ELECTRONIC SUPPORTING INFORMATION

Redox Responsive Efficient DNA and Drug Co-release from Micelleplexes Formed from Fluorescent Cationic Amphiphilic Polymer

Satyagopal Sahoo, Shibayan Kayal, Puja Poddar and Dibakar Dhara*
Department of Chemistry,
Indian Institute of Technology Kharagpur,
West Bengal 721302, India
**Email: dibakar@chem.iitkgp.ac.in*

EXPERIMANTAL SECTION

Synthesis of 2-(Methacryloyl)oxyethyl-2'-hydroxyethyl disulfide (MAOHD). MAOHD was synthesized following a reported procedure. Briefly, triethyl amine (TEA, 2.19 mL, 14.33 mmol) was added dropwise into a solution of BHEDS (4.00 g, 26.7 mmol) in 50 mL dry THF at 0 °C, and the resulting solution was stirred for 30 min. Methacryloyl chloride (1.33 mL, 14.33 mmol) in dry THF was added dropwise into the solution at 0 °C for 30 min. The mixture was stirred for 18 h at room temperature. After filtration, the solution was washed using 0.3 M HCl solution, followed by saturated $NaHCO_3$ solution and water. The organic layer was concentrated by rotary evaporation to obtain crude product which was further purified by silica gel column chromatography (ethyl acetate : hexane = 10 : 90 v/v) to obtain the final product (1.7 g, yield: 58%). The ^{1}H NMR and ^{13}C-NMR spectrum of MAOHD are shown in Figure S1 and S2 in Supporting Information. ^{1}H-NMR: δ (400 MHz, $CDCl_3$, TMS, ppm): 5.62, 6.14 (CH_2=CCH_3, s,

2H), 4.43 (CH_2CH_2OH, t, 2H), 3.90 ($COOCH_2$, t, 2H), 2.98 CH_2CH_2OH, t, 2H), 2.89 ($COOCH_2CH_2$, t, 2H), 1.95 ($CH2=CCH_3$, s, 3H). ^{13}C-NMR: δ (400MHz, $CDCl_3$, TMS, ppm): 167.5, 136.2, 126.3, 62.8, 60.4, 41.9, 37.2, 18.5.

Synthesis of 2-(Methacryloyl)oxyethyl-2'-hydroxyethyldisulfide cholate (MAODCA) monomer (Scheme 1). MAODCA was synthesized by coupling between cholic acid (CA) and MAOHD in the presence of DCC and DMAP (Scheme 1). To a solution of CA (5.066 g, 12.3 mmol) in 60 mL dry THF, DMAP (0.37 g, 3.02 mmol) and MAOHD (3.0 g, 12.6 mmol) were added under a N_2 atmosphere. After 30 min stirring, DCC (3.3 g, 16.0 mmol) in 15 mL THF was added dropwise to the reaction mixture under stirring at 0 °C. The reaction mixture was brought into room temperature and stirred for 1 day. After filtration to separate out insoluble N,N′-dicyclohexylurea (DCU), the filtrate was concentrated by rotary evaporation and then dissolved in ethyl acetate. After that, it was washed successively with 0.1 N HCl, saturated $NaHCO_3$ and brine solution. The organic layer was dried over anhydrous $MgSO_4$ and purified by column chromatography (ethyl acetate : hexane = 60 : 40 v/v) to obtain the final product with a yield of 52%. ^{1}H-NMR: δ (400 MHz, $CDCl_3$, TMS, ppm): 6.13 and 5.59 (2H, s, 1-H), 4.41 (2H, t, c-H), 4.33 (2H, t, d-H), 3.97 (1H, s, 7'-H),11 3.85 (1H, s, 12'-H), 3.45 (1H, s, 3'-H), 2.95(4H, m, e & f–H), 1.95 (3H, s, 3-H), 0.98 (3H, d, 21'-H), 0.89 (3H, s, 19,-H) and 0.68 (3H, s, 18'-H). ^{13}C-NMR: δ (400 MHz, $CDCl_3$, TMS, ppm): 174.07, 166.88, 135.86, 126.23, 72.66, 71.50, 67.70, 46.68, 46.11, 41.81, 41.60, 39.64, 39.08, 36.87, 36.76, 35.34, 35.10, 34.47, 30.93, 30.60, 29.79, 28.19, 27.23, 26.48, 22.82, 21.76, 18.09, 16.24, 11.59.

Synthesis of fluorescent 2-(methacryloyloxy)ethyl-1-pyrenebutyrate (HEA-PBA) (Scheme 1). HEA-PBA was synthesized by a coupling reaction of 1-pyrenebutyric acid (PBA) with HEA in the presence of DCC and DMAP. PBA (1.0 g, 3.46 mmol), DMAP (0.0485 g, 0.692 mmol) and

HEA (0.405 g, 3.48 mmol) were dissolved in 15 mL dry DCM under N_2 atmosphere. The reaction flask was kept in an ice-water bath and to this solution DCC (0.745 g, 3.61 mmol) in 5 mL DCM was added dropwise for 30 min. It was then stirred for 18 h at room temperature. Then it was filtered to separate out insoluble N,N′-dicyclohexylurea (DCU) and the filtrate was washed successively with 0.1 N HCl, saturated $NaHCO_3$ and brine solution and dried over anhydrous $MgSO_4$. The solvent was removed on a rotary evaporator and the resulting crude was purified by column chromatography using ethyl acetate/hexanes (1: 10) as the eluting solvent to give 6.2 g of pure pale yellow solid (yield: 51%). ^{1}H-NMR: δ (400 MHz, $CDCl_3$, TMS, ppm): 7.24-8.32 (9H,m, pyrene moiety), 6.42, 6.14 and 5.81 (3H, m, b, a & c- H), 4.36 (4H, m, d-H), 3.40(2H, t, e-H), 2.50(2H, t, g-H), 2.21(2H, m, f-H). ^{13}C-NMR: δ (400 MHz, $CDCl_3$, TMS, ppm): 173.39, 166.05, 135.74, 131.56, 131.04, 130.15, 128.90, 128.09, 127.63, 127.58, 127.50, 126.90, 126.01, 125.25, 125.14, 125.09, 124.95, 124.42, 62.45, 62.24, 33.81, 32.83, 31.05, 26.84, 26.33, 24.72.

