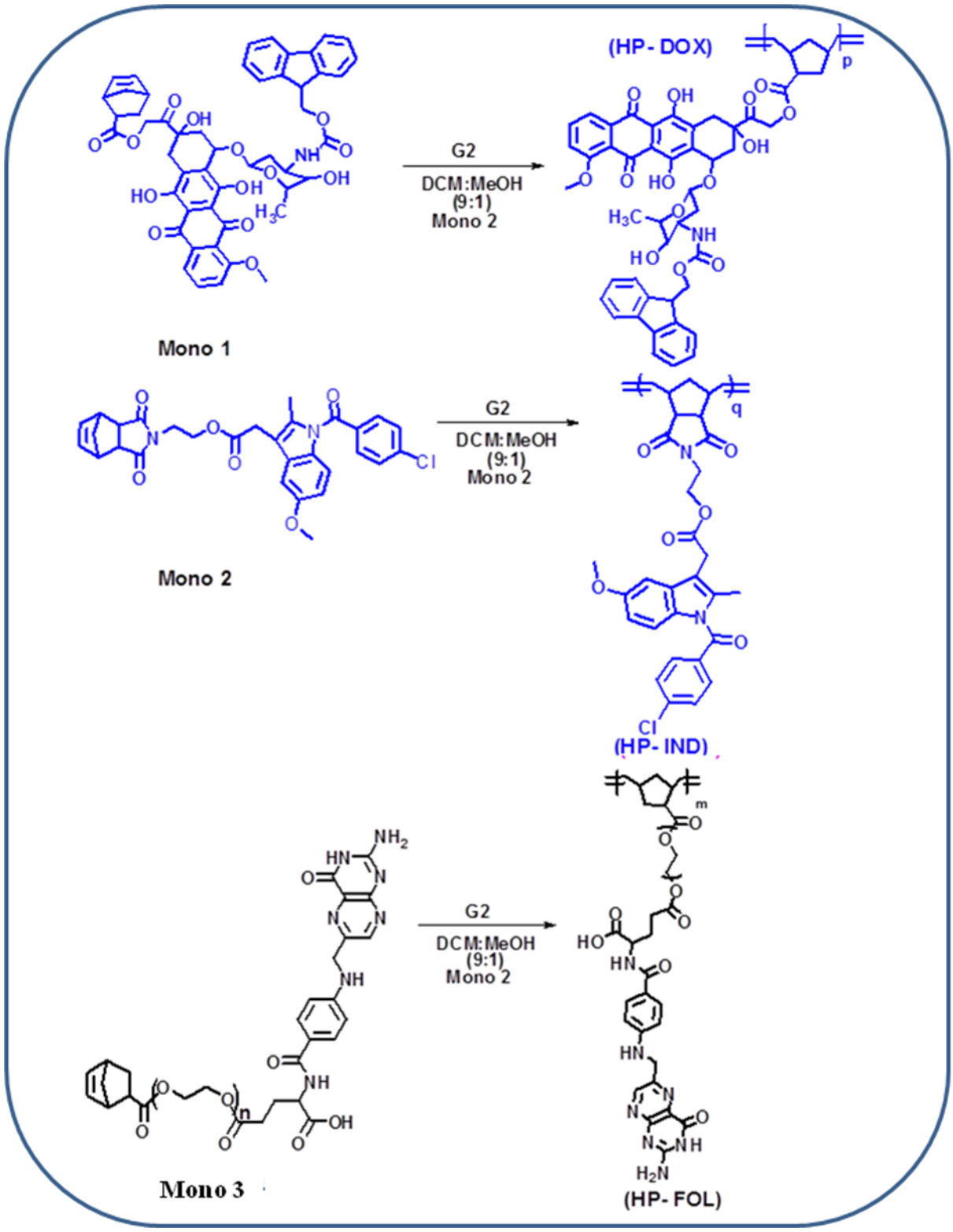
(m, 2H), 4.27 - 4.4 (m, 2H), 3.80 - 3.84 (m, 4H), 3.17 - 3.20 (m, 2H), 2.90 (s, 2H), 2.0-2.07 (m, 2H), 2.3 (s, 3H), 1.20 - 1.25 (s, 3H).). ^{13}C NMR ($CDCl_3$, 400 MHz), δ (ppm): 177.7, 170.4, 169.3, 155.9, 137.6, 136.0, 131.1, 130.61, 129, 114, 112, 111, 101, 61, 55, 47, 45, 42, 37, 29.6, 13.0 MS (ESI) calculated for $C_{30}H_{27}ClN_2O_6$ $[M + H]^+$: 546 observed 547.20

Synthesis of poly (ethyleneglycol) grafted-folate (HO-PEG-FOL)^2: Polyethylene glycol (100 mg, 0.161 mmol) and (71.0 mg, 0.161 mmol) of folic acid were dissolved in 5 ml of dry DMSO. Dicyclohexyl carbodiimide (180 mg, 0.87 mmol) and catalytic amount of (2.2 mg, 0.016 mmol) dimethyl amino pyridine were added to the mixture at room temperature. Reaction mixture was allowed to stir for 15 h under nitrogen condition. The byproduct, dicyclohexylurea, was removed by filtration. The filtrate was precipitated by diethyl ether to obtain a yellow solid, after washing with diethyl ether several times (3 x 10 ml) the material was used immediately for the next step, yield (90%) (**Figure** S9, S10, S11 & S12). ^{1}H NMR (DMSO-D_6, 500 MHZ) (**Figure** S12) : δ 11.2 - 11.5 (s, 1H), 8.624 (s, 1H), 8.6(s, 2H), 8.0 - 8.1 (d, 2 H), 7.36 - 7.7.38 (d, 2 H), 6.55 -6.70 (d, 2H), 6.50 (s, 2H), 4.50 - 4.54 (m, 2H), 4.43 - 4.4 (m, 2H), 2.30 - 2.33 (t, 2H), 2.0-2.07 (m, 2H), 1.86- 1.94(m, 2H).

Synthesis of norbornene grafted poly(ethyleneglycol)-Folate (NOR-PEG-FOL, mono 3): 5-Norbornene-2-exo-carboxylic acid (100 mg, 0.728 mmol) and poly (ethyleneglycol) grafted-folate **(HO-PEG-FOL)** (755 mg, 0.728 mmol) of were dissolved in 5 ml of dry DMSO. Dicyclohexyl carbodiimide (298 mg, 1.44 mmol) and catalytic amount of (10 mg, 0.072 mmol) dimethyl amino pyridine were added to the mixture at room temperature. Reaction mixture was allowed to stir for 15 h under nitrogen condition. The by-product, dicyclohexylurea, was removed by filtration. The filtrate was precipitated by diethyl ether to obtain a yellow solid, after washing with diethyl ether several times (3 x 10 ml) the material was used immediately for the next step, yield (90%). ^{1}H NMR (DMSO-D_6, 500 MHZ) (**Figure** S13) : δ 11.24-11.53 (broad singlet, 1H), 8.7(m, 2H), 8.62 (s, 1 H), 8.61(s, 1H), 8.06 - 8.07 (d, 2H), 7.64 - 7.65 (m, 2 H), 7.40 - 7.42 (d, 2H), 6.88 - 6.89 (d, 2H), 6.59 - 6.6 (d, 2H), 6.05 - 6.07 (m, 1H), 6.13 - 6.14 (m, 1H), 5.55 -5.57 (d, 1H), 3.16 - 3.20 (m, 4H, PEG), 2.90 (s, 2H), 2.19 - 2.28 (m, 2H), 2.30 - 2.33 (t, 2H), 2.0 - 2.07 (m, 2H), 1.86- 1.94 (m, 2H), 1.50 - 1.56 (m, 2H), 1.2 - 1.6 (m. 2H). IR (KBr, cm^{-1}): 3384, 2920, 1696, 1603, 1532, 1406, 1346, 1292, 1186, 1100. MS (ESI) observed 1162.0 (**Figure** S14).

Homopolymerization of monomers

After control synthesis of monomers, its homopolymerization conditions were explored. Homopolymerization of **mono 1** was carried out by using second generation Grubbs' catalyst, at room temperature in dry DCM and methanol (9:1 v/v %) solvent system. The polymerization was monitored by ^{1}H NMR spectroscopy. New signals were observed at 5.0-5.4 ppm and norbornene olefinic protons were disappeared at 6.10-6.14 ppm, indicating the formation of the product. The molecular weight of homopolymer of **mono1, HP-DOX**, was measured by gel permeation chromatography (GPC) using polystyrene standards. The observed molecular weight (Mn) and polydispersity index (PDI) from the GPC analysis suggested the polymerization of mono **1** was done in a very controlled fashion. Similarly, the homopolymerization of **mono 2, HP-IND (Figure S16),** was carried out by using second generation Grubbs' catalyst with feed ratios ([M]/[I] = 15, at room temperature in dry DCM and Methanol (9:1 v/v %) solvent system. It was observed the polymerizations were well-controlled resulting in narrow polydispersitiy index (PDI), with good yield (65-70%).



Scheme S2. Synthesis of Homopolymers of **mono 1, mono 2 and mono 3.**

Triblock copolymer (TBCP-1): Known amounts of monomers **1, 2** and **3** were weighed into three separate Schlenk flasks, placed under an atmosphere of nitrogen, and dissolved in anhydrous dichloromethane and methanol (9:1 v/v %). Into another Schlenk flask, 0.90 mg (25 mol %) of second generation Grubbs' catalyst was added, flushed with nitrogen, and dissolved in a minimum amount of anhydrous dichloromethane and methanol (9:1 v/v %). All three flasks were degassed three times by freeze−pump− thaw cycles. Monomer **1** (20 mg) was transferred to the flask containing the catalyst via a cannula. The reaction was allowed to stir for 1 h at room temperature until the polymerization was complete. An aliquot of the sample was taken for gel permeation chromatography. GPC analysis was done in tetrahydrofuran (flow rate = 1 mL/ min). The molecular weight of the macro initiator **1** was measured as Mn = 10000, using polystyrene standards. Then the second monomer **2** (14.6 mg) was added to the flask via a cannula. The polymerization was allowed to continue for another 2 h, until the polymerization was complete, an aliquot of the sample was taken for GPC analysis. The molecular weight of the macro initiator **2** was measured as Mn = 17000 using polystyrene standards. Finally the third monomer **3** (30 mg) was added to the flask via a cannula. The reaction was allowed to stir at room temperature until the polymerization was complete. Then the reaction mixture was quenched with ethyl vinyl ether (0.5 ml). An aliquot was taken for GPC analysis, and the remaining product was precipitated from pentane, dissolved it again in THF, passed it through neutral alumina to remove the catalyst, and precipitated again from pentane to get a pure **TBCP-1**. The molecular weight of the final triblock was measured as Mn = 29000, PDI = 1.20. ^{1}H NMR (CD_3OD, 500 MHZ) : δ 7.90 - 7.93 (m, DOX aromatic protons, FMOC aromatic protons), 7.8 - 7.88 (m, DOX aromatic protons), 7.64 - 7.70 (m, IND aromatic protons), 7.64 - 7.65 (m, FA aromatic protons), 7.6 - 7.7(m, FMOC protons), 7.486 - 7.48 (m, IND aromatic protons), 7.40 - 7.42 (d, FA aromatic protons), 7.36 - 7.42 (m, FMOC aromatic protons), 7.28 - 7.30 (m, FMOC aromatic protons), 6.88 - 6.89 (d, FA aromatic protons), 6.90 - 6.95 (m, IND aromatic protons), 6.80 - 6.87 (m, IND aromatic protons), 6.59 - 6.6 (d, FA aromatic protons), 6.30 - 6.40 (s, DOX aliphatic protons), 5.44 - 5.48 (DOX aliphatic protons), 5.20 - 5.26 (polymer backbone protons), 4.93 - 4.96 (DOX aliphatic protons), 4.83 - 4.88 (DOX aliphatic protons), 4.70 - 4.74 (DOX aliphatic protons), 4.50 - 4.58 (m, DOX aliphatic protons), 4.27 - 4.4 (m, IND aliphatic protons) 3.98 - 4.0 (s, DOX aliphatic protons), 3.16 - 3.20 (m, PEG protons), 2.10 - 2.20 (m, DOX aliphatic protons), 1.85-1.89 (m, DOX aliphatic), 1.46-1.49 (m, DOX aliphatic), 1.10-1.13 (s, DOX aliphatic protons).

