Supplementary Information

Multicolored pH-Tunable and Activatable Fluorescence Nanoplatform Responsive to Physiologic pH Stimuli

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Materials

2-(Pentamethyleneimino) ethanol (C6A) and N,N,N',N",N"-pentamethyldiethylenetriamine (PMDETA) were purchased from Sigma-Aldrich. 2-(Hexamethyleneimino) ethanol (C7A) and 2-(dibutylamino) ethanol (DBA) were purchased from Alfa Aesar Company and TCI America Inc., respectively. 4,4-Difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene-8-propionic acid, succinimidyl ester (BDIPY-NHS), 7-diethylaminocoumarin-3-carboxylic acid succinimidyl ester (Coumarin-NHS),1-(3-(succinimidyloxycarbonyl)benzyl)-4-(5-(4-methoxyphenyl)oxazol-2-yl)pyri dinium bromide (PyPMO-NHS) and tetramethyl rhodamine NHS ester (NHS-TMR) were purchased from Invitrogen Company. Cy5.5 and Cy7.5 NHS esters were purchased from Lumiprobe Company. 2-(Diisopropyl amino)ethyl methacrylate (DPA) and 2-aminoethyl methacrylate (AMA) were purchased from Polyscience Company. AMA was recrystallized twice with isopropanol and ethyl acetate (3:7). Monomers of 2-(dibutylamino) ethyl methacrylate (DBA), 2-(hexamethyleneimino) ethyl methacrylate (C7A), 2-(pentamethyleneimino) ethyl methacrylate (C6A), and PEG macroinitiator (MeO-PEG114-Br) were prepared according to procedures in the literature.[1] Other solvents and reagents were used as received from Sigma-Aldrich or Fisher Scientific Inc.

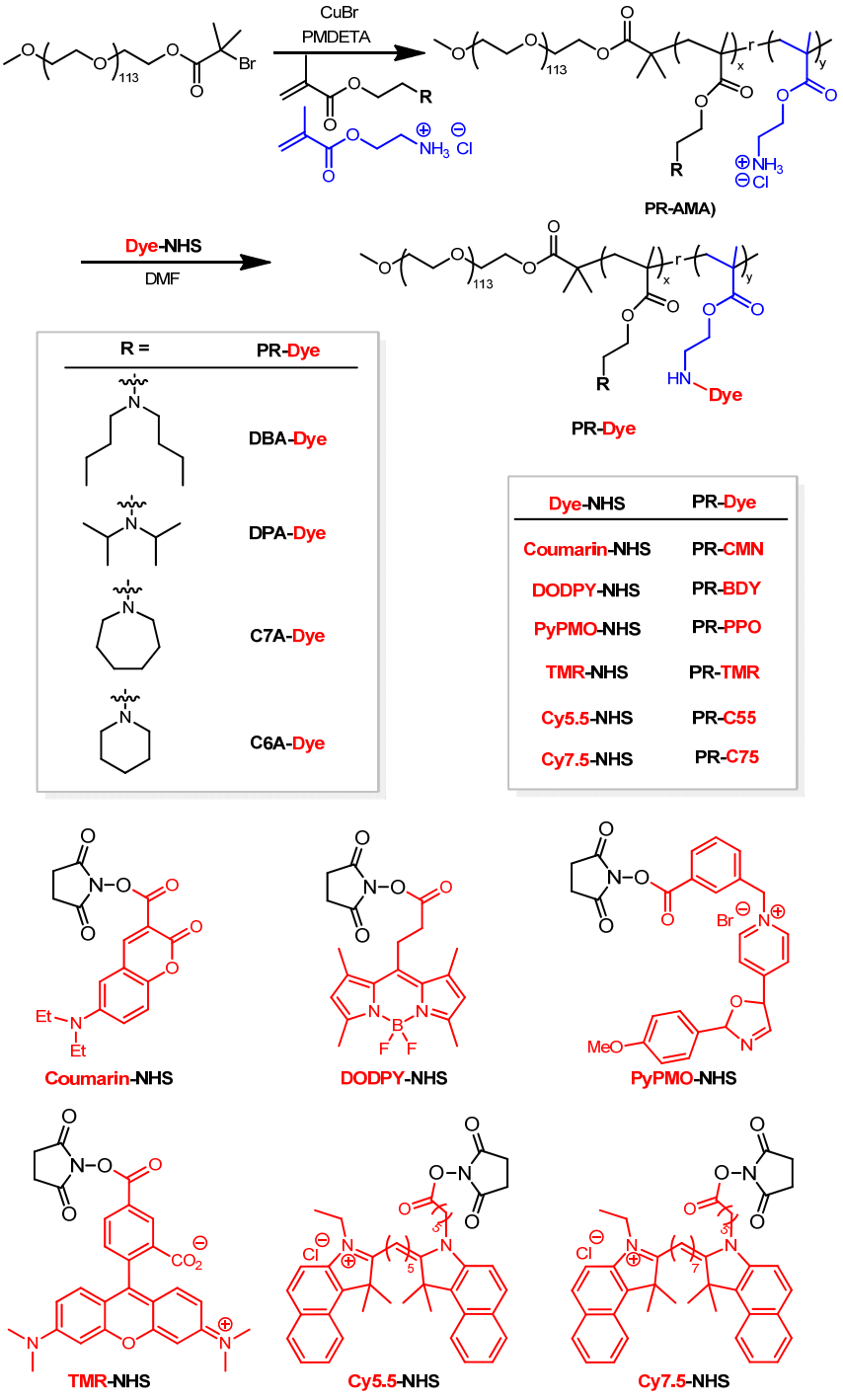
Syntheses of PEO-(PR-Dye) block copolymers