METHODS

NMR Spectroscopy. ^{1}H and ^{13}C NMR spectra were recorded using Bruker DPX-400 MHz NMR spectrometer. Spectra were calibrated using signal of residual solvent as the internal standard.

*Gel Permeation Chromatography (GPC)***.** GPC analysis provided the number-average (M_n), weight-average molecular weight (M_w) and dispersity ($Đ = M_w/M_n$) of the linear polymer and copolymers. The measurements were done using Viscotek TDAmax system fitted with a Viscotek TDA 305 detector system which is comprised of a number of detectors including refractive index detector, differential pressure viscometer detector, and dual-angle light scattering (λ = 670 nm, RALS operated at 90° and LALS operated at 7°) detectors plumbed in series. Additionally, a Viscotek UV Detector 2600 (setting λ_{abs} = 342 nm) was externally added

with the detector system of the instrument. The light scattering detector was calibrated using a polystyrene standard having narrow molecular weight distribution (M_n = 105164, M_w/M_n = 1.02, [η] = 0.48 dL g^{-1} at 33 °C in THF, dn/dc = 0.185 mL g^{-1}) provided by the supplier Viscotek. The instrument operated with an Agilent 1200 model isocratic pump, measurements were carried out using HPLC grade tetrahydrofuran (THF) as mobile phase with a flow rate of 1 mL/min at 33 °C.

UV-visible Spectroscopy: The weight % of pyrene monomer present in the pyrene containing polymer was determined using Cary 5000 UV-vis-NIR spectrophotometer. We took a known concentration of pyrene containing polymer and determined the absorbance at λ_{abs} = 342 nm (pyrene absorption) compared to a pyrene free PDMAEMA polymer at the same concentration. Comparing the difference in absorption to a pyrene calibration plot we calculated the total amount of pyrene present in the copolymer.

Fluorescence Spectroscopic Studies. Steady-state fluorescence spectra were taken in RF-6000 spectrofluorophotometer provided by SHIMADZU. For *CMC* determination by pyrene fluorescence and by external dye Nile red, the excitation wavelengths were 339 nm and 480 nm respectively. The excitation slit and emission slit were fixed at 10 nm and 5 nm respectively. For the ethidium bromide (EB) dye exclusion assay, and the DNA−EB complex/micelles were excited at 480 nm and the emission spectra were recorded from 500 -700 nm wavelength.

Dynamic Light Scattering (DLS) Measurements. The size of the prepared micelles, DNA/PDMAEMA and DNA/micelles complexes were measured by dynamic light scattering measurements at two pH - pH 4.2 and pH 7.4 using a Malvern Nano ZS instrument with a thermostated sample chamber employing a 4 mW He−Ne laser operating at a wavelength of 632.8 nm and an avalanche photodiode (APD) detector. After addition of micelles or polymer

into the solution of DNA in a cuvette, mixture was kept for 15 min for equilibration. Autocorrelation functions were deconvoluted using CONTIN software.

Zeta (ζ) Potential Measurements: Zeta potential of the prepared micelles, DNA/PDMAEMA and DNA/micelles complexes prepared in phosphate buffer (pH = 4.2 and 7.4) were determined using a Malvern Nano ZS instrument fitted with a 15 mV solid-state laser running at a wavelength of 635 nm.

Transmission Electron Microscopy (TEM). The size of the self-assembled micelles were also measured using a TEM instrument (JEOL-JEM 2100, Japan) running at a voltage of 80 kV. A drop of micellar solution was dripped onto the carbon-coated copper grid and then it was air-dried overnight naturally. The morphology of the micellar system was performed in TEM instrument at room temperature.

AFM Measurement. AFM images of complexes were recorded using an Agilent 5500 microscope. The contact mode in air had been used for measurements on mica. The typical scanning rate was less than 2 Hz to acquire high quality tracing of the surface morphology. A drop of the complex solution prepared at N/P ratio 1.0 was dripped on the mica and the sample was air-dried for overnight. For the study of disassembly of the redox-responsive complexes, the solution was treated with GSH for 48 h before dripping on the mica. The morphologies were further examined using Agilent PicoView software.

Agarose Gel Electrophoresis. The DNA binding capability of linear PDMAEMA and cationic micelles was inspected by gel electrophoresis using 0.8% agarose gel containing ethidium bromide (1 μg/mL). The complexes formed at different N/P ratios were injected into the wells of the gel. The gel running buffer contained 40 mM tris acetate (pH 7.4 and pH 4.2) and 1 mM EDTA. For the study of disassembly of the redox-responsive complexes, the solution was

injected into wells after 48 h treatment of GSH. The electrophoresis were carried out at 80 V for 35 min, after which the images of migrated DNA was captured on a UV transilluminator (254 nm).

Ethidium bromide (EB) exclusion assays. For the measurements of fluorescence of complexes, the solution was excited at 480 nm and the emission spectra were taken in the range of 500 -700 nm. Two separate solutions of DNA and EB was mixed (1 EB:1 bp) at both pH and incubated for 15 min. To this mixture an appropriate volume of micellar solution or PDMAEMA stock solution was added to obtain various N/P ratios ranging from 0 to 3.0 following which fluorescence spectra were recorded.

Preparation of micelleplexes. To prepare complexes of DNA-DOX loaded micelles (micelleplexes) at various N/P ratios, an appropriate volume of micellar solution was mixed with fixed volume of 0.5 µM solution of DNA. After mixing the complexes were allowed to stabilize for 10 min at room temperature.