Deprotection of FMOC in triblock copolymer (DTBCP-1): 25 mg of **TBCP-1,** was dissolved in 2 ml of DMF and 300 µl of piperidine was added under stirring. After 15 min, the solvent was evaporated under vacuum and was further purified by precipitation in diethyl ether and then dried. ^{1}H NMR (CD_3OD, 500 MHZ) **Figure 1(c).**

Cell culture: Hela cells, a human uterine cervical cancer cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) with penicillin (100 U/mL) and streptomycin (100 µg/mL) plus 10% fetal bovine serum (FBS). Whereas, a human embryonic kidney cell lines were maintained in MEM containing 10% FBS (fetal bovine serum) with penicillin (100 U/mL) and streptomycin (100 µg/mL) and 4T, a mouse mammary gland cancer cell lines were maintained in RPMI with containing 10% FBS (fetal bovine serum) with penicillin (100 U/mL) and streptomycin (100 µg/mL). All the cell lines were cultured for a month time with 10 passages and maintained at 37 °C with 5% CO_2 in their respective medium.

Cytotoxicity assay of COPY-DOX. Cytotoxicity of **COPY-DOX, DTBCP-1**in 4T cell lines were quantitatively determined using MTT enzymatic and colorimetric assay. All cell lines were seeded at 1×10^4 cells/well in 96 well plates and maintained in culture for 24 hrs at 37 °C in their respective medium. After 24 hrs medium was removed, cells were washed with PBS and medium containing different concentration of **COPY-DOX,** and **DTBCP-1** (25 µg-200 µg) were added to the designated wells. The whole experimental plate was incubated for 24 hrs. Fresh 20 µl of MTT from 5 mg/ml stock solution were added to each well, followed by incubation for 4 hrs at 37 °C. After 4 hrs, medium from the wells were removed and 100 µl of DMSO were added to each well and incubated for 15 mins to completely solubilise the cells. The absorbance of the resulting solution was measured at 515 nm, and cell survivals were determined by comparison of optical density with untreated respective control cell cultures.

***In Vitro* Drug Release: Dialysis study**.

Having proven the aggregation behaviour of these unique triblock copolymers, the reservoir capabilities were tested by doing dialysis studies. For a dialysis experiment, 1 mg of **DTBCP-1** was dissolved in 1 ml of distilled water and loaded in dialysis tube (3,500; Dalton cut-off) and they were immersed in a 100 mL beaker. The solution was dialyzed at 37 °C with constant stirring at pH buffer 5.5. This particular pH was chosen as a representative acidic environment similar to cancer cell. At specific time intervals, aliquot of the sample was removed and its absorbance at 220, 260, and 480 nm (**Figure** S17) were measured as an

indication of the release of folic acid, indomethacin and doxorubicin respectively. The sample was then added back to the solution to maintain the initial volume. This procedure was repeated for every 1 hr and results were observed up to 12 hrs. It was observed that after 12 hrs, there was no significant increase in the intensity of absorption. It was observed that at pH 5.5, **DOX** release was observed 64% and release of indomethacin 60%. (**Figure3a)**

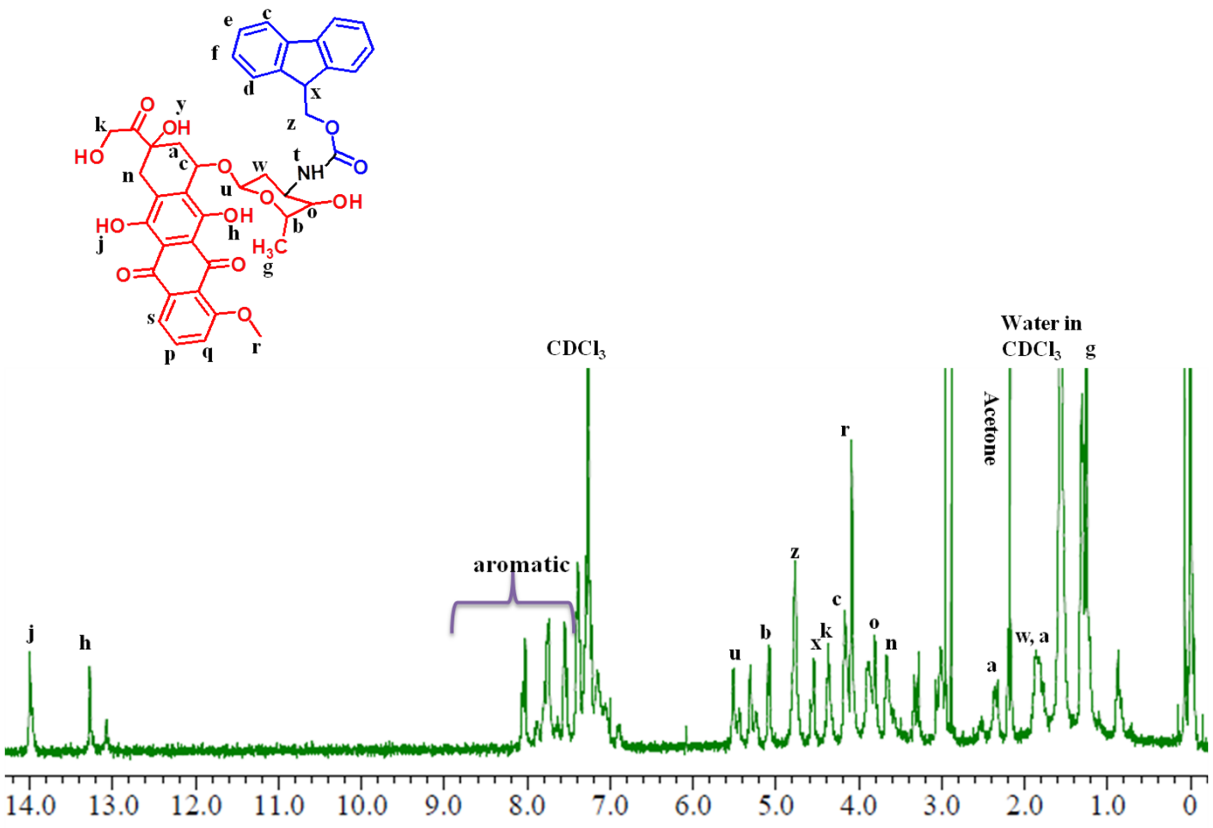


Figure S1. ^{1}H NMR spectrum of **Fmoc-DOX.**

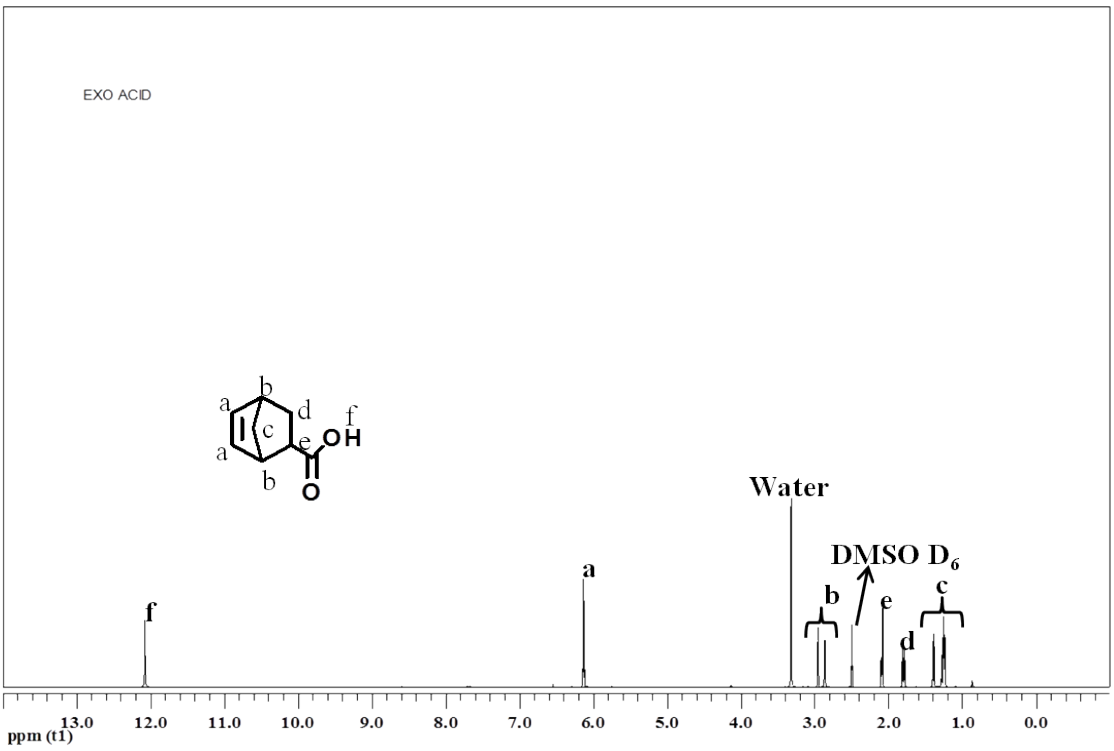


Figure S2. ^{1}H NMR spectrum of Exo-5-norbornene-2-carboxylic acid.

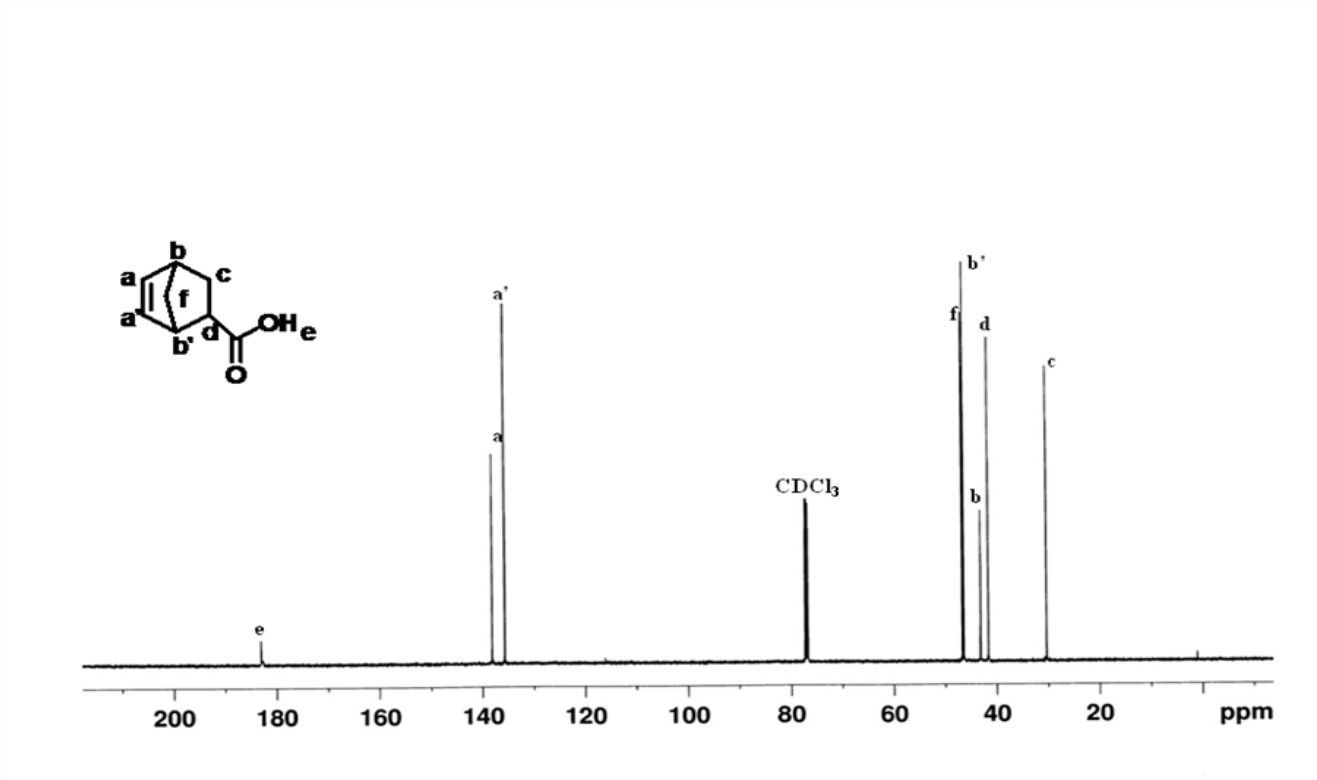


Figure S3. ^{13}C NMR spectra of exo-5-norbornene-2-carboxylic acid.