PEO-*b*-(PR-*co*-AMA) copolymers (Scheme 1) were first synthesized by atom transfer radical polymerization (ATRP) method. The primary amino groups were introduced into each polymer chain by controlling the feeding ratio of the AMA monomer to the initiator (ratio =1, 3, or 6). The dye-free copolymers were used in polymer characterizations (Table S1). PEO-*b*-P(DPA-co-AMA) was used as an example to illustrate the procedure. First, DPA (1.7 g, 8 mmol), AMA (51 mg, 0.31 mmol), PMDETA (25 μL, 0.12 mmol), and MeO-PEG_{114}-Br (500 mg, 0.1 mmol) were charged into a polymerization tube. Then a mixture of 2-propanol (2 mL) and DMF (2 mL) was added to dissolve the monomer and initiator. After three cycles of freeze-pump-thaw to remove oxygen, CuBr (16 mg, 0.11 mmol) was added into the reaction tube under nitrogen atmosphere, and the tube was sealed *in vacuo*. The polymerization was carried out at 50 ^{o}C for 8 hours. After polymerization, the reaction mixture was diluted with 10 mL THF, and passed through an Al_2O_3 column to remove the catalyst. The THF solvent was removed by rotovap. The residue was dialyzed in distilled water and lyophilized to obtain a white powder. The resulting PEO-*b*-PR copolymers were characterized by 500 MHz ^{1}H NMR, gel permeation chromatography (Viscotech GPCmax, PLgel 5μm MIXED-D columns by Polymer Labs, THF as eluent at 1 mL/min). Table S1 enlists molecular weights (M_n and M_w) and polydispersity index (PDI) of each copolymer.

Syntheses of dye-conjugated copolymers followed a representative procedure described below. For TMR conjugation, PEO-*b*-(PR-*co*-AMA) (50 mg) was first dissolved in 2 mL DMF. Then NHS-TMR ester (1.5 equivalents to the molar amount of the primary amino group) was added. The reaction mixture was stirred at room temperature for two days. The copolymers were purified by preparative gel permeation chromatography (PLgel Prep 10μm 10E3Å 300×25mm columns by Varian, THF as eluent at 5 mL/min) to remove the free dye molecules. The produced

PEO-(PR-TMR) copolymers were lyophilized and kept at -20 ^{o}C for storage.

Scheme 1



Preparation of the micelle nanoparticles

Micelles were prepared following a solvent evaporation method as previously published.[2] In the example of PDPA-TMR micelle solution, 10 mg of the copolymer was first dissolved in 0.5 mL THF and then added into 4 mL distilled water dropwise under sonication. The THF was removed through ultrafiltration with (100 kD) membrane for several times. Then the distilled water was added to adjust the polymer concentration to 5 or 10 mg/mL as a stock solution. After micelle formation, the nanoparticles were characterized by transmission electron microscopy (TEM, JEOL 1200 EX model) for micelle size and morphology, and dynamic light scattering (DLS, Malvern MicroV model, He-Ne laser, λ= 632 nm) for hydrodynamic radius (R_h).

For the preparation of molecularly mixed micelle, a mixture of PDPA-TMR and PEO-*b*-P(DPA-co-AMA) with 1:1 weight ratio was used as an example. PDPA-TMR copolymer (5 mg) and PEO-*b*-P(DPA-co-AMA) (5 mg) were first dissolved in 0.5 mL THF and then added into 4 mL distilled water dropwise under sonication. The THF was removed through ultrafiltration with (100 kD) membrane for several times. Then the distilled water was added to adjust the polymer concentration to 5 or 10 mg/mL as a stock solution for subsequent studies.