Determination of critical micellar concentration (CMC) of pyrene-labeled copolymer by fluorescence spectroscopy. Conjugation of pyrene (a hydrophobic fluorescent probe) into the polymer allowed us to measure the *CMC* of pyrene-labeled copolymer without taking help from any external fluorescent dye. Different amounts of the stock solution of copolymer in THF were added into a series of 2 mL eppendorfs. These copolymer solution containing eppendorfs were kept open for 24 h at room temperature for complete evaporation of THF following which 1 ml of buffer solution of pH 4.2 and 7.4 was added to every eppendorf. After incubation of the resulting mixtures for 30 min followed by stabilization for overnight, fluorescence spectra of these solutions were recorded from 360 to 600 nm (excitation wavelength 339 nm). Based on the pyrene emission spectra and an increasing I_{383}/I_{372} i.e., I_3/I_1 with increasing concentration of

copolymer, the *CMC* values were calculated by the crossover point at which I_3/I_1 began to increase rapidly.

To cross check the observed *CMC* values obtained from above, *CMC* of the block copolymers were also determined by using Nile Red as an external fluorescence probe. A measured amount (10 μL) of stock solution of Nile Red prepared in methanol (2.0 mM) was taken in different eppendorf. To each of these eppendorf, varying amounts of copolymer solutions prepared in THF were added and the solvent evaporated. Then, the final volume (2 mL) was adjusted with an appropriate amount of water to get a series of solution with varying polymer concentration (0 to 0.15 mg/ml) in which Nile Red concentration remained constant. After sonication of 5 min, each vial was allowed to stand for 1 h, and emission spectra were recorded at an excitation wavelength of 550 nm while monitoring the emission from 570 to 800 nm. Emission intensity at 620 nm was plotted against concentration of polymer. The observed inflection point of this plot was considered as *CMC*.

In vitro drug loading and release. Doxorubicin (DOX) was loaded to the core of the micelles by the following way: DOX•HCl was first neutralized to DOX by mixing with nearly three times mole ratio of TEA in DMSO. The polymer PDMAEMA-*b*-(PMAODCA-*r*-PPBA) taken in 2 mL of DMSO was mixed with previously prepared DOX/DMSO solution. Afterwards, this mixed solution was dropwise added to 10 mL buffer solution with vigorous stirring for 15 h. The solution was dialyzed against same buffer solution for 2 days to exclude the unloaded DOX and DMSO. The external buffer was changed regularly in 6 h intervals. This DOX-loaded micellar solution was stored at low temperature for further studies. A small part of this DOX-loaded micellar solution was lyophilized and dissolved in DMSO. The solution in DMSO was excited at 480 nm and emission was taken in between 500 to 700 nm. The concentration of loaded DOX

was determined by using a previously made calibration graph of DOX in DMSO. The drug loading capacity (LC) and loading efficiency (LE) were calculated by using the following equations:

$$\mathrm{LC}(\%) = \frac{\text{Mass of DOX loaded in micelles}}{\text{Mass of polymer}} \times 100$$

$$\mathrm{LE}(\%) = \frac{\text{Mass of DOX loaded in micelles}}{\text{Mass of DOX supplied}} \times 100$$

To investigate the reduction responsive *in vitro* release of DOX from the micelles in the presence of 10 mM and 10 μM GSH or in the absence of GSH at both pH, 10 mL of DOX-loaded micellar solution were kept in a dialysis bag (MWCO 3500 Da), which was then dipped in 200 mL of buffer solution with regular shaking. At appropriate time intervals, 5 μL of solution from the dialysis bag was taken out for fluorescence measurements in DMSO and released DOX concentration was calculated from calibration graph.

Determination of reduction responsive DNA release from micelleplexes. To study the reduction responsive release of DNA from the micelleplexes in the presence of 10 mM and 10 μM GSH or in the absence of GSH, 5 mL solution of micelleplexes were treated with GSH of appropriate concentration (or without GSH) with continuous stirring. For the DNA release study through EB exclusion assays and DLS measurements, 1 mL of GSH-treated and untreated sample were taken out at appropriate time intervals. After fluorescence and DLS measurement the solution was returned back to previous solution.