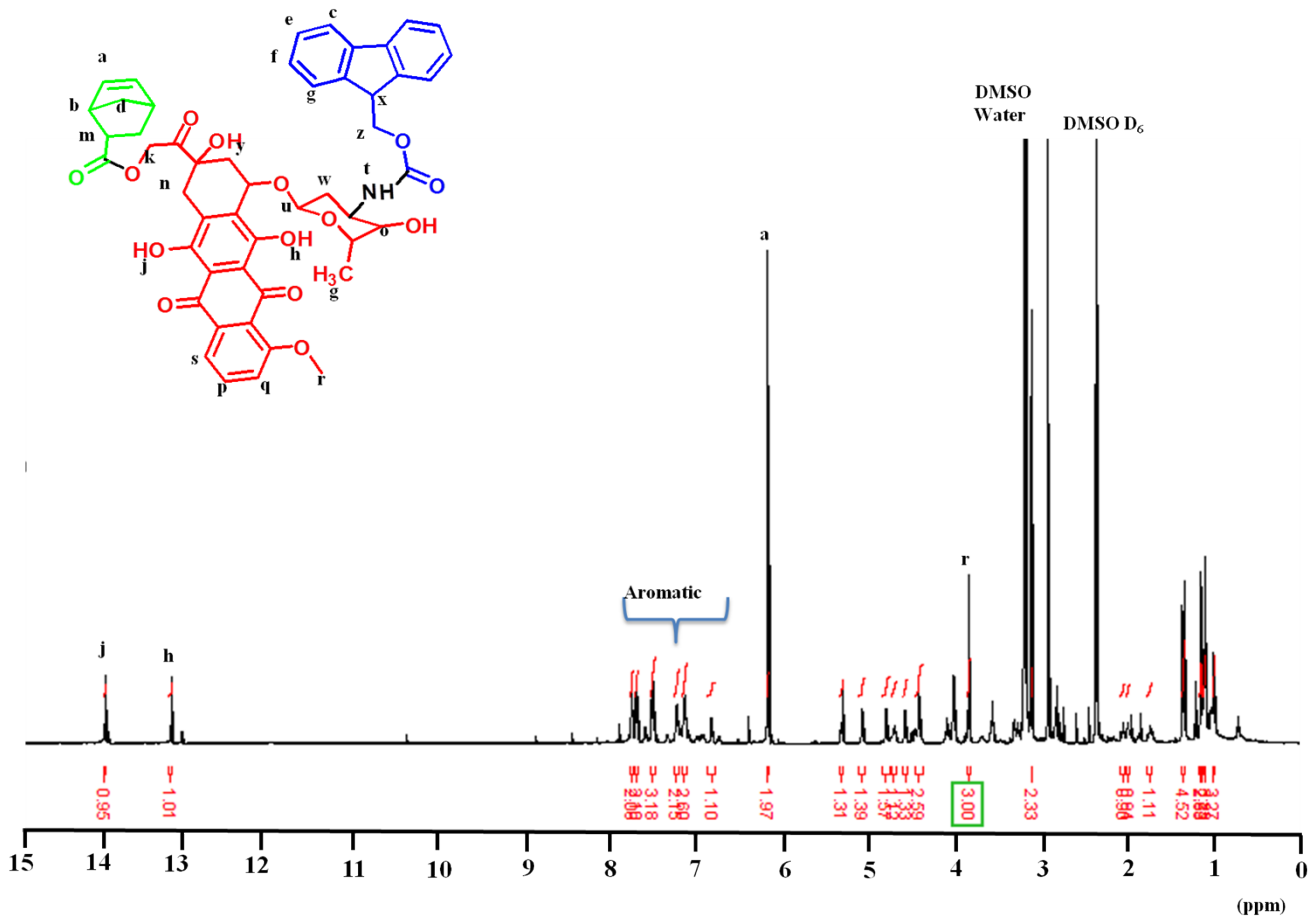


Figure S4. ^{1}**H NMR** spectra of **mono 1.**

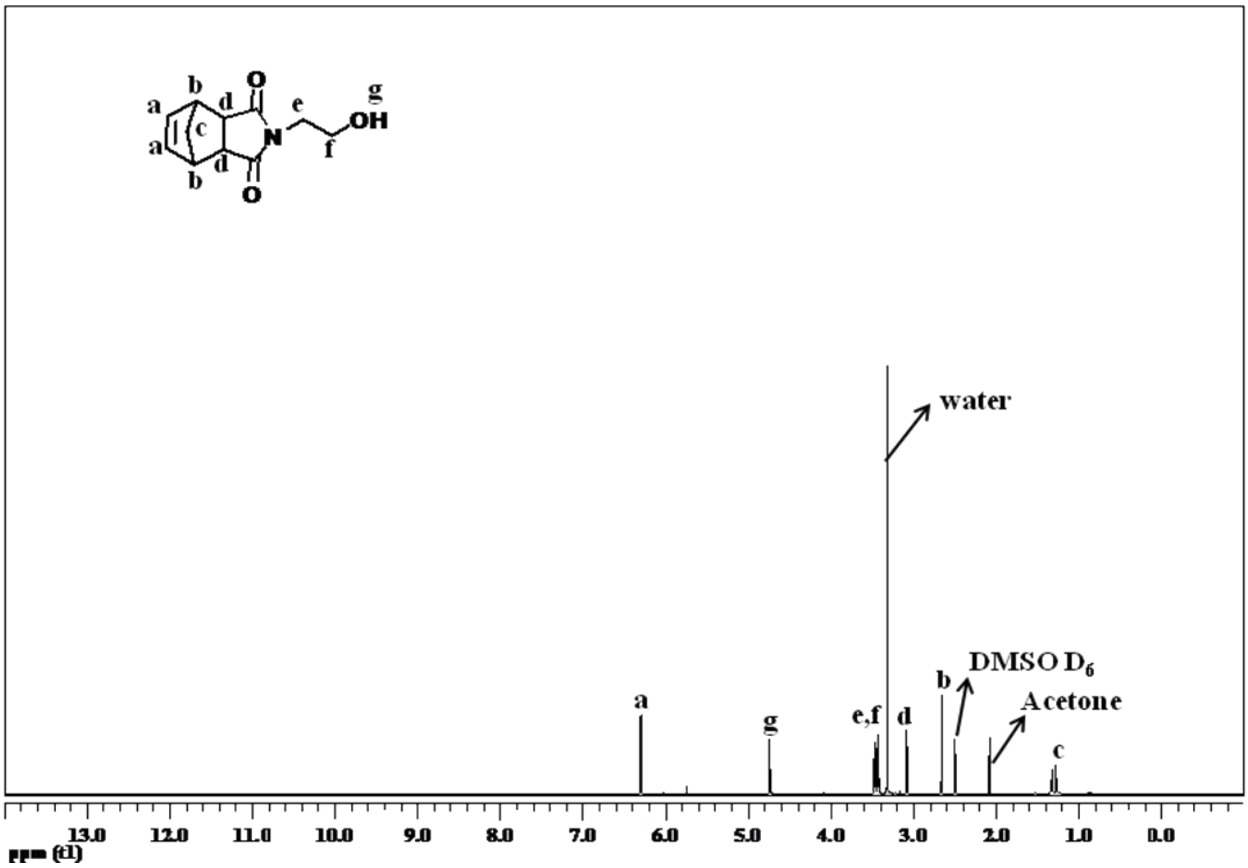


Figure S5. ^{1}H NMR spectrum of N-(hydroxyethyl)-cis-5-norbornene-exo-2,3-dicarboximide.

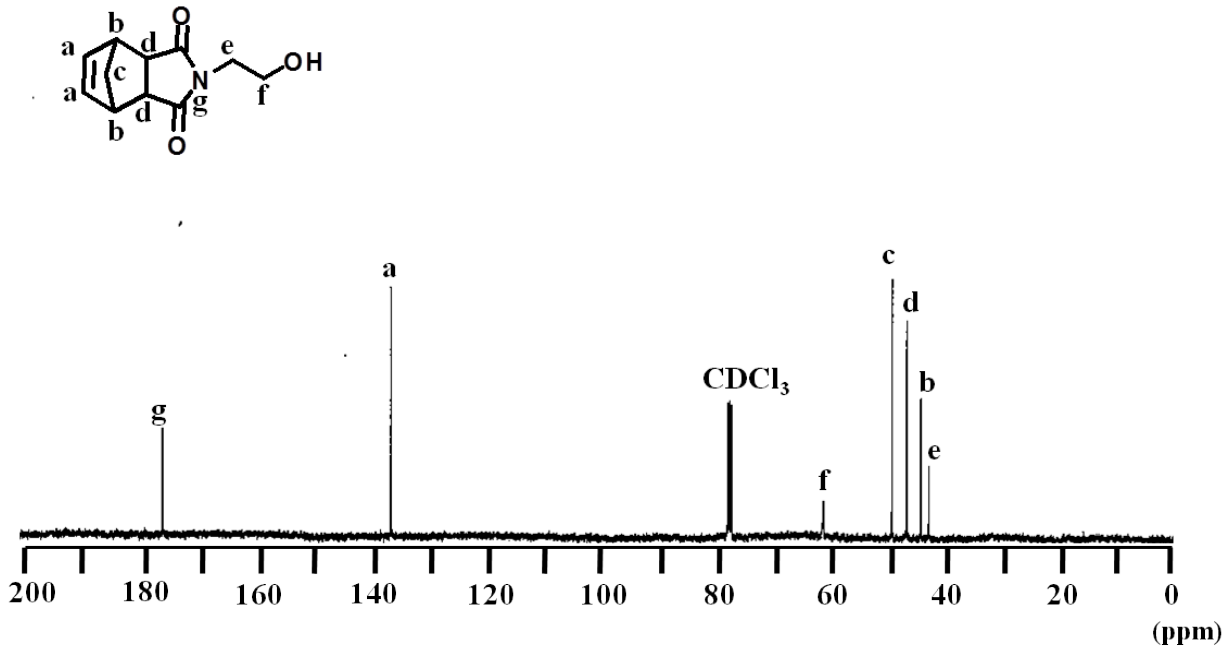


Figure S6. ^{13}C NMR spectrum of N-(hydroxyethyl)-cis-5-norbornene-exo-2,3-dicarboximide.