CMC measurement of PEO-(PDPA-AMA)

Critical micelle concentration (CMC) of PEO-(PDPA-AMA) copolymer was measured in the 0.2 M sodium phosphate buffer at pH 7.4. First, the copolymer stock solution (5 mg/mL) was diluted to different concentrations with the same buffer. In each solution, 5 μL pyrene in THF solution (2×10^{-4} mol/L) was added to 2 mL polymer solution to produce the final pyrene concentration at 5×10^{-7} mol/L. The fluorescence spectra were recorded on a Hitachi fluoremeter (F-7500 model) with the excitation wavelength of 339 nm and the excitation and emission slits at 10.0 nm and 1.0 nm, respectively. The I_1 and I_3 values were measured as the maximum emission intensity at ca. 372 and 382 nm, respectively. I_1/I_3 ratio was plotted as a function of polymer concentration at different pH values. I_1/I_3 ratio reflects the polarity of the pyrene environment where partition of pyrene in the hydrophobic micelle core leads to decreased I_1/I_3 values.[3,4] CMC values were measured as the threshold polymer concentration at which micelles were formed in solution.[3,4] To avoid TMR interference, PEO-*b*-PR copolymers without TMR conjugation were used in these studies.

pH titration of PDPA-TMR

PDPA-TMR copolymer (80 mg) was first dissolved in 5mL 0.1 M HCl and diluted to 2 mg/mL with DI water. The pH titration was carried out by adding small volumes (0.05-0.1 mL increments) of 0.02 M NaOH solution under stirring. The pH increase in the range of 2 to 11 was monitored as a function of total added volume of NaOH (V_{NaOH}). The pH values were measured using a Mettler Toledo pH meter with a microelectrode. During the titration experiment, ~ 1 mL solutions of a series of pH points was taken and filtered with 0.45 μm Nylon filter. Then its hydrodynamic radius was measured by DLS. At pH 5.8 and 6.8, the solutions were characterized by TEM.

Fluorescence and UV-Vis characterization

The fluorescence emission spectra were obtained on a Hitachi fluorometer (F-7500 model). The UV-Vis spectroscopy study was performed on a Shimadzu UV-Vis spectrophotometer (UV-1800 model). For each copolymer, the sample was initially prepared in MilliQ water at the concentration of 6 mg/mL. Then the stock solution was diluted in 0.2 M citric-phosphate buffers with different pH values. The terminal polymer concentration was controlled at 0.2 or 0.5 mg/mL.

For fluorescence lifetime measurements, the fluorescence decays of PDPA-TMR at 0.1 mg /mL were measured at pH = 7.4 and 5.5 (above and below the pH_t, respectively) in sodium phosphate/citric acid buffers. The fluorescence decays of free TMR dye (5 μg/mL) was also measured at pH = 7.4 and 5.5. These studies were carried out on a LaserStrobe fluorescence lifetime instrument (Photon Technology International, Inc., Birmingham, NJ), which consists of a nitrogen laser (GL-3300) linked to a dye laser (GL 302) and a stroboscopic detector. C-540A (Exciton, Inc., Dayton, OH) dye solution was used to generate an excitation wavelength of 540 nm. The decay curves were analyzed at the wavelength of 580 nm. The emission monochromator slit was at 4 nm. All measurements were conducted at room temperature. The IRF (instrument response function) was determined by measuring scattered light from a solution of glycogen. The fluorescence intensity decay data were analyzed by using the software supplied with the PTI instrument.

For the fluorescent images of PDBA-BDY, PDPA-TMR, C7A-C55 and C6A-C75 solutions at different pH values (100 μg/mL for each sample), the Maestro imaging system (CRI, Inc., Woburn, MA) was used by choosing a proper band pass excitation filter and a proper long-pass emission filter according to the instrument manual.

Cell culture and Confocal imaging study of micelle uptake

Human lung carcinoma H2009 cells were cultured in RPMI 1640 medium (Invitrogen, CA) supplemented with 5% fetal bovine serum (FBS), 100 IU/mL penicillin and 100 μg/mL streptomycin at 37 °C in 5% CO_2 atmosphere.

Prior to confocal imaging studies, H2009 cells were plated in glass bottom cell culture dishes (MatTek, MA) in 2 mL complete RPMI medium and incubated with mixed nanoparticles of PDBA-BDY, PDPA-TMR and C7A-C55 where each nanoparticle concentration was at 0.2 mg/mL in phenol-free RPMI 1640 medium. The medium was changed after one-hour incubation. Confocal images were captured at different time points. The images were processed and analyzed under identical conditions by the Image-J software. Five independent measurements were analyzed and averaged as the mean ± standard deviation. Images were captured at designated time points by a Nikon ECLIPSE TE2000-E confocal microscope with a 100× objective lens. PDBA-BDY, PDPA-TMR and C7A-C55 were excited at 488, 543 and 623 nm, respectively. The FITC, TRITC and Cy5 filters were used for PDBA-BDY, PDPA-TMR and C7A-C55 imaging, respectively.

