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Electronic Supporting Information

Highly Efficient Gene Release in Spatiotemporal Precision Approached by Light and pH Dual Responsive Copolymers

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Materials.

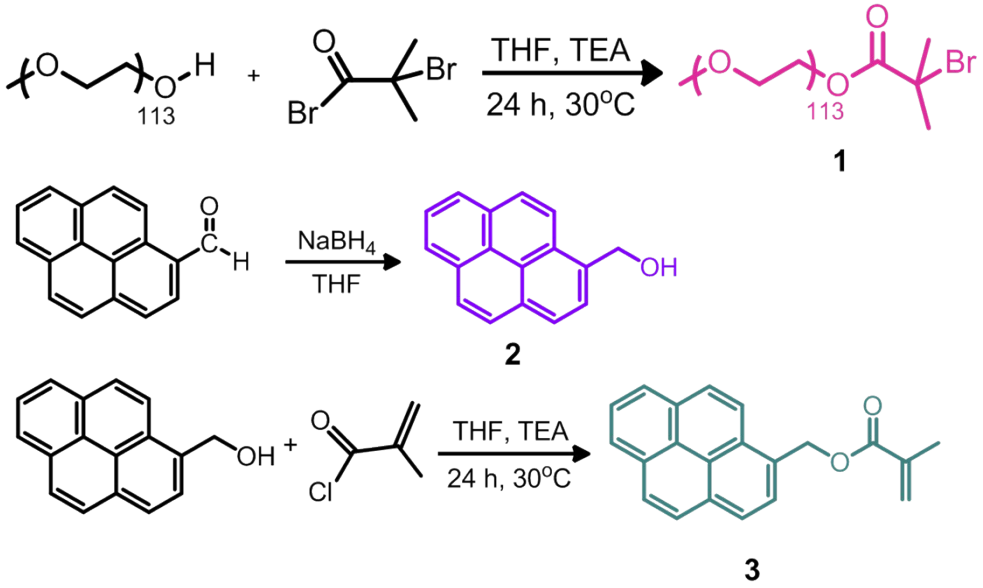
The 2-(Dimethylamino) ethyl methacrylate (DMAEMA, Aldrich, 98%) was passed through basic Al_2O_3 to remove inhibitors. Poly(ethylene glycol) methyl ether (M_n = 5000 g/mol) (Aldrich), α-bromoisobutyryl bromide (BIBB, Alfa Aesar, 97%), triethylamine (TEA, Alfa Aesar, 99%), *N,N,N′,N″,N″*-pentamethyldiethylenetriamine (PMDETA, Aldrich, 99%), methacryloyl chloride (Alfa Aesar, 97%), 1-pyrenecarboxaldehyde (Alfa Aesar, 99%), copper(I) bromide (CuBr, Aldrich, 97%), and sodium borohydride ($NaBH_4$, Alfa Aesar, 98%) were used as received. Dry tetrahydrofuran (THF, >99%, MARCON) were prepared by distillation with CaH_2 under nitrogen. Dry dichloromethane (DCM, Merck, 99%) was prepared by distillation with sodium powder under nitrogen. OrDial D14b (nominal MWCO: 12 000 − 14 000, Orange Scientific) was used for dialysis. RNase/DNase-free water was prepared by adding diethyl pyrocarbonate (DEPC, purchased from MDBio Inc., Taiwan) into DIW (0.1% v/v). The solution was stirred for 1 day and then was autoclaved to remove DEPC. Negative control siRNA oligo duplex, FAM-labeled negative control siRNA oligo duplex and GAPDH siRNA were purchased from MDBio Inc., Taiwan.

Instrument.

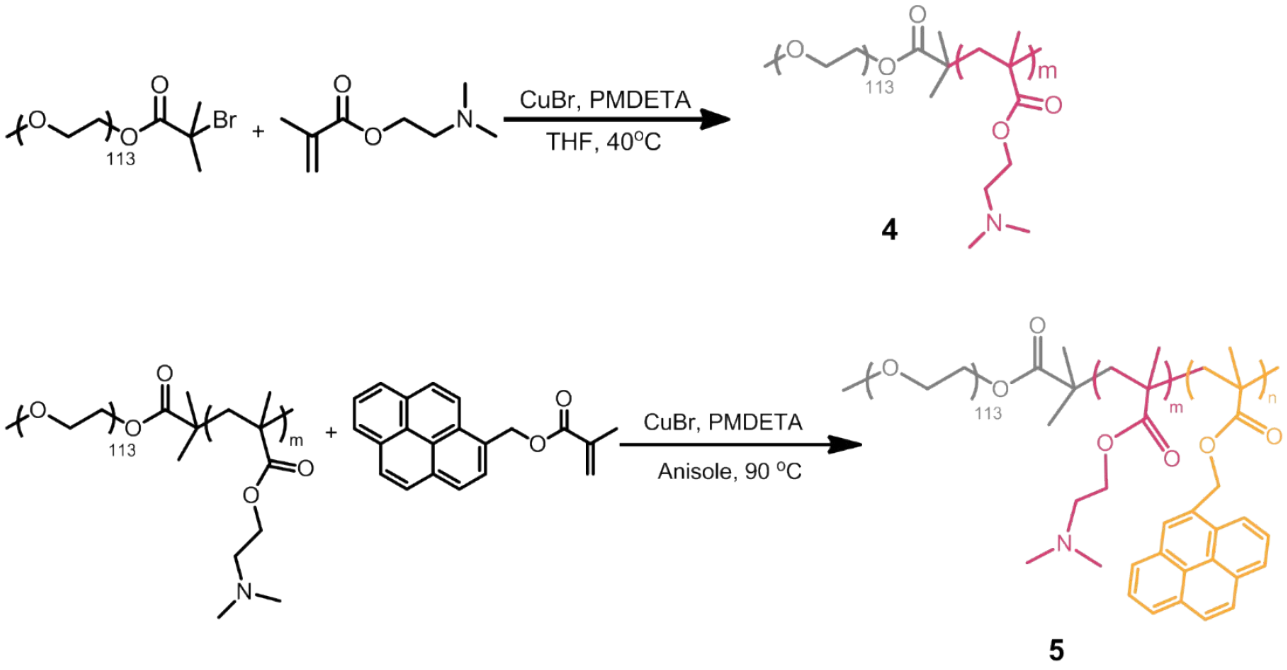
NMR spectroscopy was used for characterization of chemical structure and calculation of monomer conversion. The spectra were recorded on a Bruker-400 spectrometer at 298 K. The chemical shifts in ^{1}H NMR were shown in ppm refer to residual solvent in $CDCl_3$ as δ 7.24 ppm. Molecular-weight distribution of PEG-*b*-PDMAEMA were determined via gel permeation chromatography (GPC) (UltiMate® 3000 HPLC system, Thermo Scientific Dionex) with Waters columns (Styragel® HR 2 DMF, Styragel® HR 4 DMF, and Styragel® HR 5 DMF) using DMF with LiBr (0.1M) as the eluent at 80 °C with flow rate equal of 1 mL /min and Refractive Index Detector (RI-101, Thermo Scientific Dionex). The calibration was based on linear poly(ethylene glycol) (PEG) with molecular weight (2000, 5000, 17800, 31400, 72750 g/mol) with low polydispersity ($Đ$ = 1.01~1.05). Molecular-weight distribution of PEG-*b*-PDMAEMA-*b*-PPy were determined by gel permeation chromatography (GPC) (UltiMate® 3000 HPLC system, Thermo Scientific Dionex) with Shodex columns (Shodex KF-802, Shodex KF-803, and Shodex KF-805) using THF as the eluent at 30 °C with flow rate equal to 1 mL /min and Refractive Index Detector (RI-101, Thermo Scientific Dionex). The calibration was based on linear poly(ethylene glycol) (PEG) with varied molecular