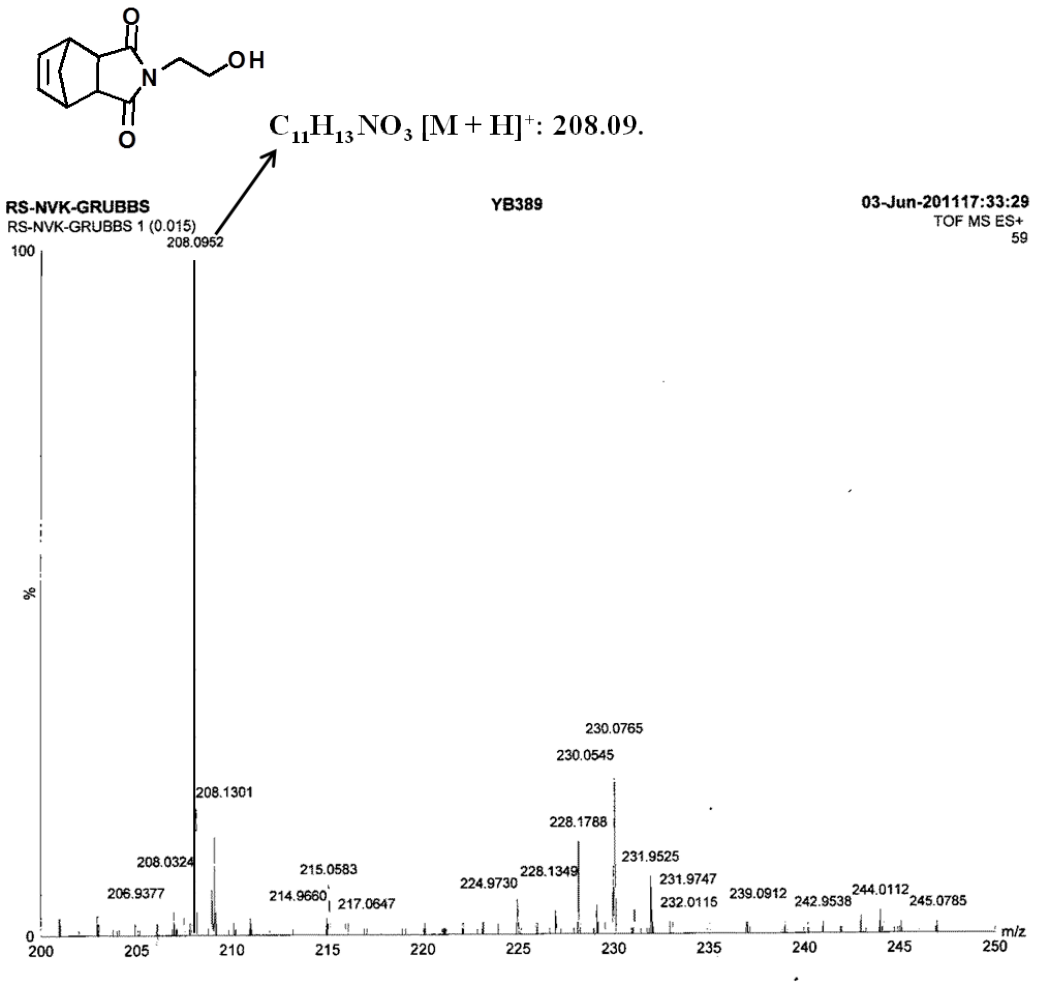


Figure S7. Mass spectrum of N-(hydroxyethyl)-cis-5-norbornene-exo-2,3-dicarboximide.

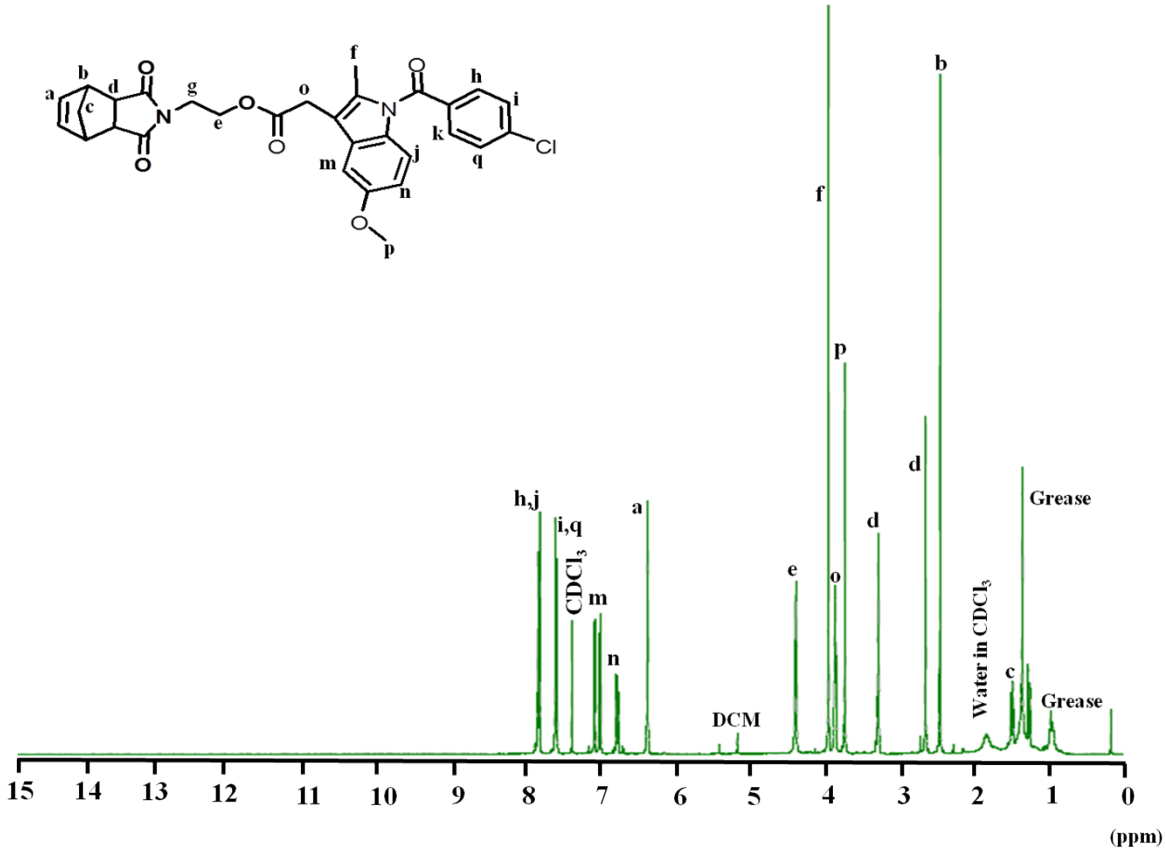


Figure S8. ^{1}H NMR spectrum of **Mono2**.

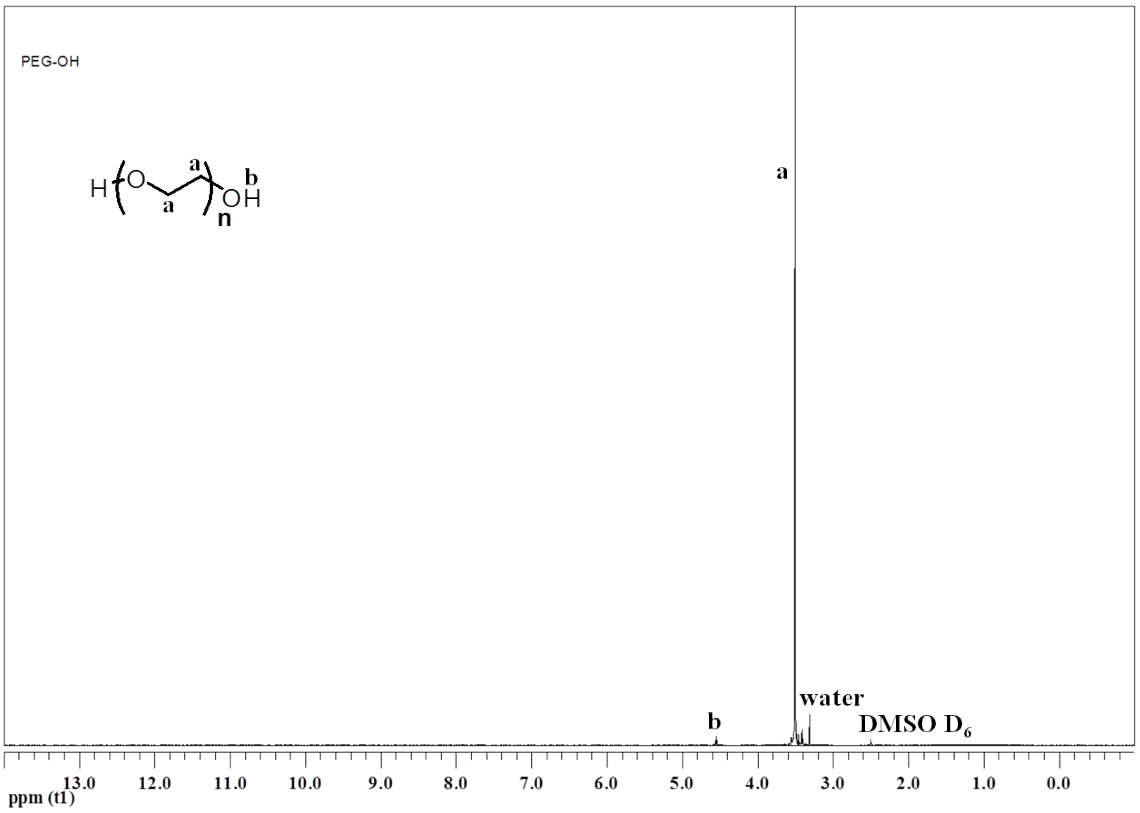


Figure S9. ^{1}H NMR spectrum of poly (ethyleneglycol).

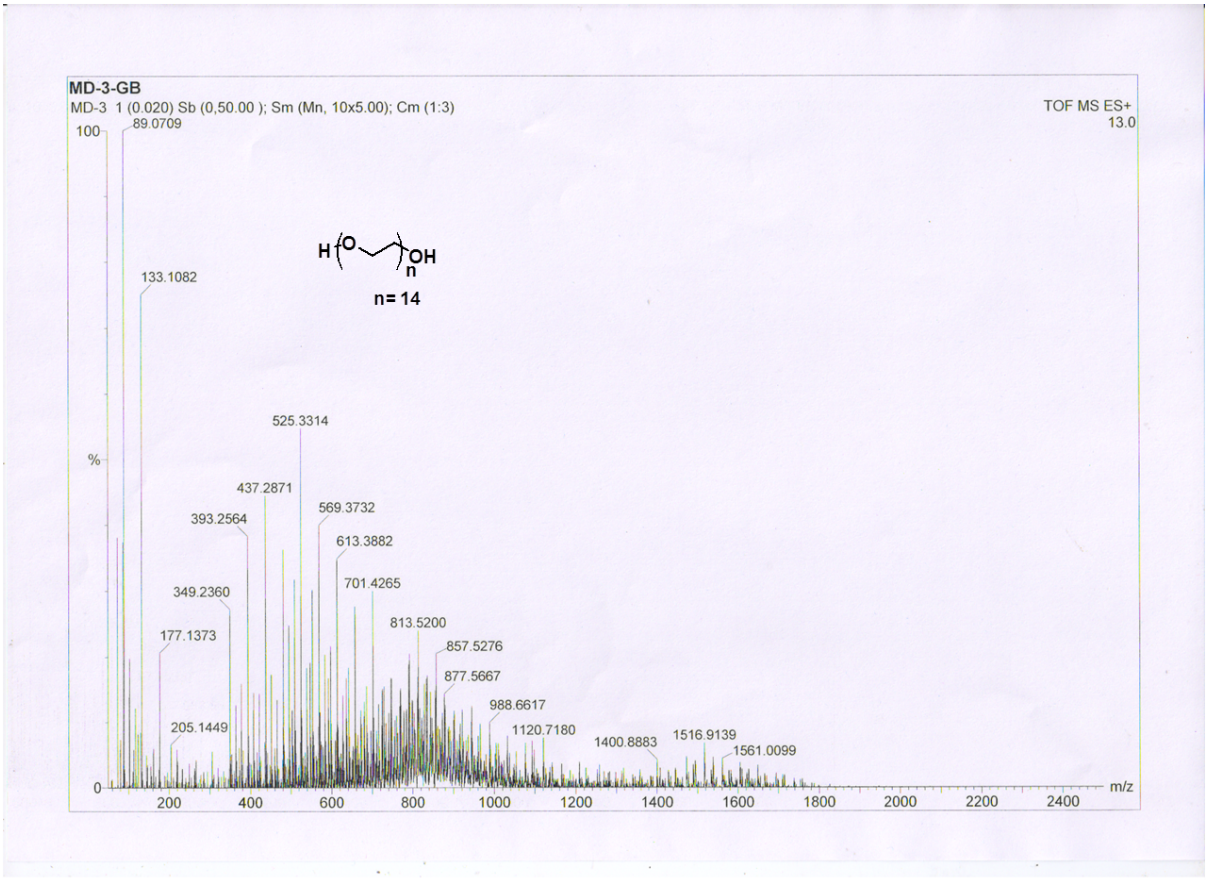


Figure S10. Mass spectrum of poly (ethyleneglycol).