Reference

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2. Nasongkla, N.; Bey, E.; Ren, J. M.; Ai, H.; Khemtong, C.; Guthi, J. S.; Chin, S. F.; Sherry, A. D.; Boothman, D. A.; Gao, J. M., Multifunctional polymeric micelles as cancer-targeted, MRI-ultrasensitive drug delivery systems. Nano. Lett. 2006, 6, 2427-2430.
3. Kalyanasundaram, K.; Thomas, J. K., Environmental Effects on Vibronic Band Intensities in Pyrene Monomer Fluorescence and Their Application in Studies of Micellar Systems. J. Am. Chem. Soc. 1977, 99, 2039-2044.
4. Winnik, F. M., Photophysics of Preassociated Pyrenes in Aqueous Polymer-Solutions and in Other Organized Media. Chem. Rev. 1993, 93, 587-614.

Table S1. Characterization of PEO-*b*-(PR-*co*-AMA) diblock copolymers.

Copolymer	$M_{n,\ ^1H\ NMR}$ (kD)[a]	$M_{n,GPC}$ (kD)[b]	$M_{w,\ GPC}$ (kD)[b]	PDI[b]
PEO_{114}-*b*-P(DPA_{74}-*co*-AMA_1)	20.9	19.7	23.8	1.21
PEO_{114}-*b*-P(DPA_{77}-*co*-AMA_3)	21.9	20.2	24.2	1.20
PEO_{114}-*b*-P(DPA_{80}-*co*-AMA_6)	23.0	21.3	25.3	1.19
PEO_{114}-*b*-P(DBA_{65}-*co*-AMA_3)	21.1	20.0	23.6	1.18
PEO_{114}-*b*-P($C7A_{65}$-*co*-AMA_3)	19.6	18.4	21.5	1.17
PEO_{114}-*b*-P($C6A_{81}$-*co*-AMA_3)	21.4	20.5	25.4	1.24

[a]Number-averaged molecular weight (M_n) as determined by ^1H NMR. [b]Number-averaged molecular weight (M_n), weight-averaged molecular weight (M_w), and polydispersity index ($PDI=M_w/M_n$) as determined by GPC using THF as the eluent.

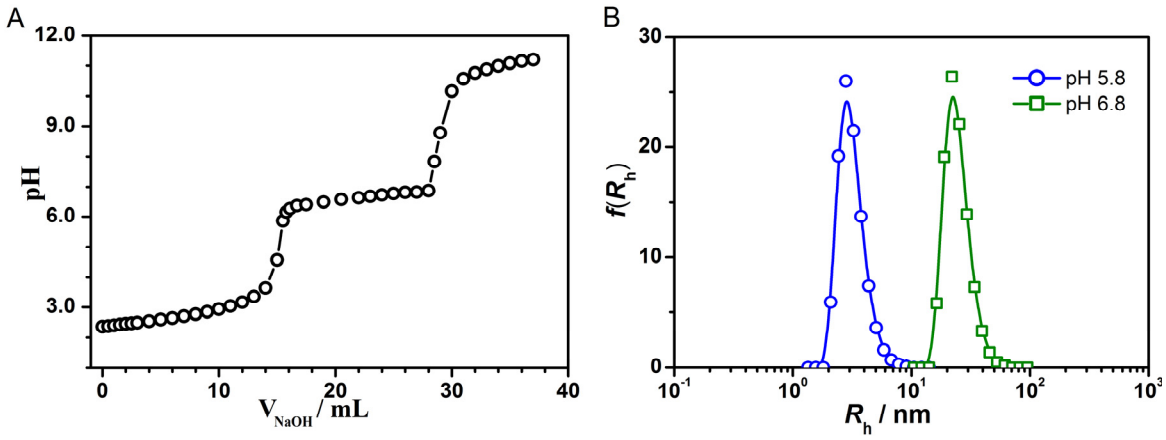

Figure S1. (A) pH titration curve of PEO-(PDPA-TMR) with 0.02 M NaOH aqueous solution. (B) Number-weighted hydrodynamic radius distribution of PEO-(PDPA-TMR) aqueous solution at pH 5.8 and 6.8 during the titration experiment.