SUPPORTING FIGURES

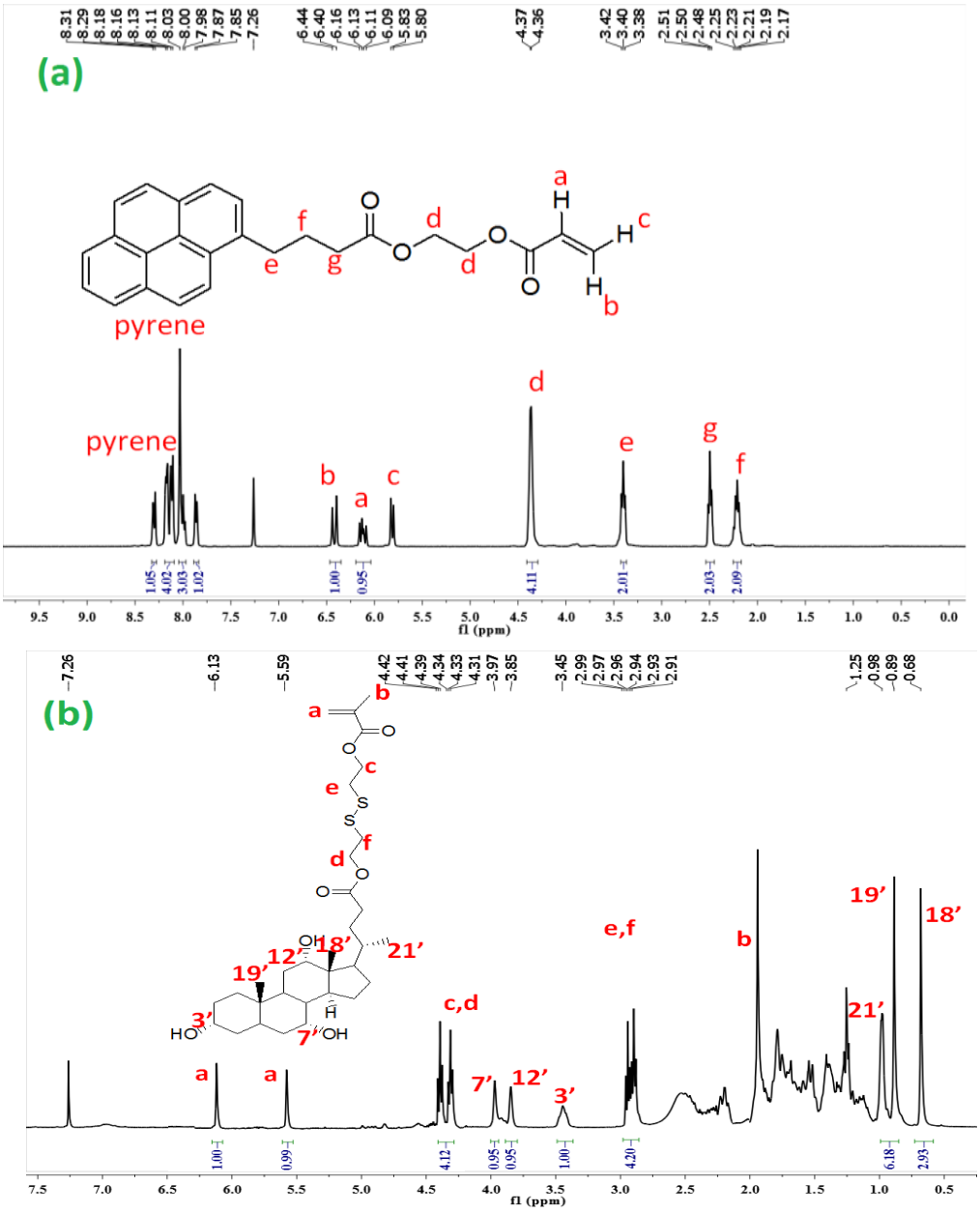

Figure S1: (a) and (b) represent ^{1}HNMR spectra of monomers HEA-PBA and MAODCA in $CDCl_3$.

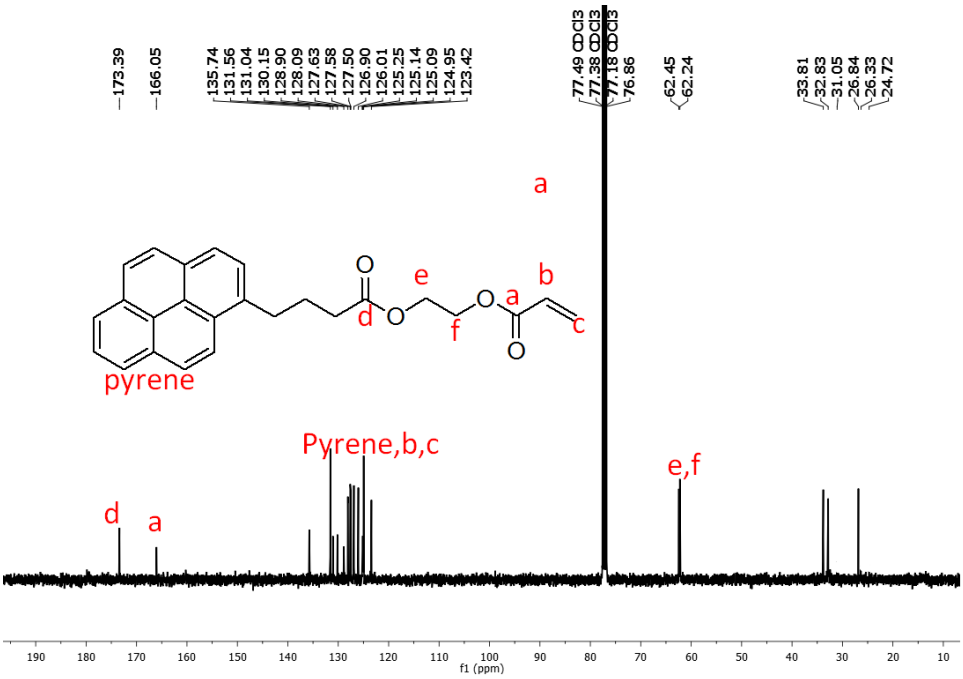

Figure S2: ^{13}C NMR spectra of monomer HEA-PBA in $CDCl_3$ in 400 MHz.