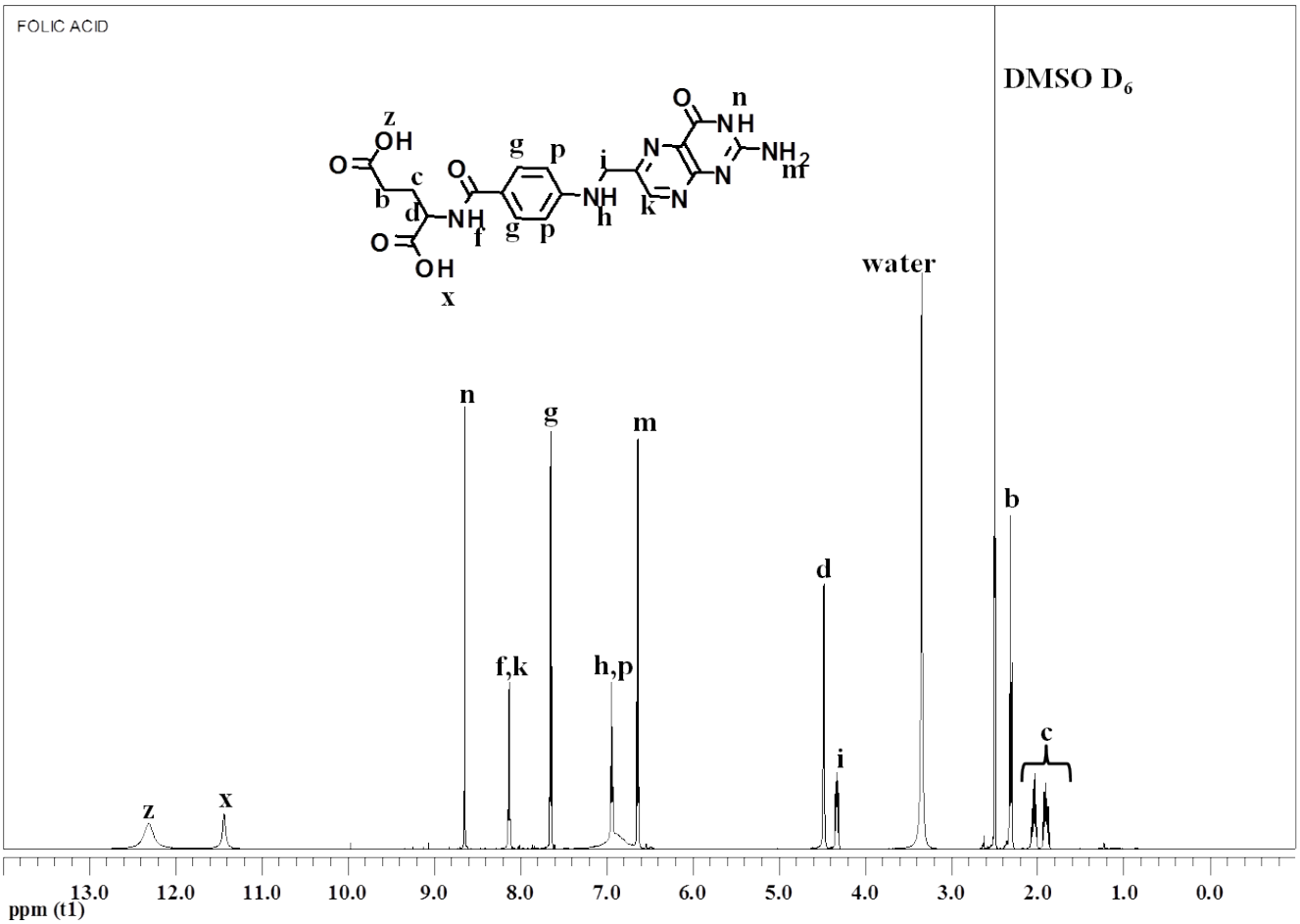


Figure S11. ^{1}H NMR spectrum of folic acid.

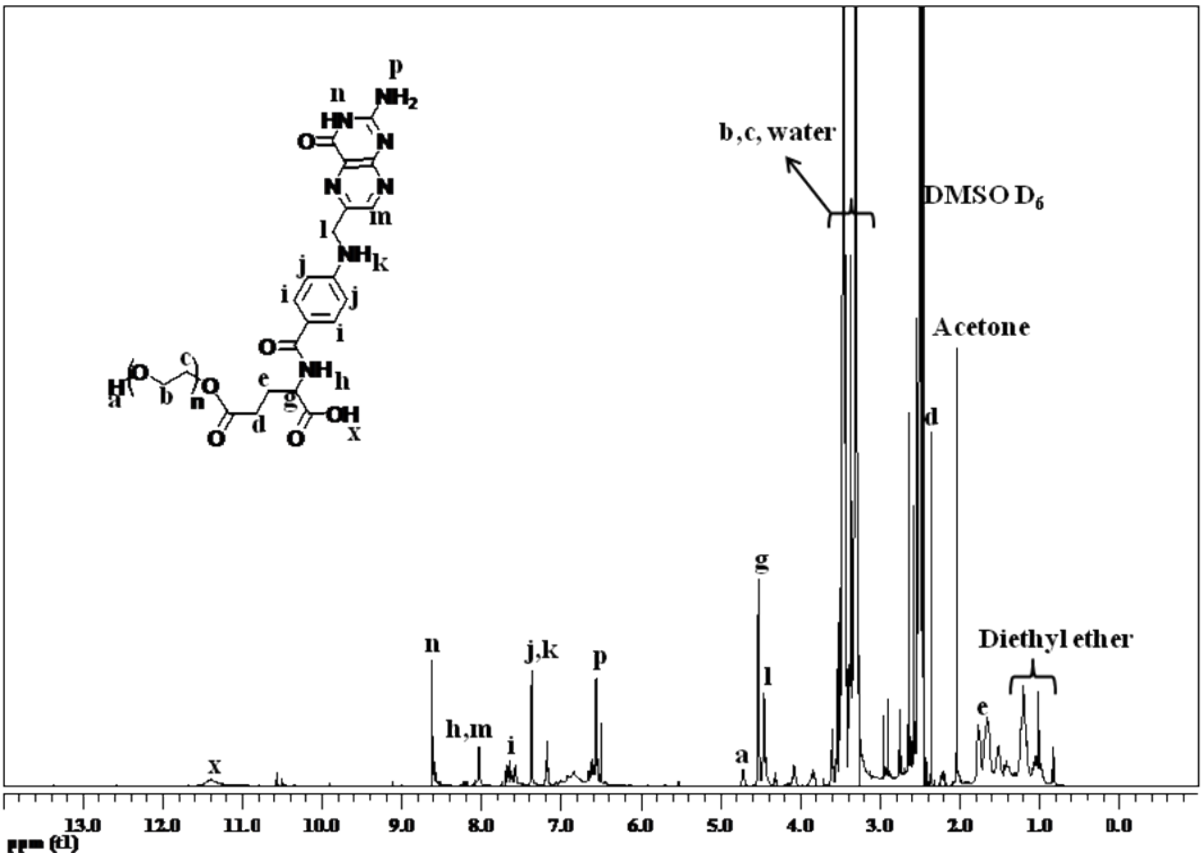


Figure S12. ^{1}H NMR spectrum of poly (ethyleneglycol) grafted-folate **(PEG-FOLATE).**

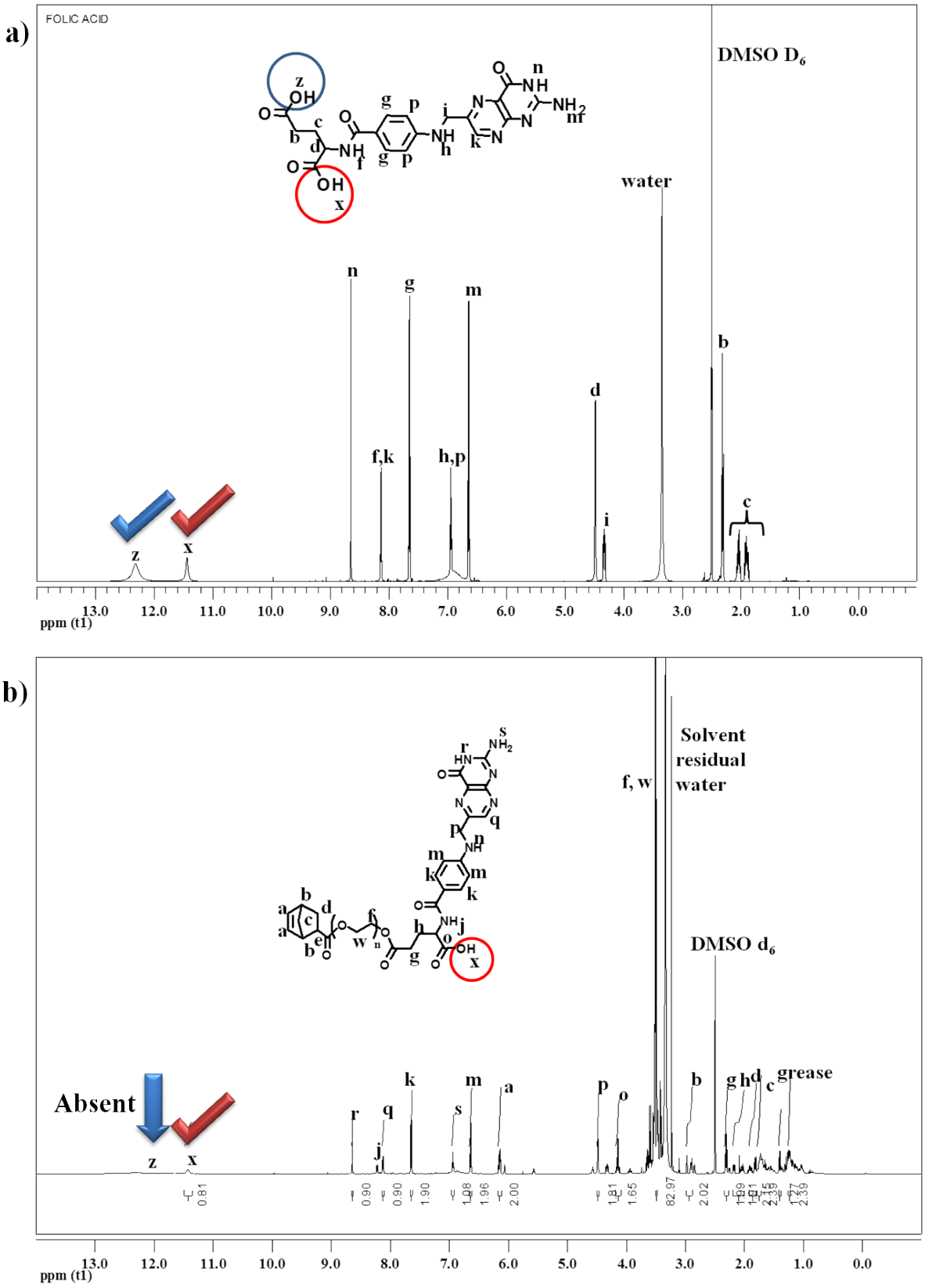


Figure S13. a) ^{1}H NMR spectrum of folic acid. b) ^{1}H NMR spectrum of **Mono3.**