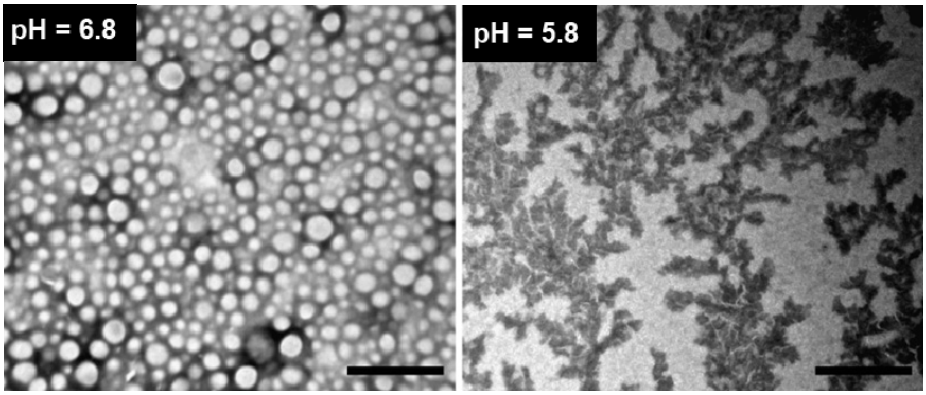

Figure S2. TEM characterization of PEO-(PDPA-TMR) aqueous solution at pH 6.8 and 5.8 from pH titration experiment. The scale bar is 200 nm in both images.

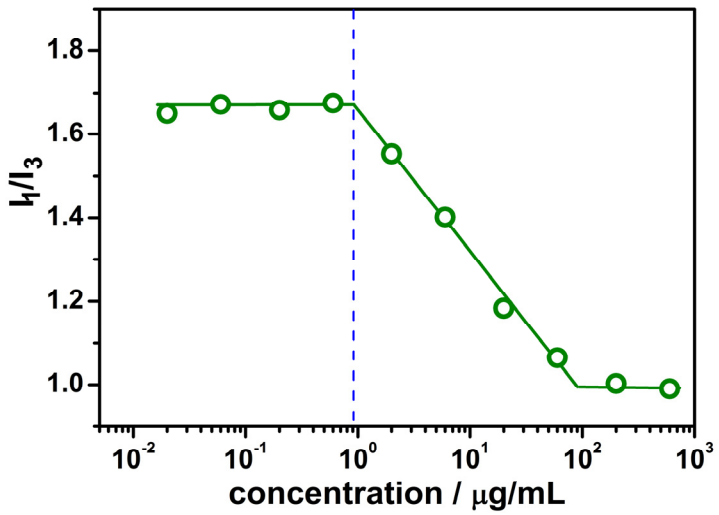

Figure S3. Measurement of critical micelle concentration (CMC) of PEO_{114}-*b*-($PDPA_{77}$-*co*-AMA_3) copolymer. The I_1/I_3 fluorescence ratio of pyrene is plotted as a function of PEO_{114}-*b*-($PDPA_{77}$-*co*-AMA_3) concentration. CMC is indicated by the dashed line at 0.9 μg/mL.

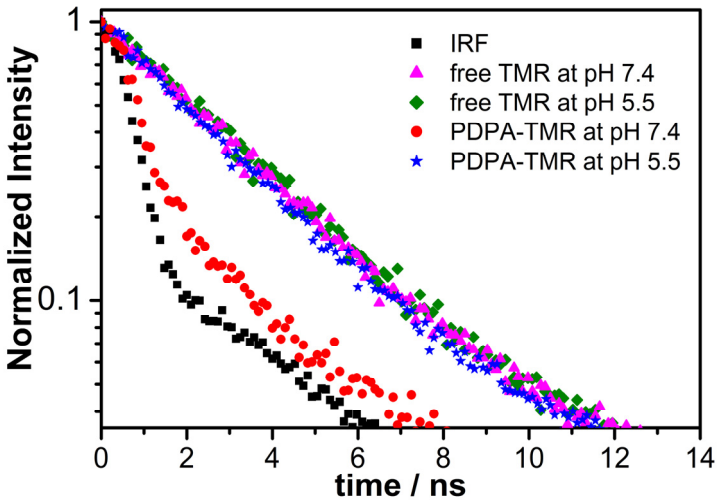

Figure S4. Fluorescence intensity decay of free TMR dye and PEO-(PDPA-TMR) in aqueous solution at pH 7.4 and 5.5.