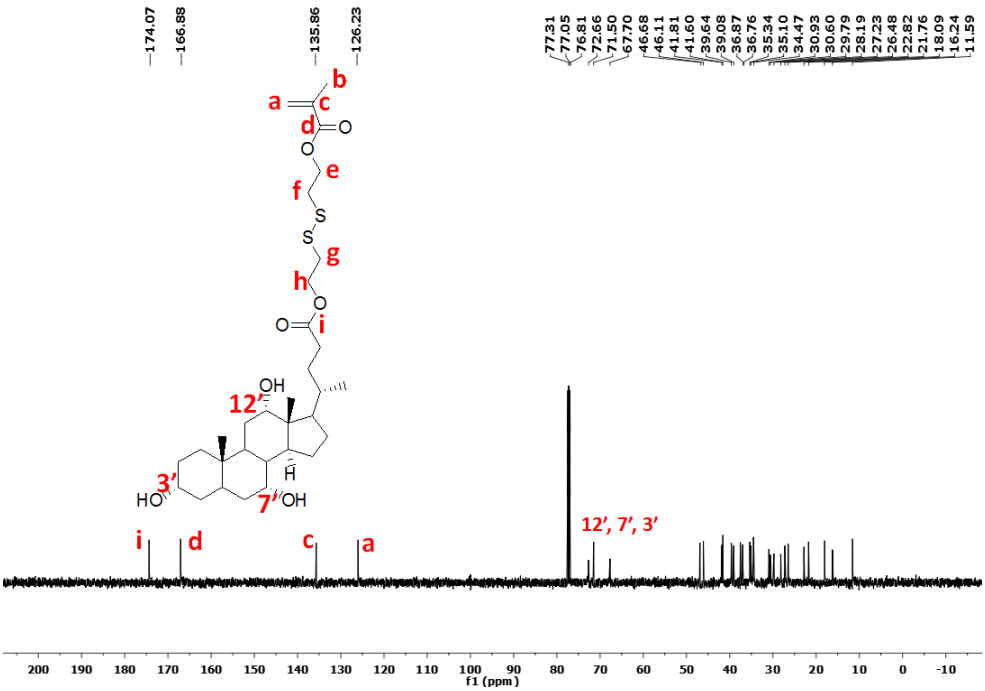

Figure S3: ^{13}C NMR spectra of monomer MAODCA in $CDCl_3$ in 400 MHz.

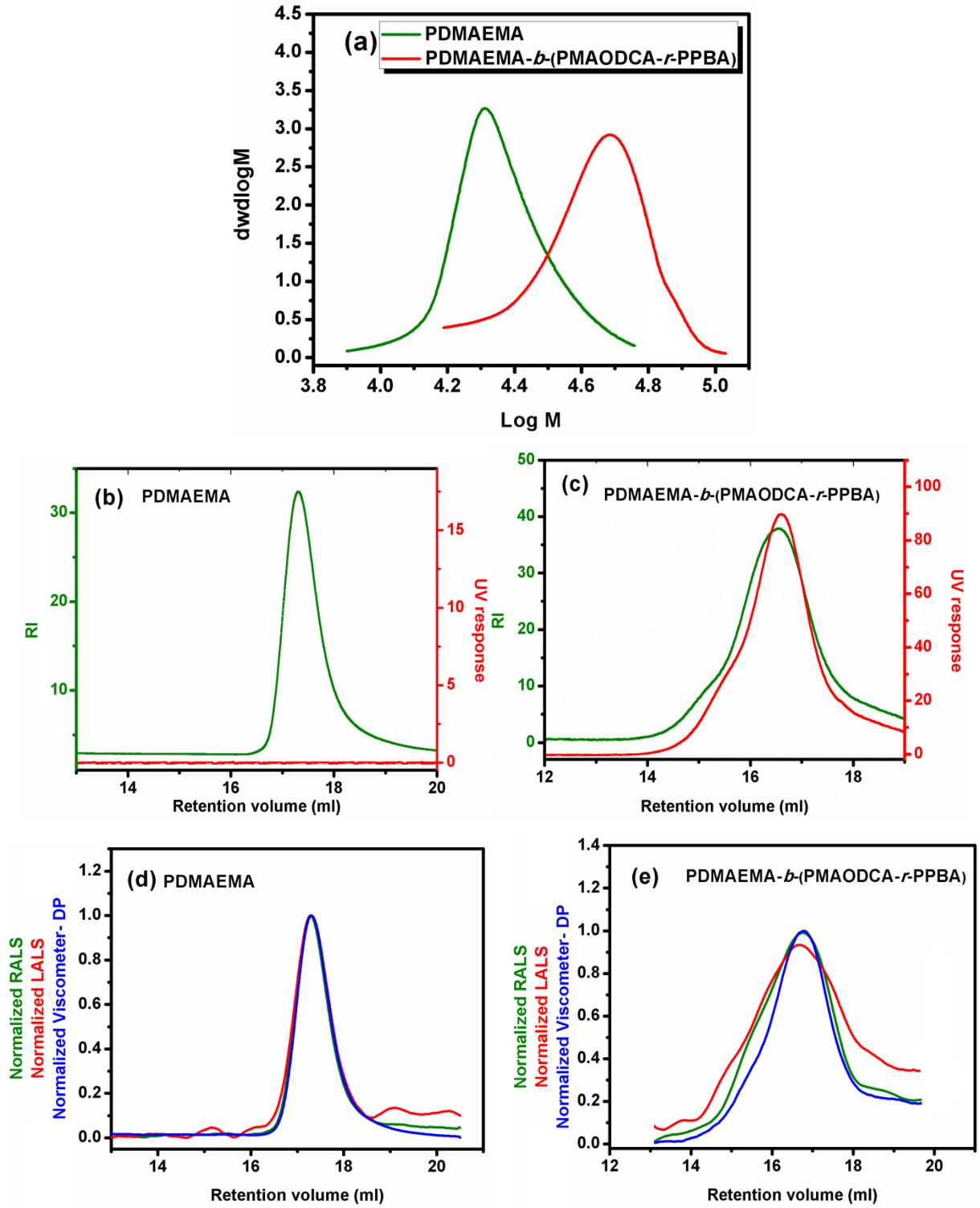

Figure S4: (a) Molecular weight distributions of PDMAEMA homopolymer and PDMAEMA-*b*-(PMAODCA-*r*-PPBA) copolymer. GPC traces involving RI and UV response of (b) PDMAEMA and (c) PDMAEMA-*b*-(PMAODCA-*r*-PPBA). GPC traces involving RALS, LALS and Viscometer-DP of (d) PDMAEMA and (e) PDMAEMA-*b*-(PMAODCA-*r*-PPBA).