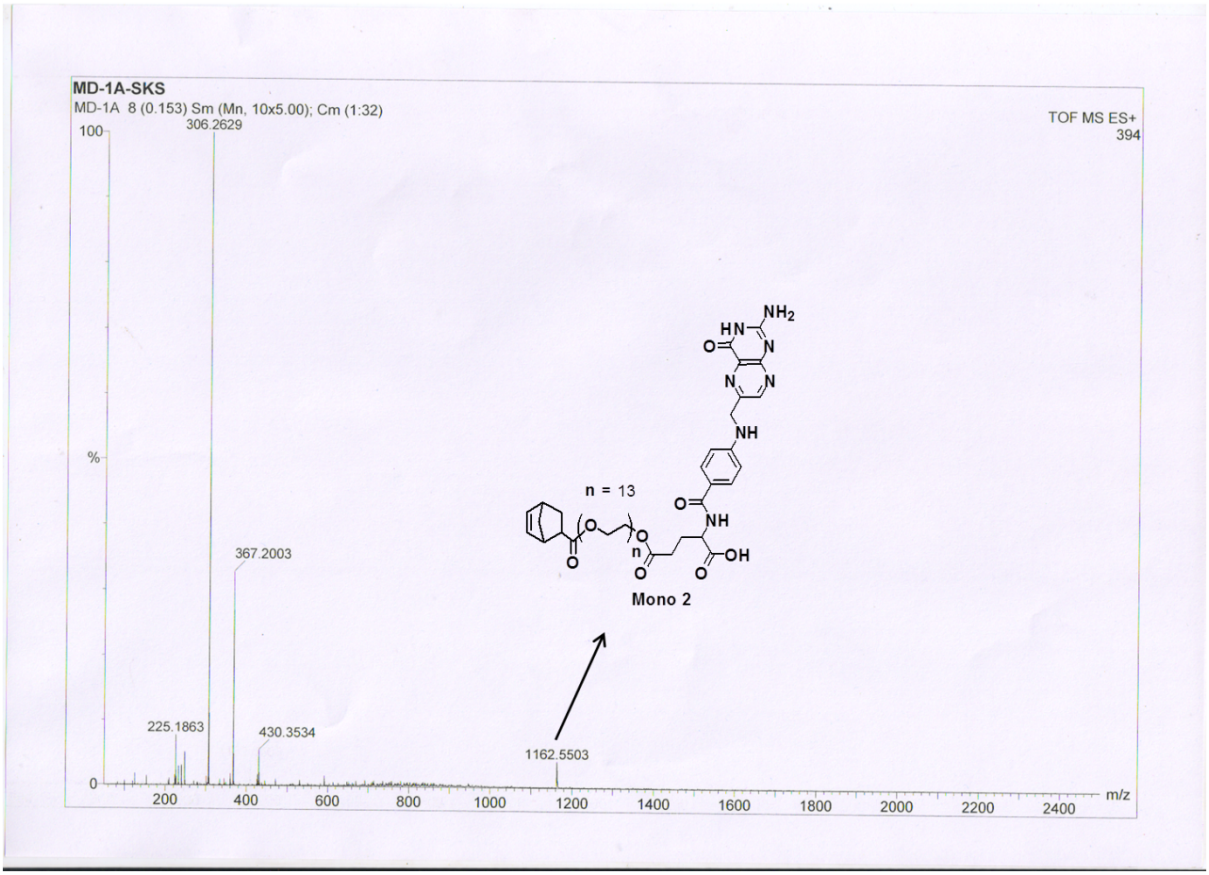


Figure S14. Mass spectrum of norbornene grafted poly(ethyleneglycol)-Folate.

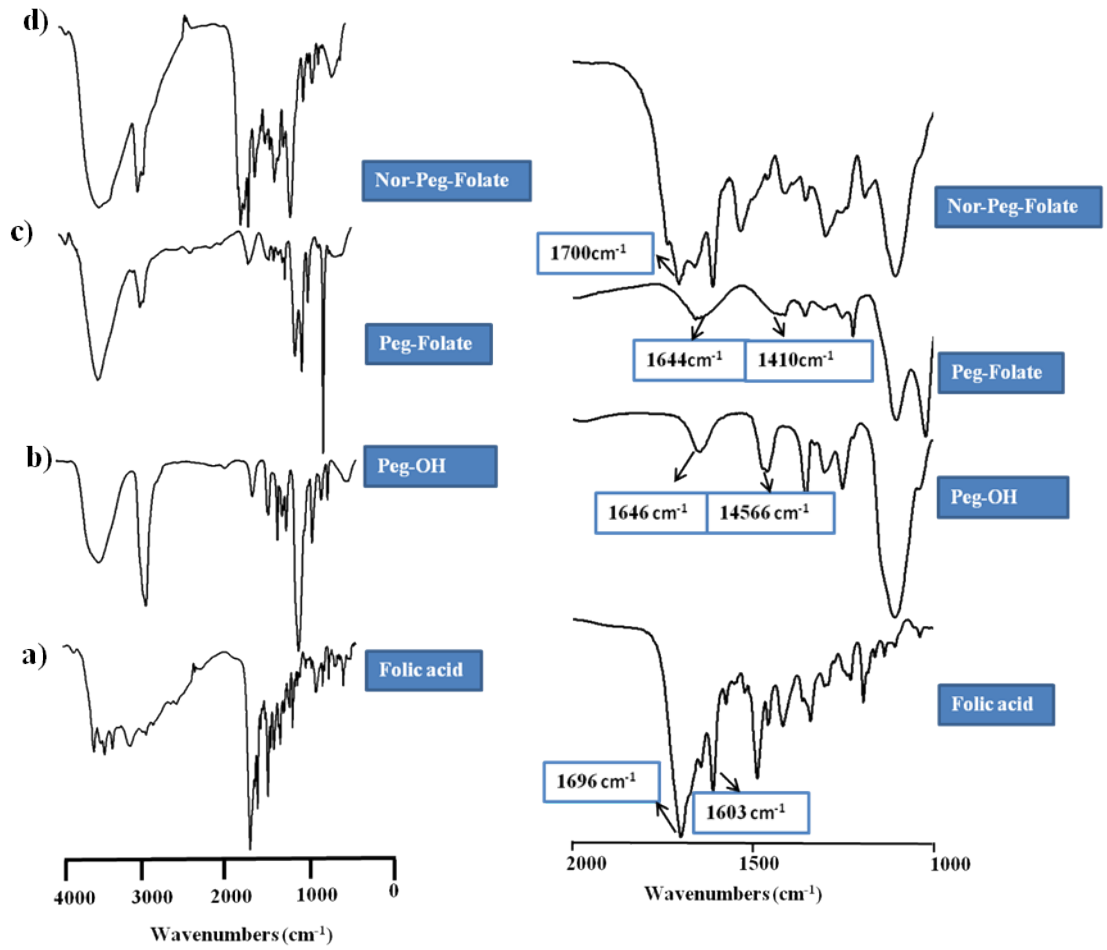


Figure S15. FTIR spectra of 1 (a) Folic acid (b) poly (ethyleneglycol) (c) poly (ethyleneglycol) grafted-folate (PEG-FOLATE) (d) norbornene grafted poly(ethyleneglycol)-Folate.

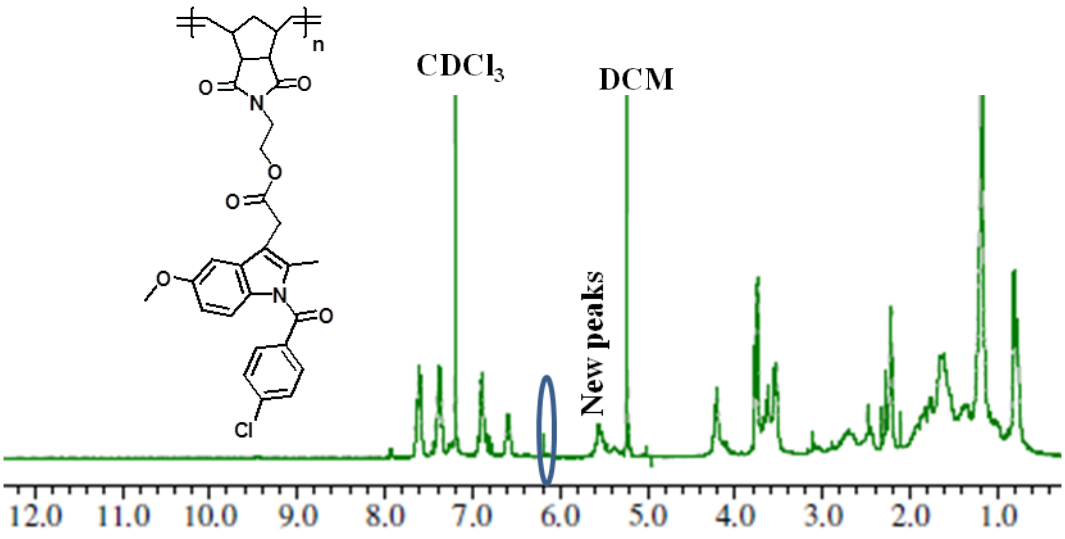
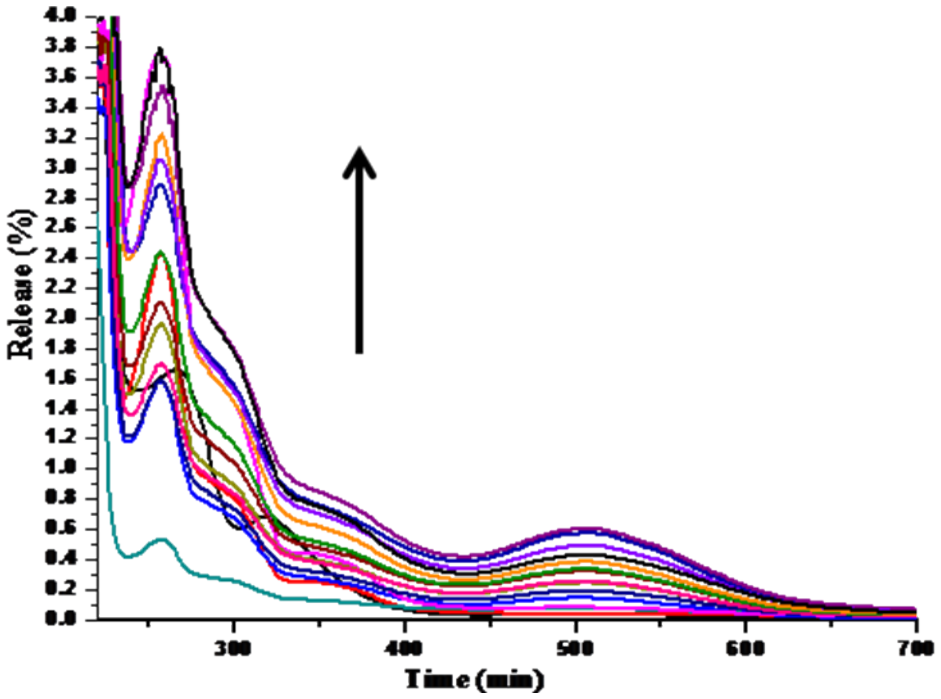


Figure S16. ^{1}H NMR spectrum of homopolymer of **mono 2**



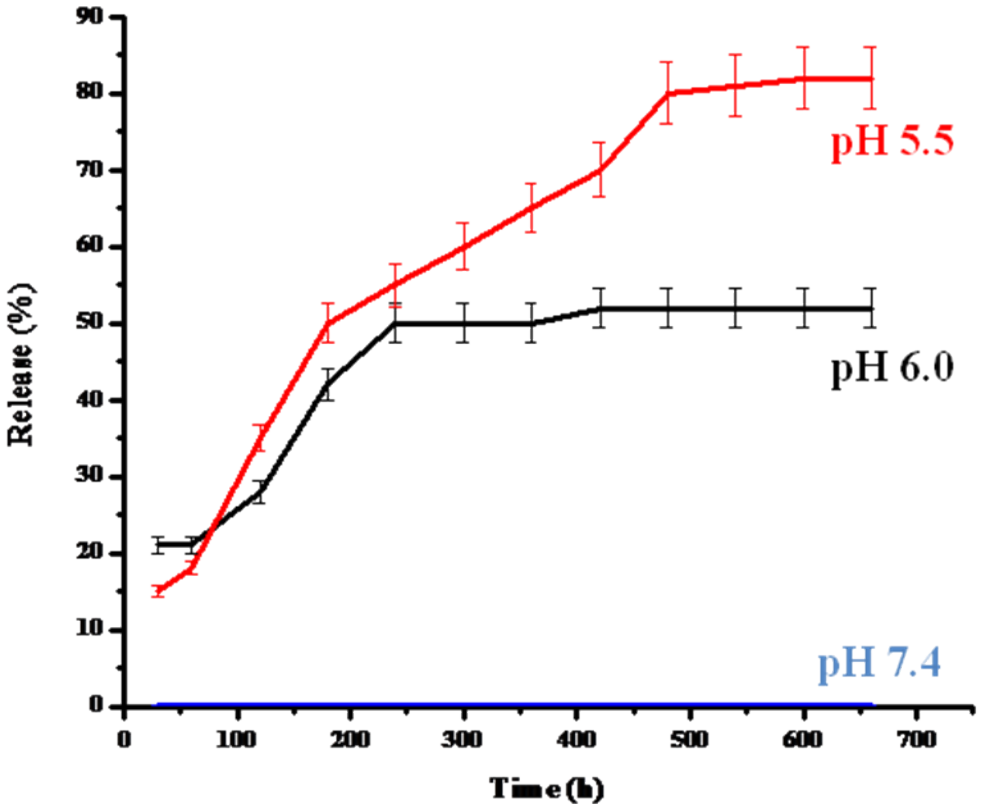


Figure S17. Drug release studies of **DTBCP-1** polymer at different pHs. UV response was measured with varying time intervals up to 700 minutes.