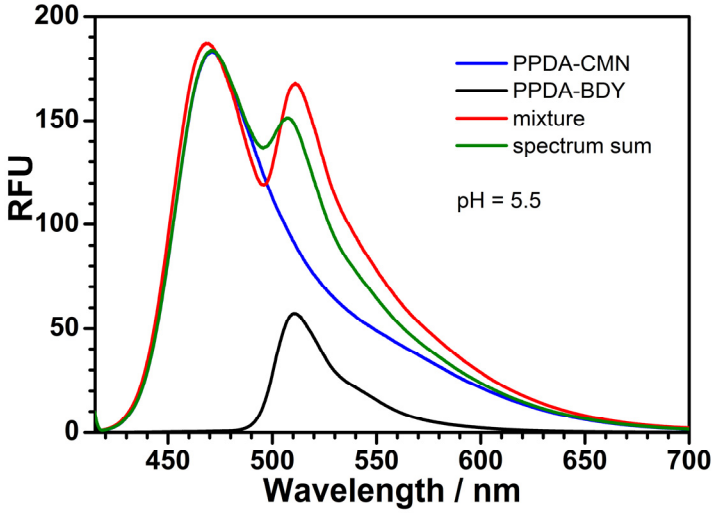

Figure S5. Fluorescence emission spectra of PDPA-CMN, PDPA-BDY and their molecular mixture at pH 5.5 (unimer states). Each polymer concentration is controlled at 200 μg/mL. The samples were excited at 408 nm and the emission spectra were collected from 420 to 700 nm.

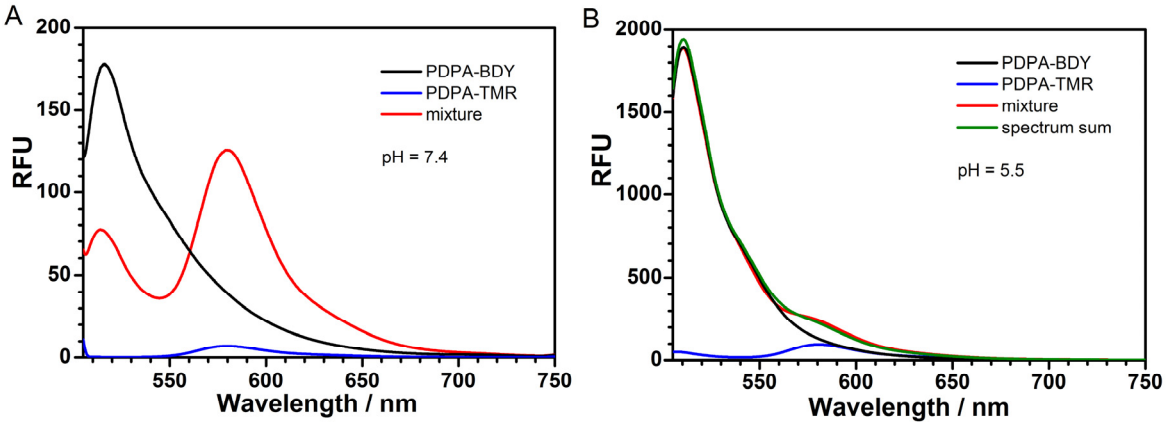

Figure S6. (A) Fluorescence emission spectra of PDPA-BDY, PDPA-TMR and their molecular mixture with 1:1 weight ratio at pH 7.4. Each individual polymer concentration is at 200 μg/mL. The samples were excited at 498 nm and the emission spectra were collected from 505 to 750 nm. (B) Fluorescence emission spectra of these solutions at pH 5.5.