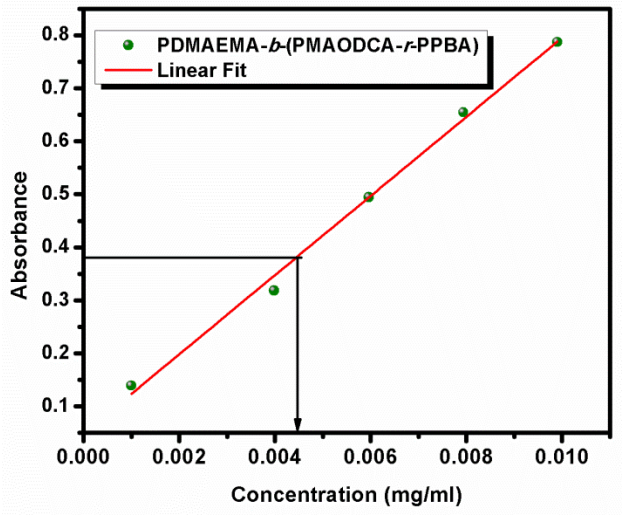

Figure S5: Calibration plot to determine the total amount of pyrene present in the copolymer.

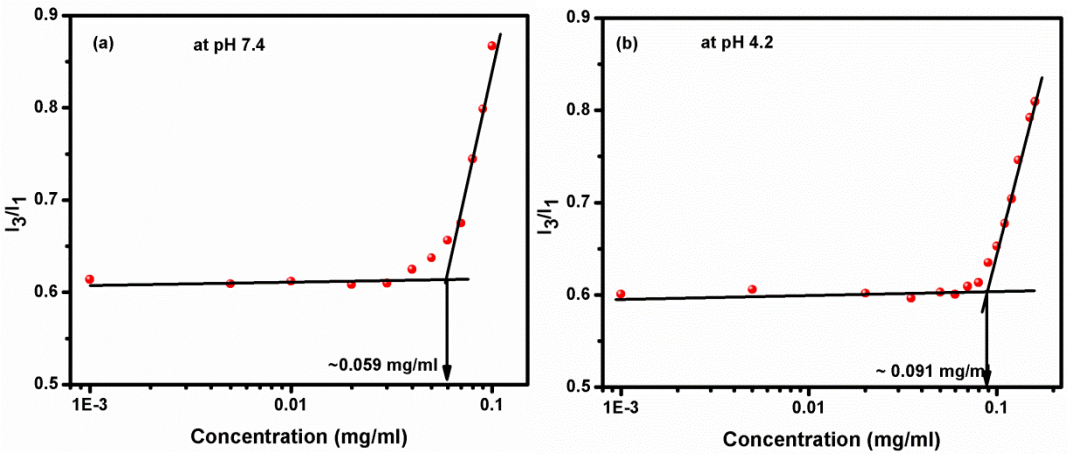

Figure S6: Determination of *CMC* of PDMAEMA-*b*-(PMAODCA-*r*-PPBA) at two solutions pH: (a) pH 7.4 and (b) pH 4.2 using pyrene fluorescence.

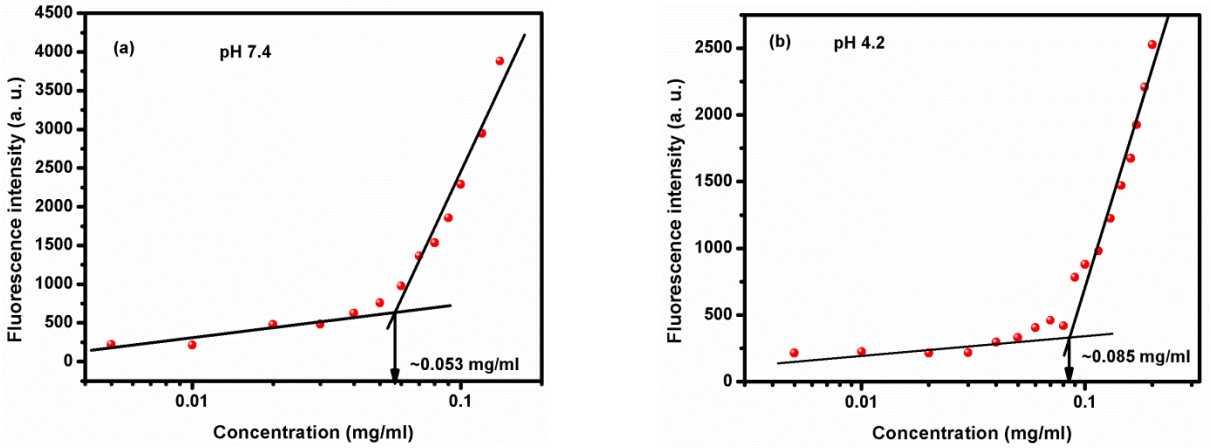

Figure S7: Determination *CMC* of Poly(DMAEMA)-*b*-Poly[(MAODCA)-*r*-(HEA-PBA)] using external dye Nile red in PBS buffer solutions of two pH - (a) pH 7.4 and (b) pH 4.2.

Table S1: *CMC* values of Poly(DMAEMA)-b-Poly[(MAODCA)-r-(HEA-PBA)]using pyrene and external dye Nile red.

pH 7.4 *CMC* (mg/ml)		pH 4.2 *CMC* (mg/ml)	
Using Pyrene fluorescence	Using Nile red fluorescence	Using Pyrene fluorescence	Using Nile red fluorescence
0.059	0.053	0.091	0.085