weight (2000, 5000, 17800, 31400, 72750 g/mol) and low polydispersity ($Đ$ = 1.01~1.05). UV-vis spectra were measured by ultraviolet-visible spectrophotometry, (SHIMADZU UV-1800). Fluorescence spectra were measured by fluorescence spectrophotometer (JASCO FP-6500). The hydrodynamic diameter was measured by dynamic light scattering (DLS) with a single-angle (173°) Malvern Zetasizer Nano S (He-Ne laser, λ = 633 nm) (Malvern Instruments, Worcestershire, U.K.). Condensation efficiency of siRNA by polyplexes and micelleplexes using ethidium bromide were quantified by microplate spectrophotometer (SPECTRAmax® *Plus*[384], Molecular Devices Corporation). Transmission electron microscope (TEM) images were captured by Philips, TECNAI 20 at 160 kV. Human epithelial breast cancer cells (MDA-MB-231) were cultured in RPMI-1640 supplemented with 10% fetal bovine serum FBS and 1% penicillin-streptomycin (HyClone, Logan, UT) and 1 mM Sodium Pyruvate.

Methods.



Scheme S1 Synthesis of the precursors used in the preparation of PEG-*b*-PDMAEMA-*b*-PPy.



Scheme S2 Preparation of PEG-*b*-PDMAEMA and PEG-*b*-PDMAEMA-*b*-PPy via ATRP.

Table S1 Characterization of PEG-*b*-PDMAEMA and PEG-*b*-PDMAEMA-*b*-PPy

Entry	$M_{n,\ NMR}$ [a] (g mol^{-1})	PDI	DP[d]	
			DMAEMA	Py
PEG_{113}-*b*-$PDMAEMA_{31}$	9,900	1.27[b]	31	--
PEG_{113}-*b*-$PDMAEMA_{31}$-*b*-PPy_{30}	18,900	1.38[c]	31	30
PEG_{113}-*b*-$PDMAEMA_{80}$	17,700	1.30[b]	80	--
PEG_{113}-*b*-$PDMAEMA_{70}$-*b*-PPy_{28}	24,400	1.40[c]	70	28
PEG_{113}-*b*-$PDMAEMA_{105}$	21,500	1.31[b]	105	--
PEG_{113}-*b*-$PDMAEMA_{105}$-*b*-PPy_{32}	31,100	1.33[c]	105	32

[a]Molecular weight determined by ^{1}H-NMR
[b]Polydipersity measured by GPC using DMF/LiBr as eluent
[c]Polydipersity measured by GPC using THF as eluent
[d]Degree of polymerization calculated by ^{1}H-NMR

copolymers.

Synthesis of PEG_{113}-Br macroinitiator (1).[1]

Typically, PEG_{113}-OH (1 g, 0.2 mmol) was dissolved in 10 mL of anhydrous DCM (10 mL) followed by the addition of TEA (84 μL, 0.6 mmol). BIBB (74.2 μL, 0.6 mmol) was then added into the solution under ice bath. This solution was stirred for 24 hours at 25 °C and then extracted by DCM with 0.1M HCl and with NaCl (5% w/w) twice. The crude product was purified by precipitation in ether to obtain the white powder (yield = 88%). ^{1}H NMR ($CDCl_3$), δ (ppm): 4.33 (d, 2 H, -OCH_2COO-), 3.73 (m, 452H, -CH_2-), 3.36 (s, 3H, -OCH_3), 1.92 (s, 6H, $(CH_3)_2CBrCOO$-).

Synthesis of 1-hydroxymethylpyrene (2). [1]

A 15 mL ethanol solution with $NaBH_4$ (290 mg, 7.67 mmol) and NaOH (20 mg, 0.5 mmol) was added to THF solution (30 mL) with 1-pyrenecarboxaldehyde (589 mg, 2.56 mmol) at 0 °C. The mixture was stirred for 3 hours at 25 °C and then quenched by dilute HCl (0.1 M) until the solution reaching neutral. The solution was futher diluted by water (50 mL) and extracted by DCM for three times. The organic phase was dried over $MgSO_4$ after the extraction with saturated $NaHCO_3$ solution (30 mL) and water (30 mL). The 1-hydroxymethylpyrene was obtained from the organic phase after the solvent removal (yield = 95%). ^{1}H NMR ($CDCl_3$), δ ppm: 7.99-8.33 (m, 9H, aromatic H), 5.41 (s, 2H, $-CH_2$).

Synthesis of 1-pyrenylmethyl methacrylate (3). [1]

The methacryloyl chloride (0.81 mL, 8.6 mmol) was added into the THF solution (10 mL) with 1-pyrenemethanol (1 g, 4.3 mmol) and TEA (1.2 mL, 8.6 mmol) in ice bath. The reaction was then conducted at 25 °C for 24 hours. The solvent was evaporated and the product was obtained by the recrystallization from methanol at 25 °C (yield = 85%).

^{1}H NMR ($CDCl_3$), δ ppm: 7.99-8.31 (m, 9H, Ar-H), 6.13 (s, 1H, CH_2=C), 5.90 (s, 2H, CH_2O), 5.55 (s, 1H, CH_2=C), 1.95 (s, 3H, CH_3).