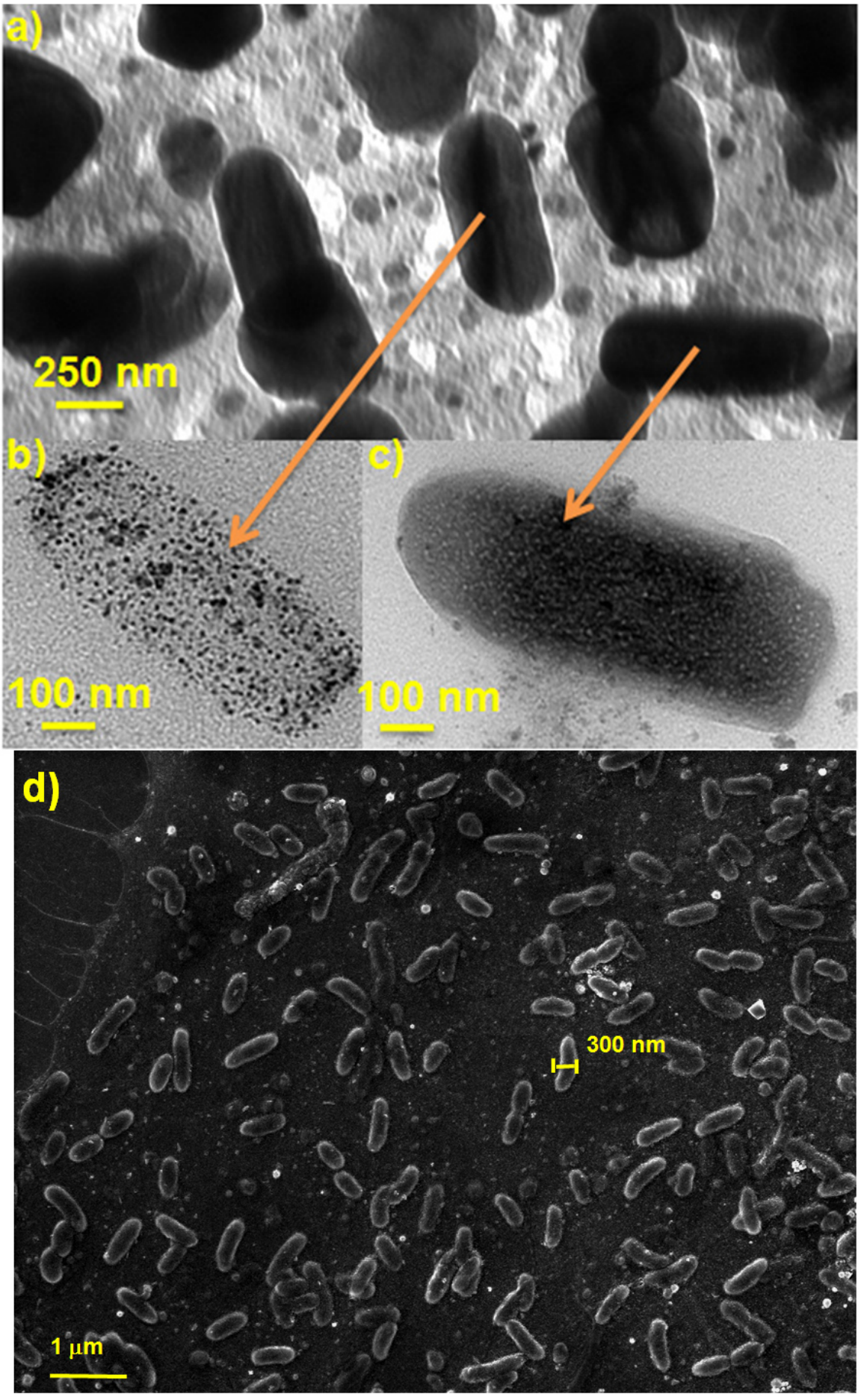

a)
250 nm
b)
100 nm
c)
100 nm
d)
300 nm
1 μm

Figure S18. a) - c) TEM images of **DTBCP-1; d) SEM** clearly shows the uniformity of the observed capsule like morphology.

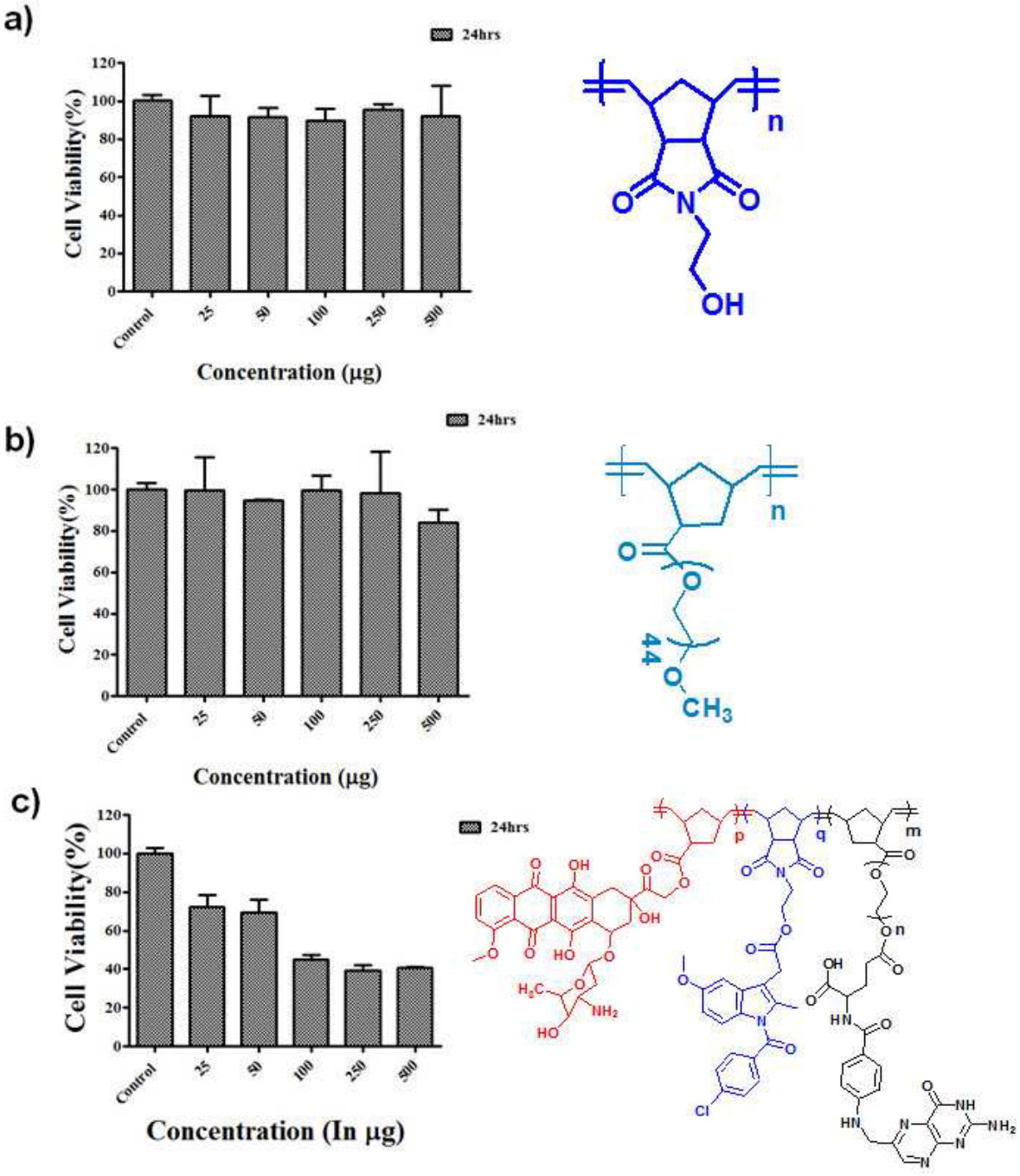
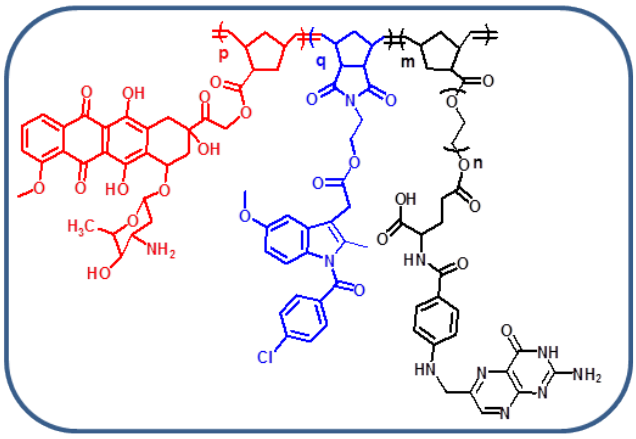


Figure S19. Control cell viability experiment on 4T cells to demonstrate the perceived Increase in the toxicity due to the unique design.