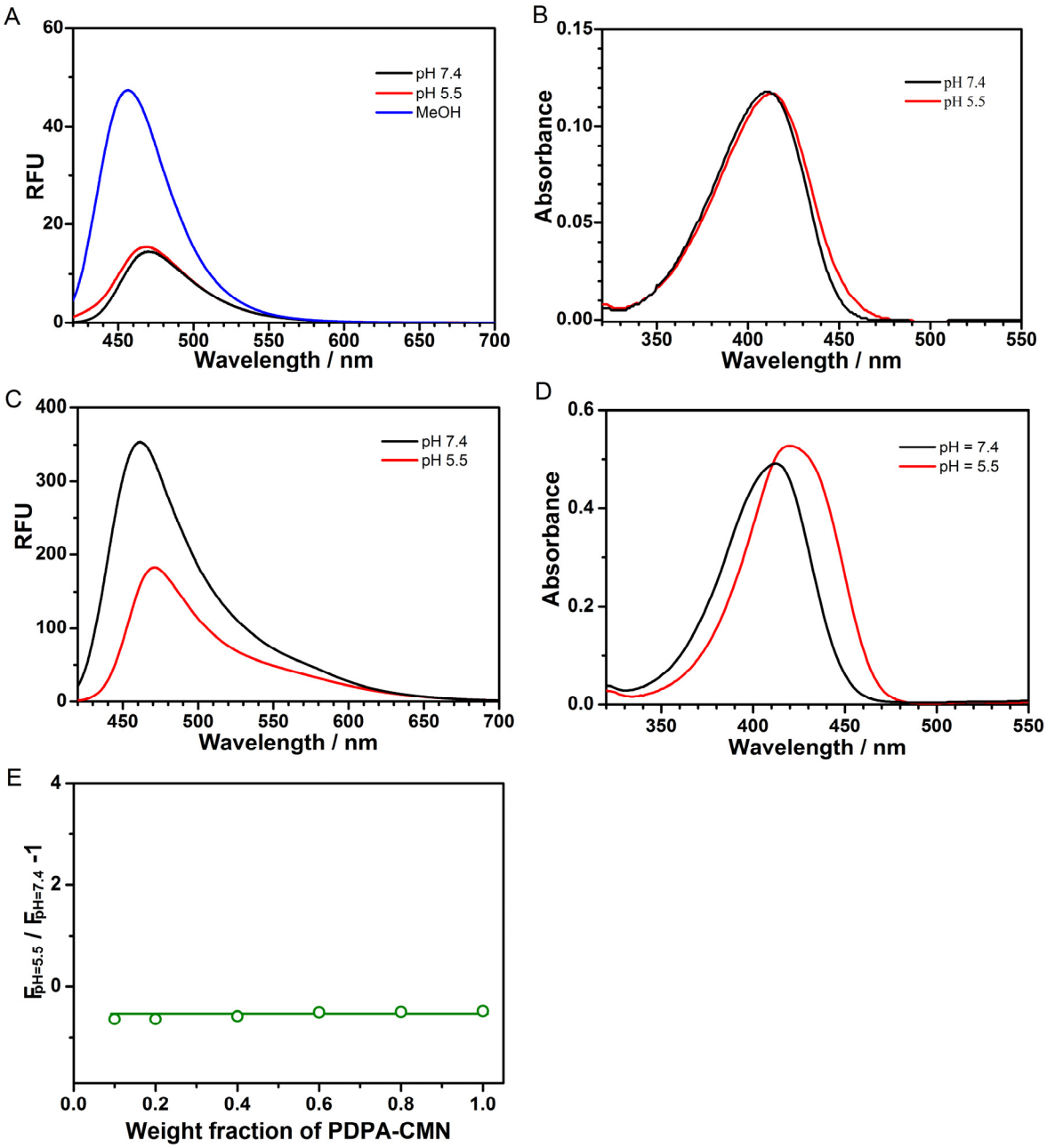

Figure S7. (A) Fluorescence and (B) UV-Vis absorption spectra of free coumarin aqueous solution at pH 7.4 and 5.5 (coumarin concentrations are 1 μg/mL). (C) Fluorescence emission and (D) UV-Vis absorption spectra of PDPA-CMN aqueous solution at pH 7.4 and 5.5 where the polymer concentration is at 200 μg/mL. (E) Change of the fluorescence intensity ratio at pH 5.5 over 7.4 as a function of weight percentage of PDPA-CMN in the molecular mixture of PDPA-CMN and its corresponding synthetic precursor (total polymer concentrations are at 200 μg/mL). For measurement of fluorescence emission, the samples were excited at 408 nm, and the emission spectra were collected from 420 to 700 nm.