Preparation of poly(ethylene glycol)-*b*-poly(2-dimethylaminoethyl methacrylate) (PEG-*b*-PDMAEMA) by ATRP (4).[2]

DMAEMA (0.79 g, 5 mmol), PMDETA (20.88 μL, 0.1 mmol), and THF (4 mL) were added into a schlenk flask. After three cycles of freeze-pump-thaw process, the mixture was injected into another schlenk flask with PEG_{113}-Br (500 mg, 0.1 mmol) and CuBr (17.21 mg, 0.12 mmol) under nitrogen. The polymerization was carried out at 40 °C for 24 hours. The polymer product was obtained from the precipitation using cold hexane after the copper complexes was removed by chromatography using neutral alumina.

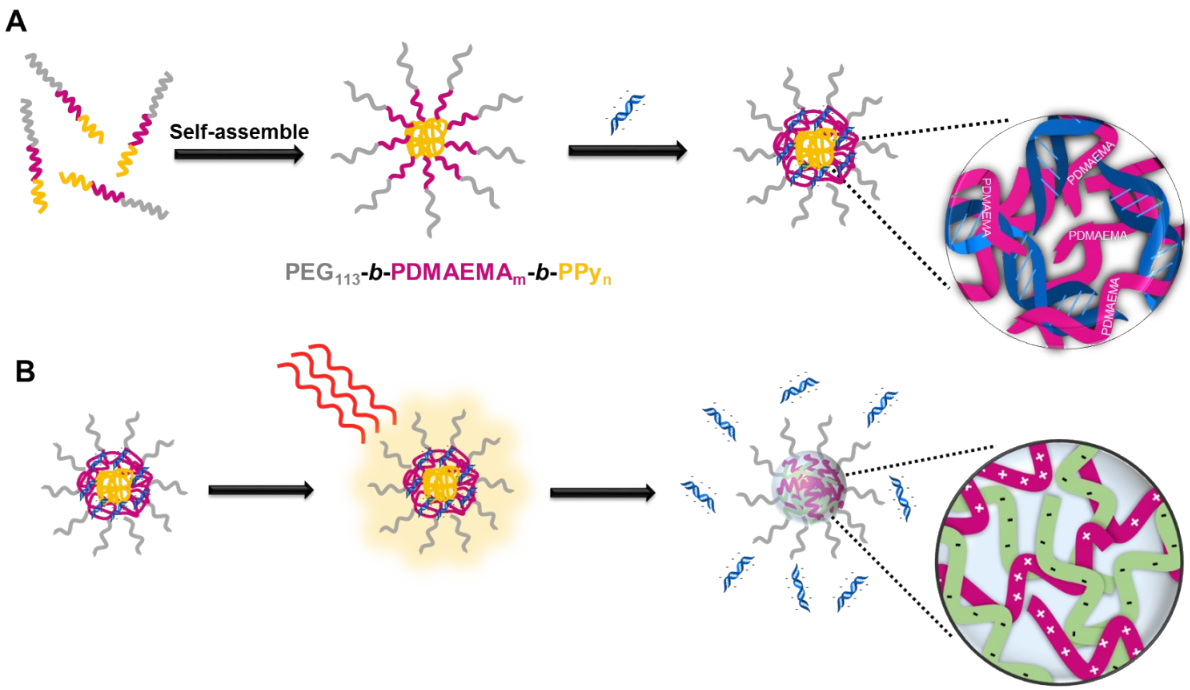
Preparation of poly(ethylene glycol)-*b*-poly(2-dimethylaminoethyl methacrylate)-*b*-poly(pyrenylmethyl methacrylate) (PEG-*b*-PDMAEMA-*b*-PPy) triblock copolymer by ATRP (5).[1]

Typically, anisole (4 mL) and PMDETA (5.6 mg, 0.032 mmol) were added to schlenk flask. After three cycles of freeze-pump-thaw process, the mixture was injected into

another schlenk flask with PEG_{113}-*b*-$PDMAEMA_{50}$ (200 mg, 0.016 mmol), 1-pyrenylmethyl methacrylate (240 mg, 0.800 mmol), and CuBr (4.6 mg, 0.032 mmol) under nitrogen. The polymerization was carried out at 90 °C for 24 hours. The mixture of polymeric product and monomers were obtained from the precipitation using cold hexane after the copper complexes was removed by chromatography using neutral alumina. The residual monomers were removed by dialysis (molecular weight cut off, MWCO: 12000-14000) against THF for two days and against water for 1 day. The polymer product was obtained as light yellow powder by lyophilization.

Fabrication of siRNA-condensed PEG-*b*-PDMAEMA polyplexes.[3]

Polyplexes were prepared by addition of 10 μL of polymer solution (PEG-*b*-PDMAEMA) to siRNA (2 μM, 10 μL) in RNase-free water to form complexes at various N/P ratios and incubated for 20 minutes.



Scheme S3 (A) Proposed image of the condensation of siRNA by self-assembled PEG_{113}-*b*-$PDMAEMA_{31}$-*b*-PPy_{30} micelles through electrostatic interaction between positively-charged PDMAEMA and negatively-charged siRNA. (B) Proposed mechanism of light-induced siRNA release from the micelleplexes: the PMAA converted from PPy binds to PDMAEMA via electreostatic interaction using the negative charge of carboxyl groups and simultaneously pushs out the encapsulated siRNA.

Fabrication of PEG-*b*-PDMAEMA-*b*-PPy triblock copolymer micelles and siRNA-condensed micelleplexes.

The triblock copolymer micelles were prepared by nanoprecipitation. PEG-*b*-PDMAEMA-*b*-PPy in THF (1 mg/mL) was added to distilled water (5 mL) under sonication, then the mixture was loaded into dialysis bag (molecular weight cut off, MWCO: 12000-14000) against distilled water to remove THF for 24 hours followed by lyophilization to obtain yellow powder. Micelleplexes were prepared by addition of 10 μL of micelle solution (PEG-*b*-PDMAEMA-*b*-PPy) to siRNA solution (2 μM, 10 μL) in RNase-free water to form complexes at various N/P ratios and incubated for 20 minutes.

Photo-triggered disruption of PEG-*b*-PDMAEMA-*b*-PPy triblock copolymer micelles.

Self-assembled micelles were re-dispersed in distilled water and adjusted to 0.1 mg/mL with distilled water or THF and DIW solution (50% v/v) for the validation of photo-triggered degradation. The time-dependent fluorescence emission spectra with

excitation at 365 nm were measured under UV light irradiation (4 W, 365nm) for predetermined time.