DTBCP (M/I = 10, polymer block ratio targeted), p = q = m = 10
DTBCP (M/I = 15, polymer block ratio targeted), p = q = m = 15
DTBCP (M/I = 20, polymer block ratio targeted), p = q = m = 20
Where M/I = Monomer to Grubbs catalyst ratio

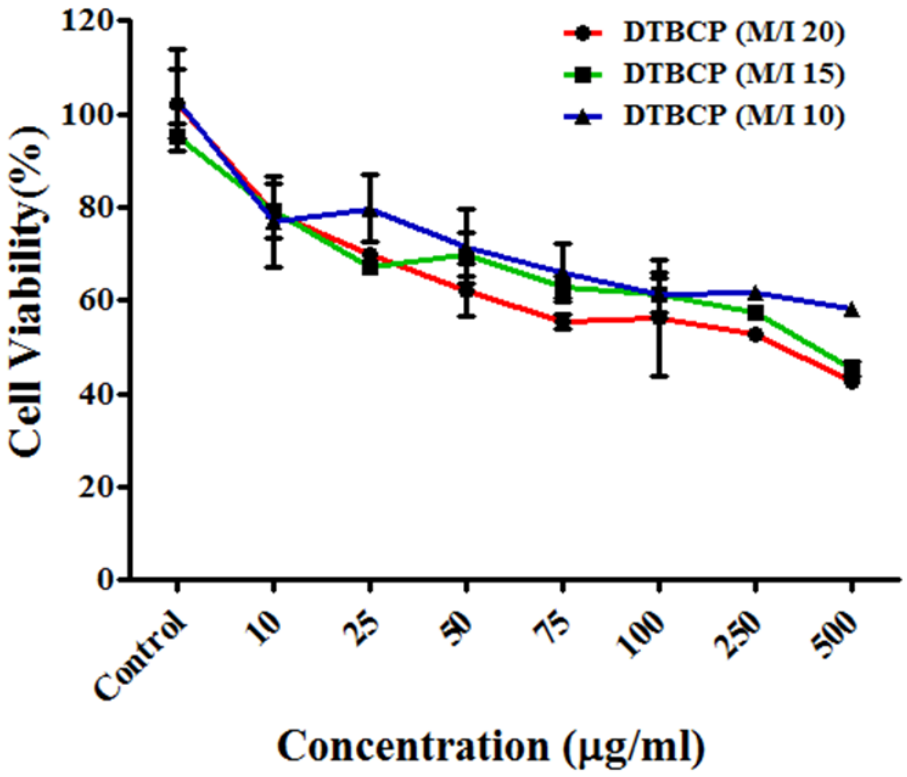
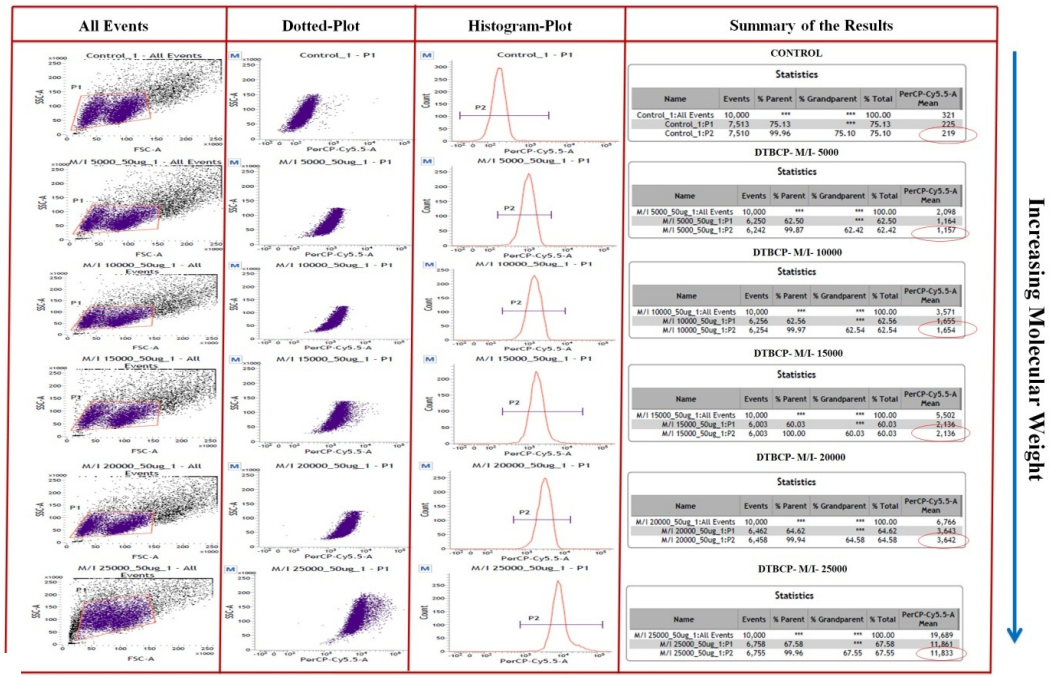


Figure S20. Control cell viability experiment on Hela cells with **DTBCP** with different feed ratios **(M/I = 10, 15, 20)** to demonstrate the perceived Increase in the toxicity due to the unique design.

a)



b)

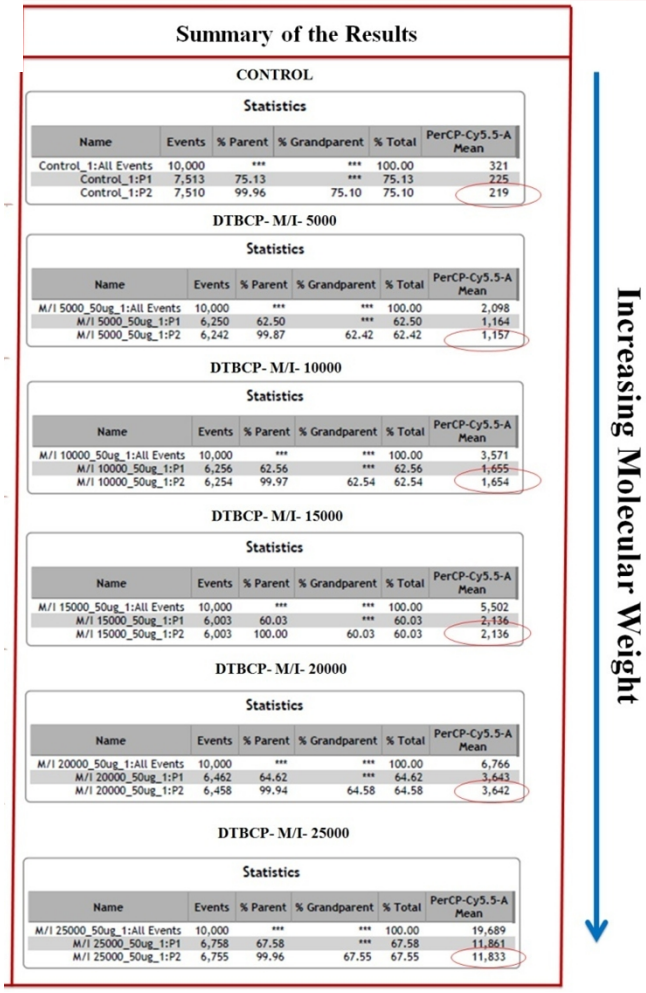


Figure S21. a) Summary of flowcytometry results of **DTBCP-1** molecules having different molecular weight (viz. **M/I- 5000, M/I- 10000, M/I- 15000, M/I- 20000** and **M/I- 25000**) applied on HeLa cells at **50 µg; b)** Zoomed in summary of the results.

Materials and Methods for Flow Cytometery Experiment:

Cells: HeLa cells (human cervical cancer cell line) have maintained in MEM (Minimum Essential Medium) medium containing with 10% fetal bovine serum (FBS), penicillium (100 µg /mL) and Streptomycin (100 µg/mL). The HeLa cells were grown in medium stated above in 5% CO_2 at 37 °C.

Chemicals and Consumables:

Penicillin, streptomycin, and fetal bovine serum (FBS) were purchased from Invitrogen.
MEM was purchased from Gibco, BD FACS Verse.
35mm cell culture plate plates are from Nunc
Cells scrapers are purchased from Corning Incorporated.

MTT-

For MTT assay 1.25×10^4 cells/0.2 mL were seeded into 96 well plate and incubated for 24 hrs in 5 % CO_2 at 37 °C. A wide range of concentration (5, 25, 50, 75, 100, 250 and 500 µg/mL) of the drug was used for MTT assay.

Viability of the cell was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) Assay. A fresh 20 µL solution of MTT from 5 mg/mL stock solution was added to each well, followed by incubation for 4 hrs at 37 °C. After 4hrs, medium was removed from the wells and 100 µL of DMSO was added to each well and shaken for 15 min, and the absorbance of the resulting solution into the cells was measured at 600 nm by ELISA Plate Reader (Bio Tek Instrument – ELx 800).

Drug preparation

For this experiment, selected dose concentration of the drug to be used for drug internalization assay on cells was 50 µg/mL. For the internalization assay drug was dissolved in MEM media and filtered through 0.22 µm filter.

In order to measure the Mean Fluorescence Intensity (MFI) of **DTBCP** polymer having different molecular weight was used for flow cytometery.
HeLa cells were seeded in 35 mm cultural dishes (1×10^6 cells/2 mL). After the cells reached 90% confluency, **DTBCP** having different molecular weight (50µg/ml and 100 µg/mL of

each drug) were applied to dishes and incubated for 24 hrs. After 24 hrs incubation cells were washed by 1X PBS (Phosphate buffered saline). Then the cells were dislodged and collected by the cell scraper (Corning) and cells were centrifuged and re-suspended in Buffer (1X PBS). Then 10^5 cells in 500µL of cells suspension were distributed in each flow tubes for flow analysis.

Since, the nanocarrier was having fluorescence property in red spectrum due to DOX, here the **DTBCP** molecule was excited by blue laser at 488nm and emission was detected by PerCP-Cy5.5 (Red) channel within the bandwidth of 673-727nm.

In order to analyze the FACS results, in SSC (Side Scatter Channel) Vs FSC (Forward Scatter Channel) dot plot, 'P1' gate was put to sort out the desired cell population for further analysis. In the very next SSC Vs PerCP-Cy5.5 dot plot, the P1 gate was applied and subsequently in PerCP-Cy5.5 histogram as well. 'P2' gate was put in the histogram to get the Mean Fluorescence Intensity of that initially gated cell population i.e., 'P1'.

Control tube exerted MFI value of 219 due to only the primary PMT voltage applied. Subsequently, **DTBCP** molecules having different molecular weight (viz. **M/I- 5000, M/I-10000, M/I- 15000, M/I- 20000** and **M/I- 25000**) applied on HeLa cells at **50 µg** concentrations and each tube exerted MFI value in increased order with ascending order of the molecular weight which was clearly shown in the flowcytometry result. All these tubes exerted high MFI value and true positive signal due to fluorescence emission.

Note- FSC voltage-79.7mv, SSC voltage- 290, PerCP-Cy5.5 voltage- 443.1, Thresold of FSC- 10,000.

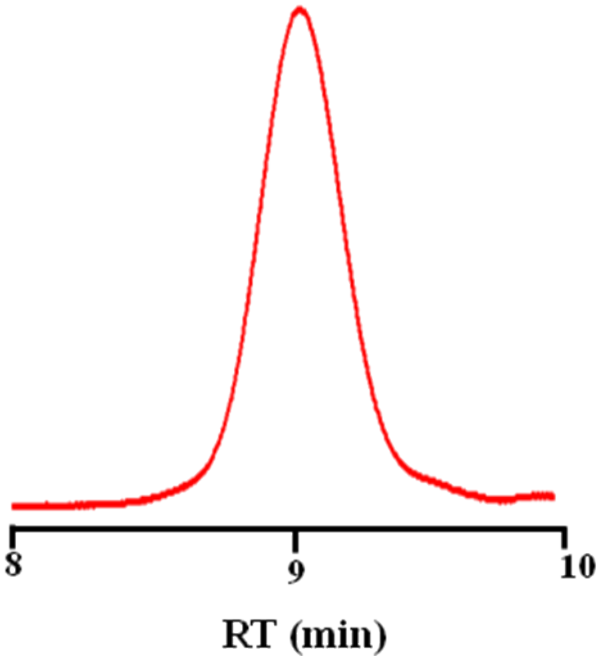


Figure S22. DMF GPC analysis of macroinitiator 1 (M_n = 10 kDa; PDI = 1.2).

References

1. C. C. Ver Nooy and C. S. Rondestvedt, *J. Am. Chem. Soc*., 1955, **77**, 3583.
2. (a) S. Dhar, Z. Liu, J. Thomale, H. Dai and S. J. Lippard, *J. Am. Chem. Soc.,* 2008, **130**, 11467; (b) J. H. Van Steenis, E. M. van Maarseveen, F. J. Verbaan, R. Verrijk, D. J. A. Crommelin, G. Storm and W. E. Hennink, *J. Control. Release.,*2003, **87**, 167; (c) W. L. Zhang, N. Li, J. Huang, S. F. Luo, M. X. Fan, S.Y. Liu, B. Muir and J.H. Yu, *J. Appl. Polym. Sci.* 2011**, 121**, 3175; (d) S. L. Kim, H. J. Jeong, E. M. Kim, C.M. Lee, T.H Kwon and M. H. Sohn, *J. Korean. Med. Sci.* 2007, **22**, 405.