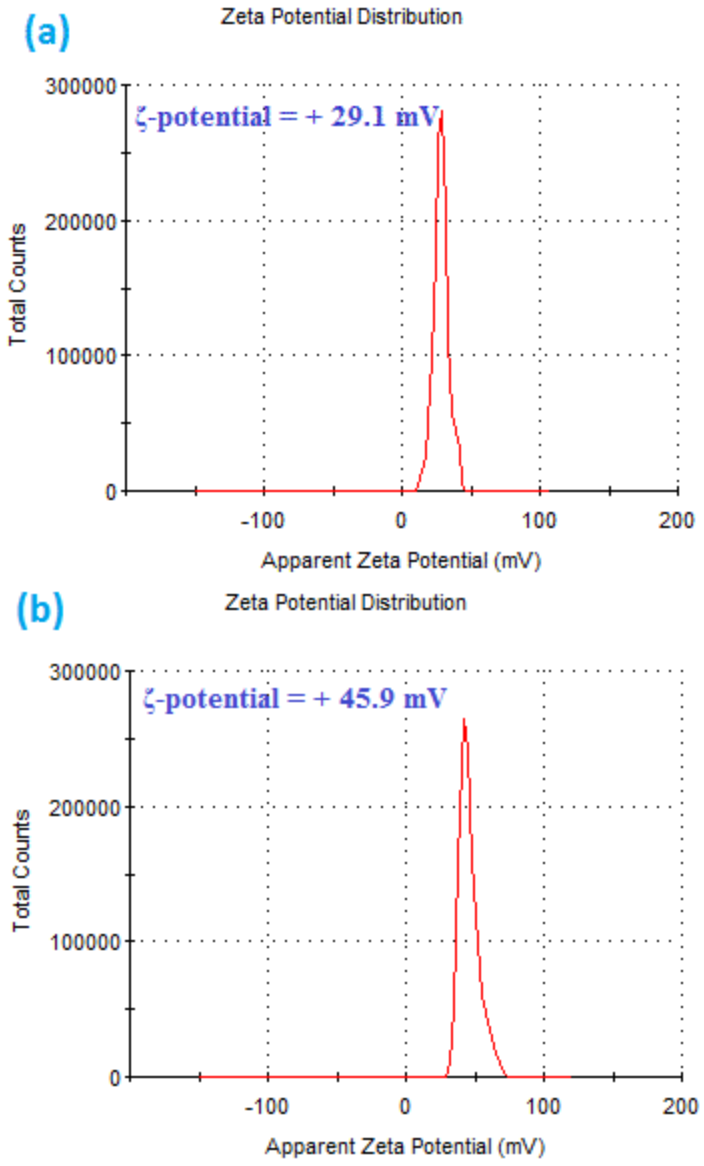

Figure S8: zeta potential values (a, b) of the copolymer PDMAEMA-*b*-(PMAODCA-*r*-PPBA) at 0.1 mg/ml concentration at pH 7.4 and 4.2 respectively.

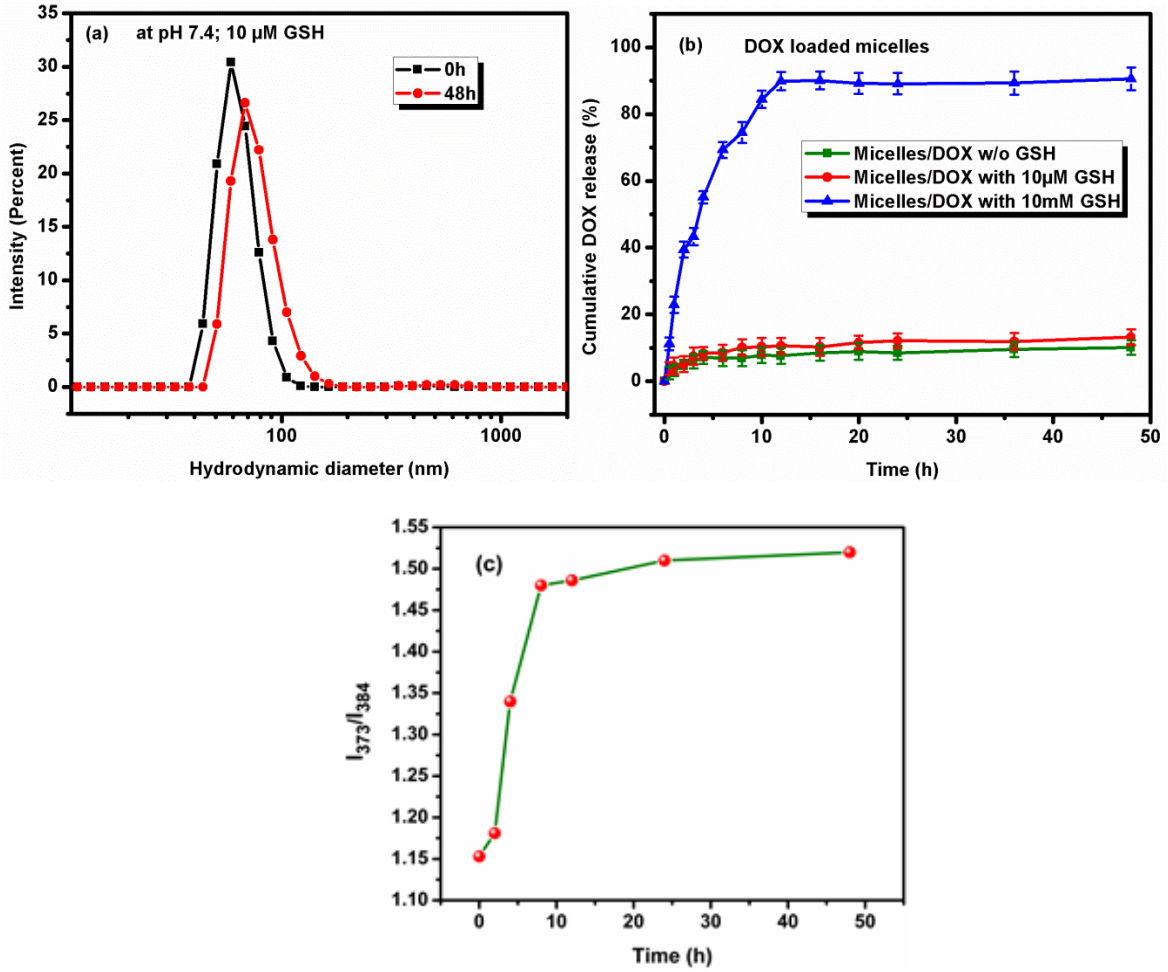

Figure S9: (a) Change in DLS size distribution of micelles after 48 h of 10 µM GSH treatment at pH 7.4; (b) cumulative DOX release from DOX-loaded micelles on treatment with GSH at 4.2; and (c) plot of I_1/I_3 with time.