Loading efficiency of polyplexes and micelleplexes at various N/P ratios ranging from 0.5 to 10 by ethidium bromide (EtBr) exclusion assay.[4]

The condensation efficiency of siRNA by micelleplexes were determined by ethidium bromide assay (EBA). The formulation was mentioned above. The complexes were formulated by addition of 10 μL of micelle solution (PEG-*b*-PDMAEMA-*b*-PPy) to siRNA solution (2 μM, 10 μL) in RNase-free water to form complexes at various N/P ratios and were diluted to 200 μL. After incubation for 20 minutes, the diluted complexes were mixed with EtBr (20 μL, 5 μg/mL) solution thoroughly and further incubation for 20 minutes. The mixture was measured by fluorescence intensity of each sample by microplate reader with excitation at 510 nm and emission at 590 nm.

Evaluation of siRNA release from micelleplexes at phosphate buffer (pH 7.4 and 6.0, *I* = 0.14 M) with or without light irradiation.[5,6]

Stock solution of micelleplexes was prepared by mixing FAM-labeled siRNA (50 μL,

2 μM) and PEG_{113}-*b*-$PDMAEMA_{31}$-*b*-PPy_{30} (25 μL, 0.5 mg/mL) at N/P ratio of 5 in 15 mL centrifuge tube under sonication for 30 seconds and then stood for 20 minutes. The siRNA condensation efficiency was approached as high as 95 %. The stock solution of micelleplexes was further diluted to 2 mL (phosphate buffer at 6.0, I = 0.14 M), followed by UV irradiation for 30 minutes, and then was incubated at 37 °C with gentle shaking (100 rpm). Release of siRNA was monitored at several time intervals (1, 2, 4, 8, 12, 24 hour). At predetermined time, the micelleplexes (200 μL) was extracted and centrifuged (centrifugal filtration, MWCO: 50 kDa, Millipore) for 10 minutes at 10,000 rcf at 4 °C, and then was washed by DIW and centrifuged for two times. The same quantity (200 μL) of fresh buffer solution was added back to sustain the volume for continued monitoring of siRNA release. The supernatant was taken and diluted to 200 μL for the measurement of FAM fluorescence (λ_{ex}= 494 nm、λ_{em}= 520 nm). The residual of siRNA in micelleplexes was quantified against FAM-labeled siRNA calibration curve to determine the release of siRNA from micelleplexes. The evaluation of siRNA release from micelleplexes in pH 7.4, pH 6.0, or DIW without light irradiation was executed by the same method.

Cell viability.

MDA-MB-231 cells were seeded at 3000 cells per well in a 96-well plate for 24 hours before transfection. Cells were treated with micelles of PEG_{113}-*b*-$PDMAEMA_{31}$-*b*-PPy_{30} at concentration ranging from 0.1 to 20 μg/mL under serum-free RPMI-1640 for 6 hours. Then serum-free RPMI-1640 medium was removed and replaced with fresh RPMI-1640 for another 24 hours incubation. Afterward, 15 μL of 5 mg/mL MTT (in phosphate buffered saline, PBS) was added to each well and the cells were incubated for 3 hours at 37 °C. The medium was then replaced with 50 μL DMSO in each well. The absorbance was measured by Multiskan (Thermo, USA) at 570 nm. All data were normalized based on background intensity. All experiments were carried out with six replicates, while control groups (without micelles) were twelve replicates. The cell viability (%) was calculated using the following equation: Cell viability (%) = (samples / mean of controls) * 100%

Cellular uptake.

MDA-MB-231 cells were cultured in complete RPMI1640 medium with 10% FBS, 1% penicillin-streptomycin and 1 mM sodium pyruvate, and were seeded 10^5 cells/well in

a volume of 1 mL/well to a 12-well plate with a coverslip each well at 37°C for 24 hours. Before treatment, medium was replaced with serum-free RPMI1640 medium. MDA-MB-231 cancer cells were respectively treated with free FAM-labled siRNA (40 nM), and micelleplexes containing FAM-labled siRNA (40 nM) or FAM-labled siRNA (80 nM) for 4 hours and then the medium was fixed with 4% paraformaldehyde, followed by washing PBS for three times. The coverslips containing the cells were mounted with 3μL of DAPI to dye cell nucleus and imaged by the confocal laser scanning microscopy (LSM 780, Zeiss, Germany). The images were analyzed using Matlab.

GAPDH knockdown efficiency.[7–10]

MDA-MB-231 cells were cultured in complete RPMI1640 medium with 10% FBS, 1% penicillin-streptomycin and 1 mM sodium pyruvate, and were seeded 5000 cells/well in a volume of 100 μL/well to a 96-well microplate at 37°C for 24 hours for futher incubation. Before treatment, medium was replaced with serum-free RPMI1640 medium. Then, the cells were respectively treated with free GAPDH siRNA (40 nM), and micelleplexes containing GAPDH siRNA (40 nM) or control siRNA (40 nM) for

4 hours and then washed by PBS for three times and replaced with complete RPMI1640 medium. Micelleplexes containing GAPDH siRNA with UV treatment for 30 minutes were excecuted after cellular internalization for 4 hours, and medium washed by PBS for three times and replaced with complete RPMI1640 medium. The cells were further incubated for 72 hours, followed by evaluation of GAPDH protein activity using KDalert™ GAPDH Assay Kit. Preparation and utilization of KDalert™ Master Mix, including cell lysis and GADPH enzyme assay, were performed according to the KDalert™ GAPDH Assay Kit User Guide. The remaining GAPDH protein of MDA-MB-231 cancer cells after transfection were evaluated by the measurement of GAPDH enzyme activity *via* increment of fluorescence intensity at excitation wavelength at 560 nm with emission at 590 nm within 4 minutes. Accroding to the User Guide, the quantitative evaluation of remaining gene expression in MDA-MB-231 cancer cells was determined from the ratio of the fluorescence increase for a given transfection condition to the fluorescence increase for cell only without treatment:

Remaining expression (%) = $(\Delta FL_{\text{given transfection condition}}/\Delta FL_{\text{cell only}}) * 100\%$

GAPDH knockdown efficiency of a given transfection condition was calculated from the percent remaining gene expression as following equation:

GAPDH knockdown efficiency (%) = 100 % – Remaining expression (%)

Supporting Figures

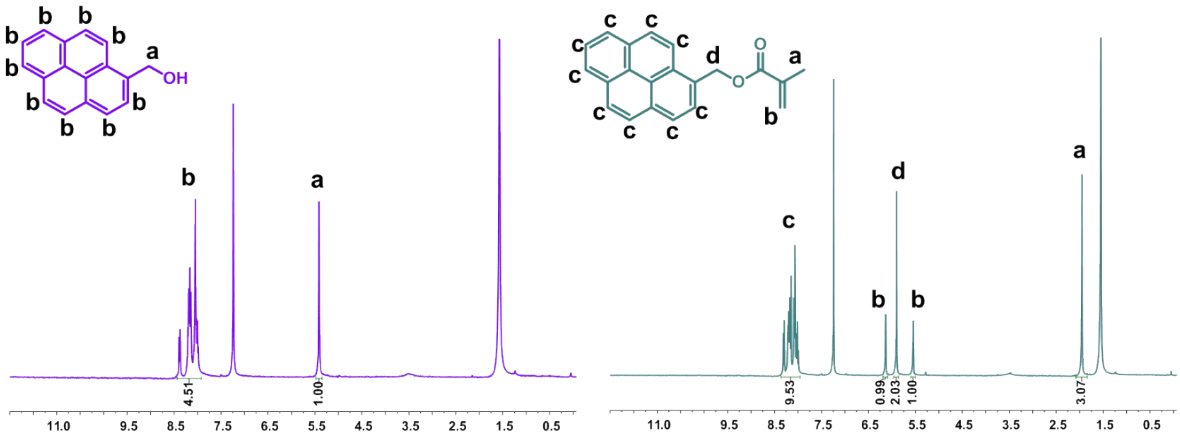


Fig. S1 ^{1}H-NMR spectra of 1-hydroxymethylpyrene (left diagram) and 1-pyrenylmethyl methacrylate (right diagram) in d-chloroform.

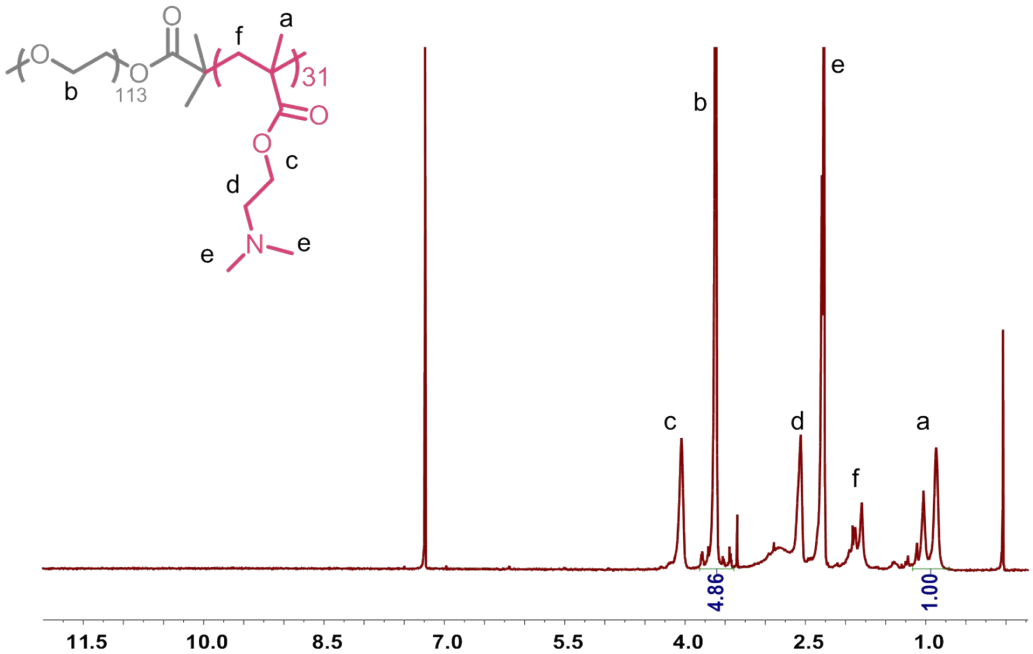


Fig. S2 ^{1}H-NMR spectrum of PEG_{113}-*b*-$PDMAEMA_{31}$ in d-chloroform.

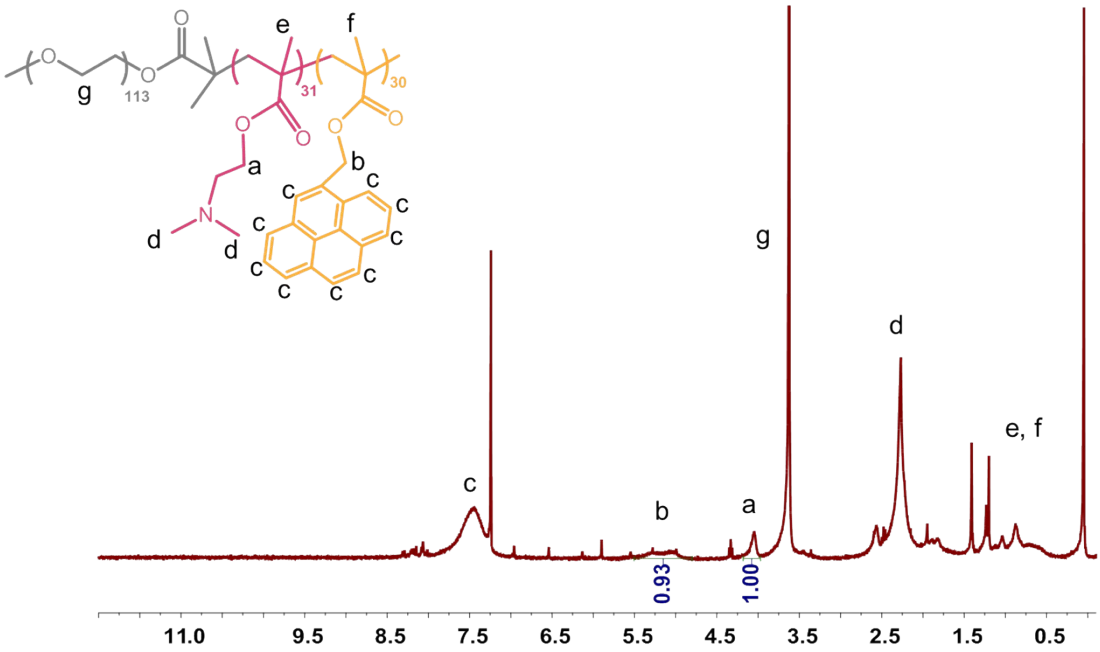


Fig. S3 ^{1}H-NMR spectrum of PEG_{113}-*b*-$PDMAEMA_{31}$-*b*-PPy_{30} in d-chloroform.