Table S2: GSH-triggered *in vitro* DOX release (%) after 24 h.

Conc. of GSH treated	pH 7.4 (% of release)		pH 4.2 (% of release)	
	DOX loaded micelles	DOX loaded micelles/DNA	DOX loaded micelles	DOX loaded micelles/DNA
w/o GSH	9%	7.5%	8.5%	7%
10µM GSH	11.5%	10.5%	12.5%	7%
10 mM GSH	88%	81%	89%	77%

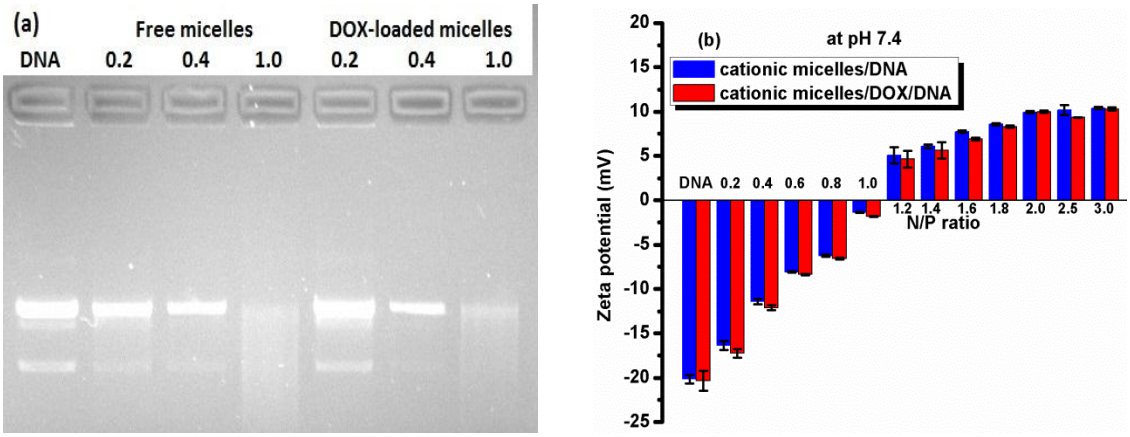

Figure S10: Comparison of Zeta potential (ζ) and gel retardation between micelleplexes formed from free micelles and DOX-loaded micelles.

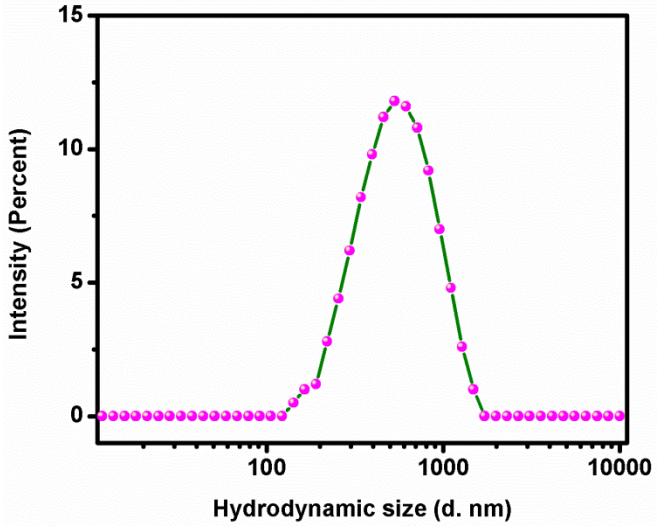

Figure S11: DLS size distribution of naked plasmid DNA.

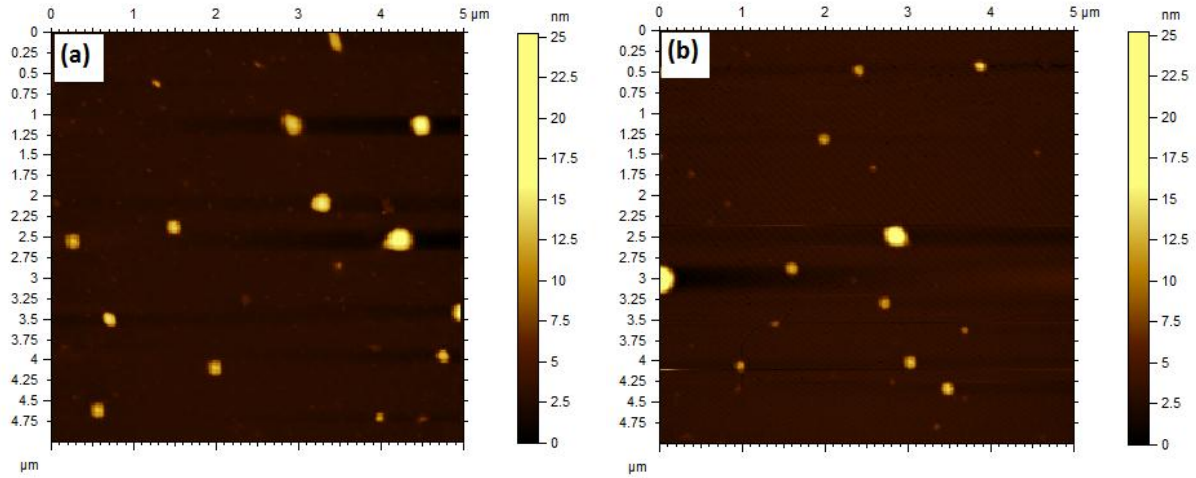

Figure S12: (a, b) represent AFM images of DOX-loaded micelleplexes and free micelleplexes respectively at N/P ratio of 1.2.