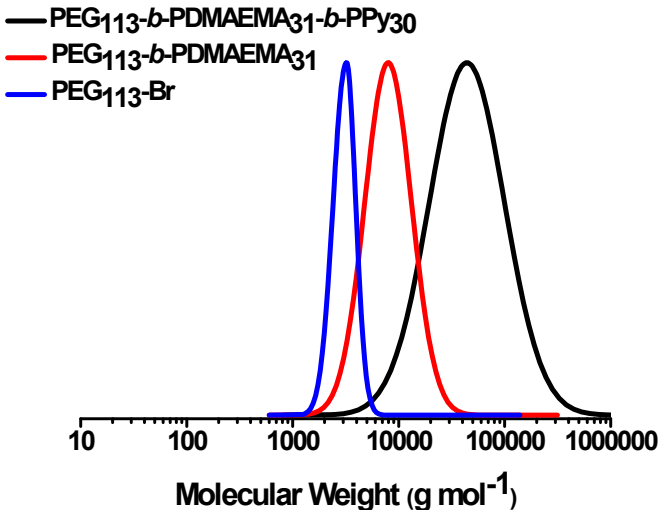


Fig. S4 GPC traces of PEG_{113}-*b*-$PDMAEMA_{31}$-*b*-PPy_{30}, PEG_{113}-*b*-$PDMAEMA_{31}$, and PEG_{113}-Br. Triblock copolymer of PEG_{113}-*b*-$PDMAEMA_{31}$-*b*-PPy_{30} was obtained

by chain extension from PEG_{113}-*b*-$PDMAEMA_{31}$ that was synthesized by PEG_{113}-Br using ATRP technique.

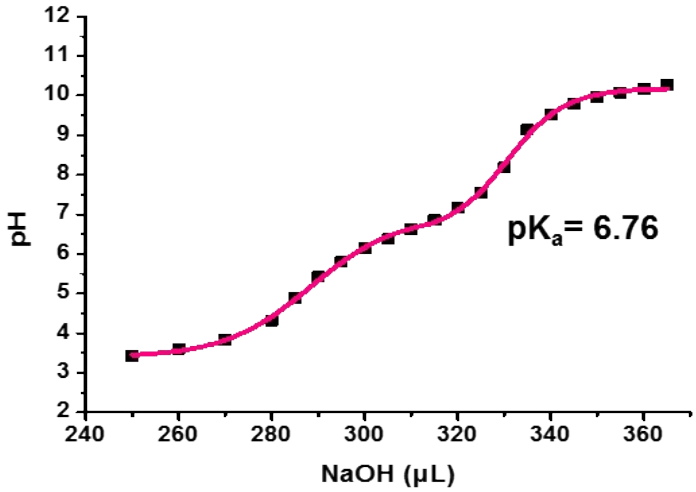


Fig. S5 The titration curve of PEG_{113}-*b*-$PDMAEMA_{31}$-*b*-PPy_{30} (50 μg/mL in water) using 0.1 M NaOH solution as the titrant showing the effective pK_a = 6.76.

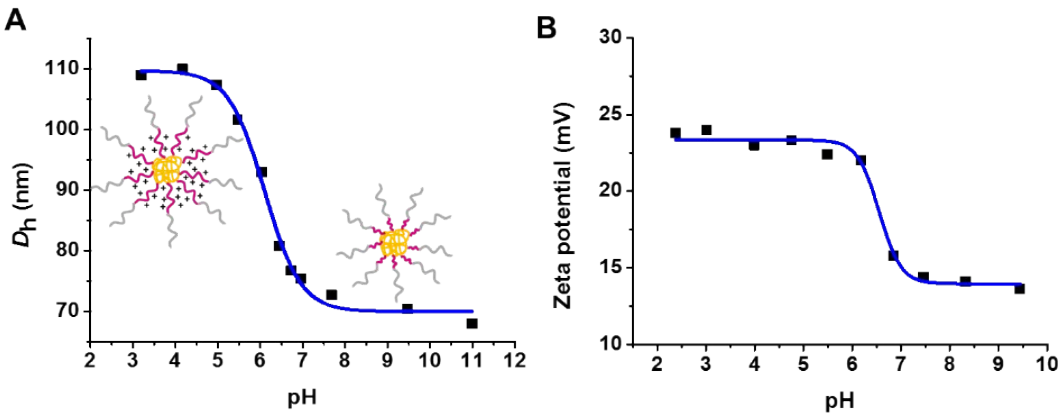


Fig. S6 (A) pH-responsively swelling behavior of PEG_{113}-*b*-$PDMAEMA_{31}$-*b*-PPy_{30} micelles in DIW (50 μg/mL) at various pH values ranging from 3 to 11 determined by DLS. (B) Zeta potential of pH-responsive PEG_{113}-*b*-$PDMAEMA_{31}$-*b*-PPy_{30} micelles at pH values ranging from 2 to 10 measured by DLS (Malvern ZS 90).

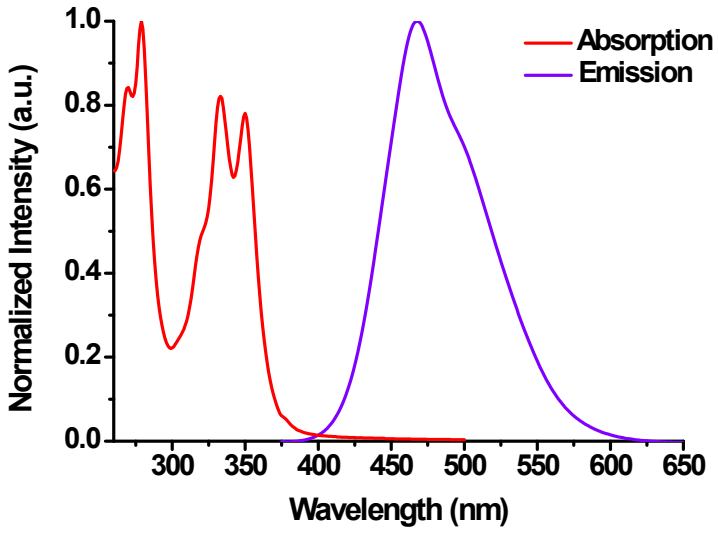


Fig. S7 The normalized UV-vis spectrum and fluorescence spectrum of PEG_{113}-*b*-$PDMAEMA_{31}$-*b*-PPy_{30} self-assembled micelles in distilled water (50 μg/mL).

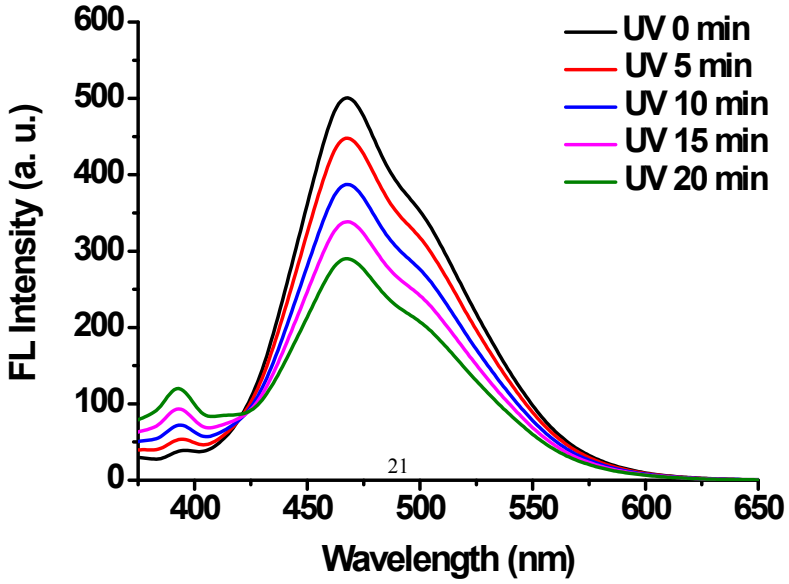


Fig. S8 Fluorescence spectra of PEG_{113}-*b*-$PDMAEMA_{31}$-*b*-PPy_{30} in water/THF (v/v = 1/1) upon UV irradiation for 0, 5, 10, 15, and 20 minutes.

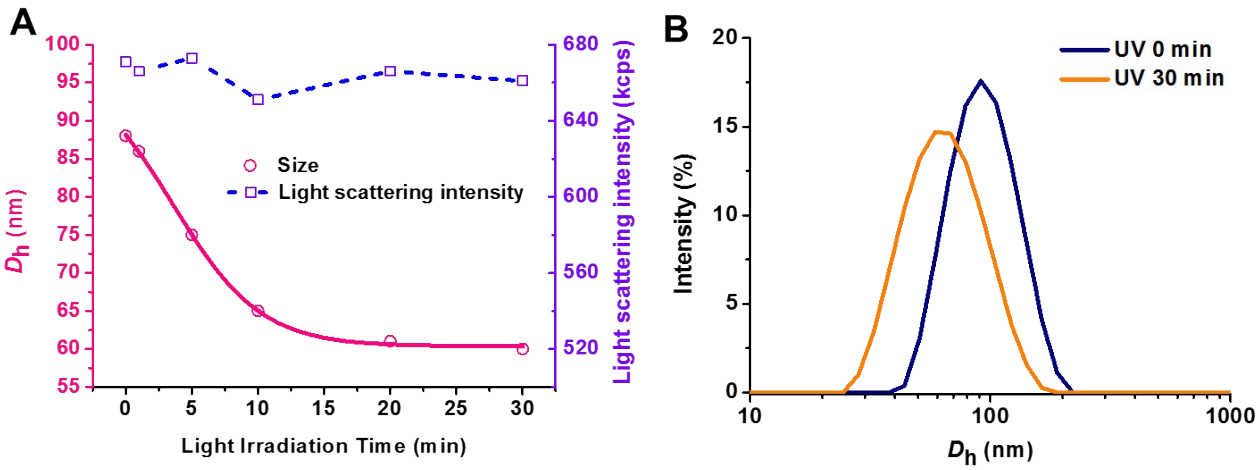


Fig. S9 The change of particle size and light scattering intensity of PEG_{113}-*b*-$PDMAEMA_{31}$-*b*-PPy_{30} with UV irradiation time determined by DLS. (A) DLS analysis of PEG_{113}-*b*-$PDMAEMA_{31}$-*b*-PPy_{30} in DIW after UV irradiation (0, 1, 5, 10, 20, and 30 minutes). (B) Size evolution of PEG_{113}-*b*-$PDMAEMA_{31}$-*b*-PPy_{30} self-assemblies before and after UV irradiation (30 minutes).

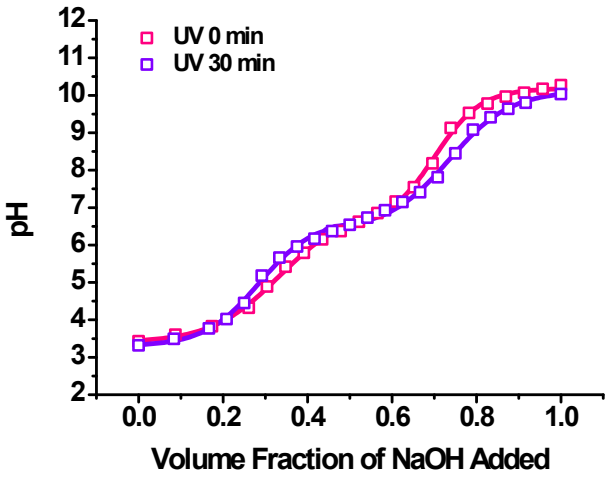


Fig. S10 Titration curves of PEG_{113}-*b*-$PDMAEMA_{31}$-*b*-PPy_{30} and PEG_{113}-*b*-$PDMAEMA_{31}$-*b*-$PMAA_{30}$ (PEG_{113}-*b*-$PDMAEMA_{31}$-*b*-PPy_{30} after UV irradiation for 30 min) using 0.1 M NaOH solution.

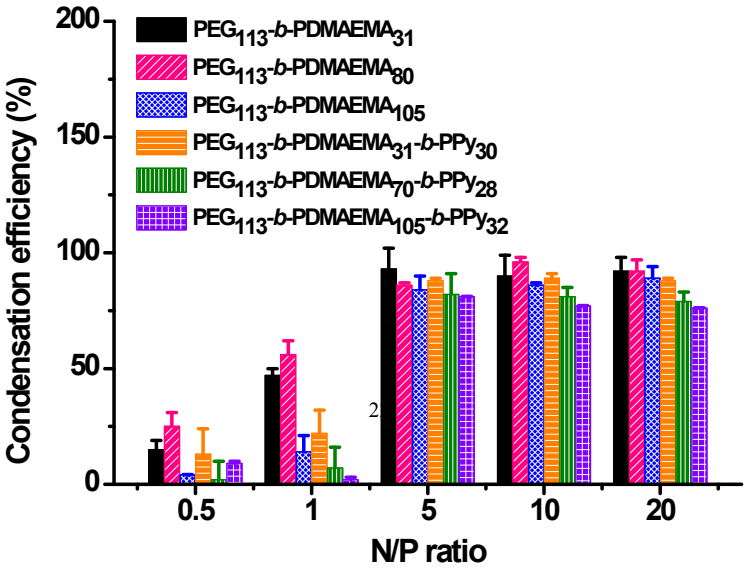


Fig. S11 siRNA condensation efficiency of PEG-*b*-PDMAEMA copolymers and PEG-*b*-PDMAEMA-*b*-PPy micelles determined by EBA with various N/P ratios.

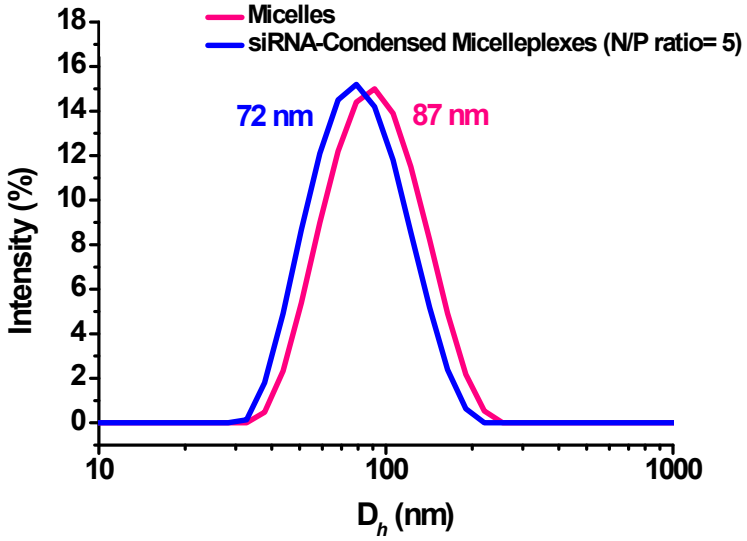


Fig. S12 DLS analysis of pristine PEG_{113}-*b*- $PDMAEMA_{31}$-*b*-PPy_{30} micelles and siRNA-condensed PEG_{113}-*b*-$PDMAEMA_{31}$-*b*-PPy_{30} micelleplexes at N/P ratio of 5.

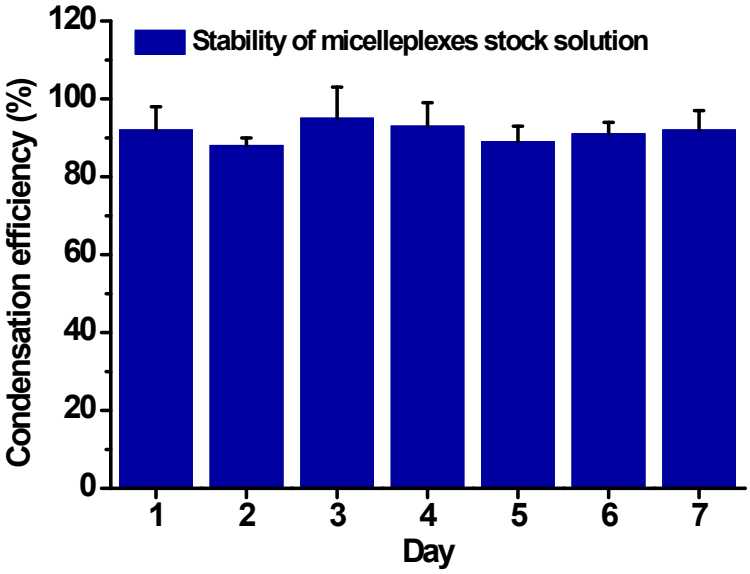


Fig. S13 siRNA condensation efficiency of PEG_{113}-*b*-$PDMAEMA_{31}$-*b*-PPy_{30} micelles in DIW at N/P ratio of 5 in 7 days.

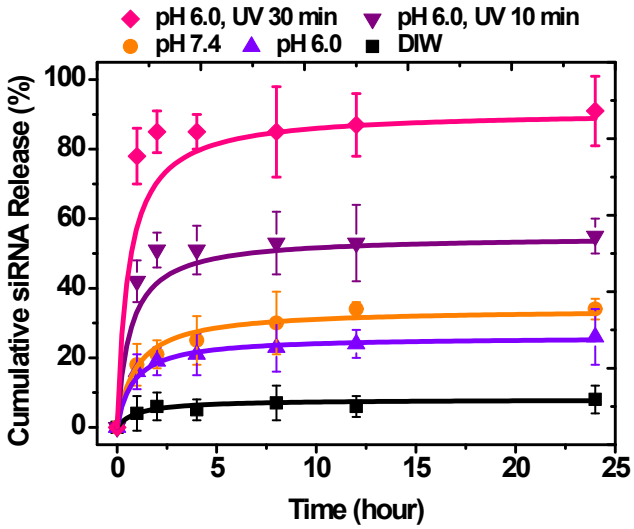


Fig. S14 Cumulative siRNA release of micelleplexes (N/P = 5) at 37 °C in DIW (formulation), phosphate buffer with pH 7.4, ionic strength = 0.14 M, and phosphate buffer with pH 6.0, ionic strength = 0.14 M before and after light-irradiation for 30 minutes.

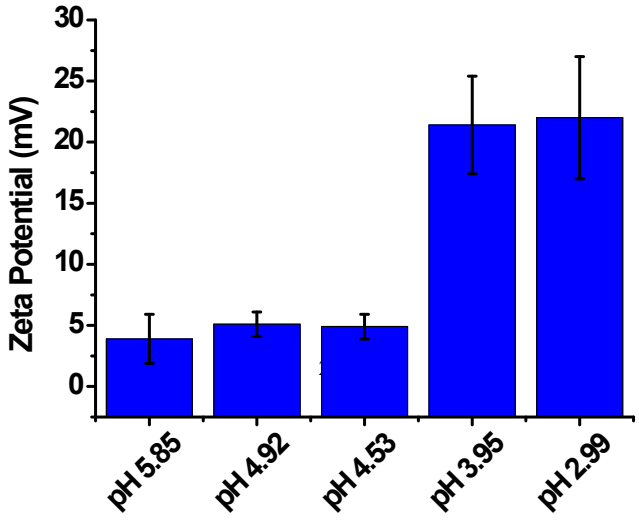


Fig. S15 Zeta potential of PEG_{113}-*b*-$PDMAEMA_{31}$-*b*-PPy_{30} self-assembled micelles in distilled water after UV irradiation for 30 minutes at various pH values.

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