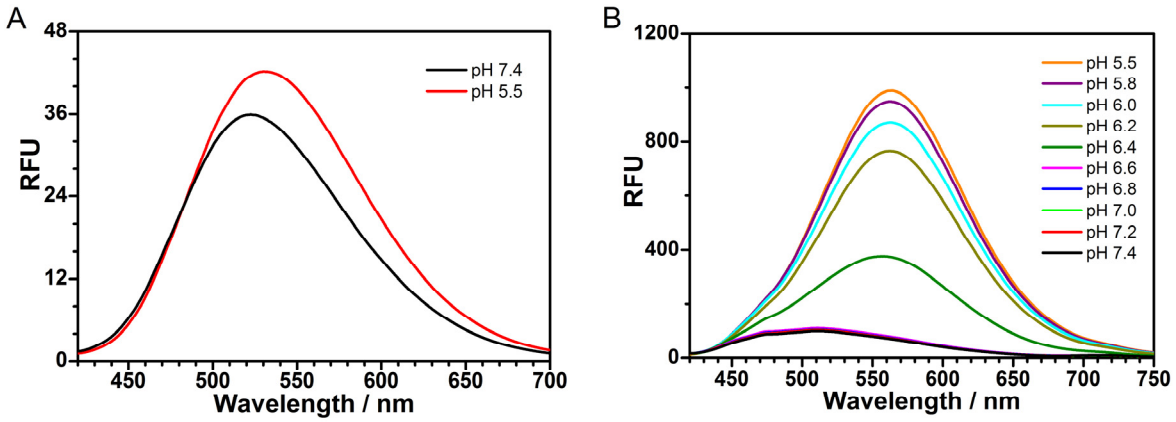

Figure S8. (A) Fluorescence emission spectra of free PPO dye in aqueous solution at pH 7.4 and 5.5 (PPO concentration is at 15 µg/mL). (B) pH dependent fluorescence emission spectra of PEO-(PDPA-PPO) copolymer in aqueous solution where the copolymer concentrations are at 500 µg/mL. For fluorescence emission experiments, the samples were excited at 408 nm.

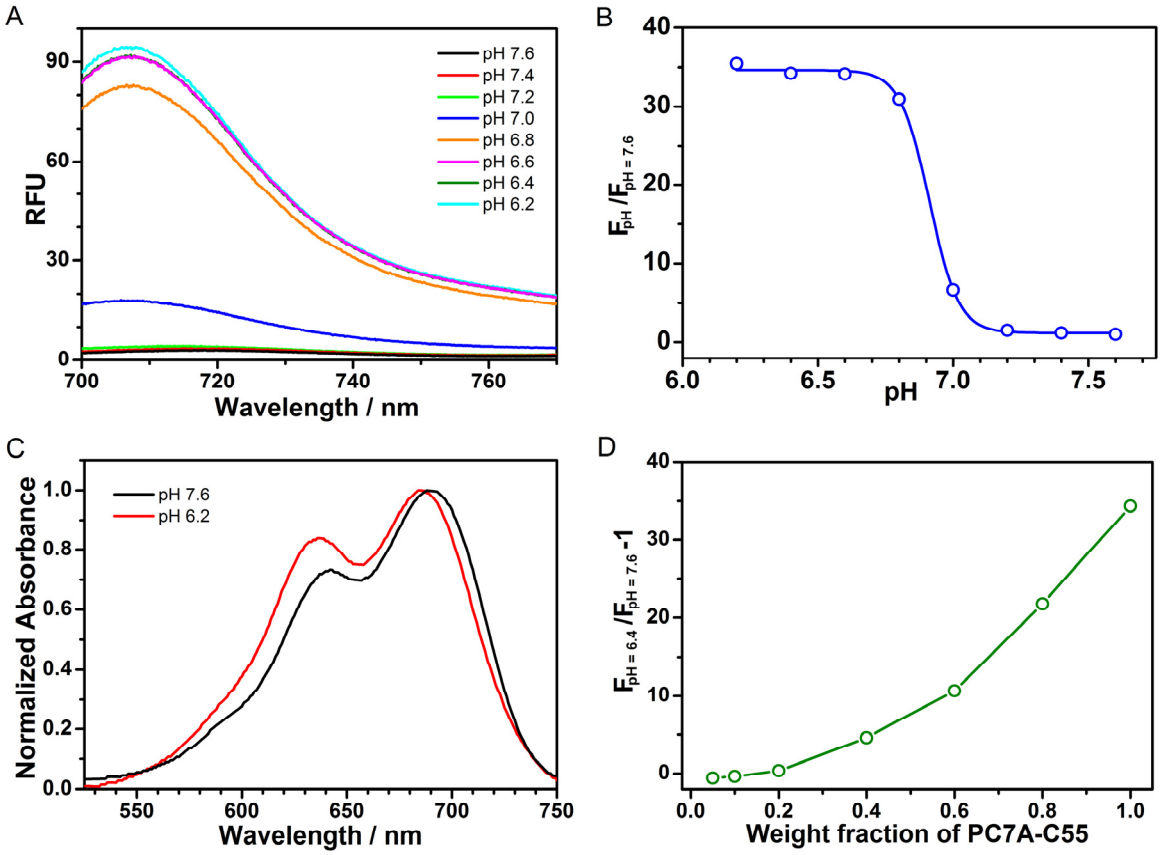

Figure S9. (A) pH dependent fluorescence emission spectra and (B) fluorescence intensity ratio of PC7A-C55 as a function of pH in aqueous solution (copolymer concentrations are at 200 μg/mL). The samples were excited at 690 nm, and the emission spectra were collected from 700 to 780 nm. (C) The absorption spectra with normalization to the monomer peak intensity of PC7A-C55 in aqueous solution at pH 7.6 and 6.2. (D) Change of the fluorescence intensity ratio as a function of weight percentage of PC7A-C55 in the molecular mixture of PC7A-C55 and its corresponding dye-free synthetic precursor.

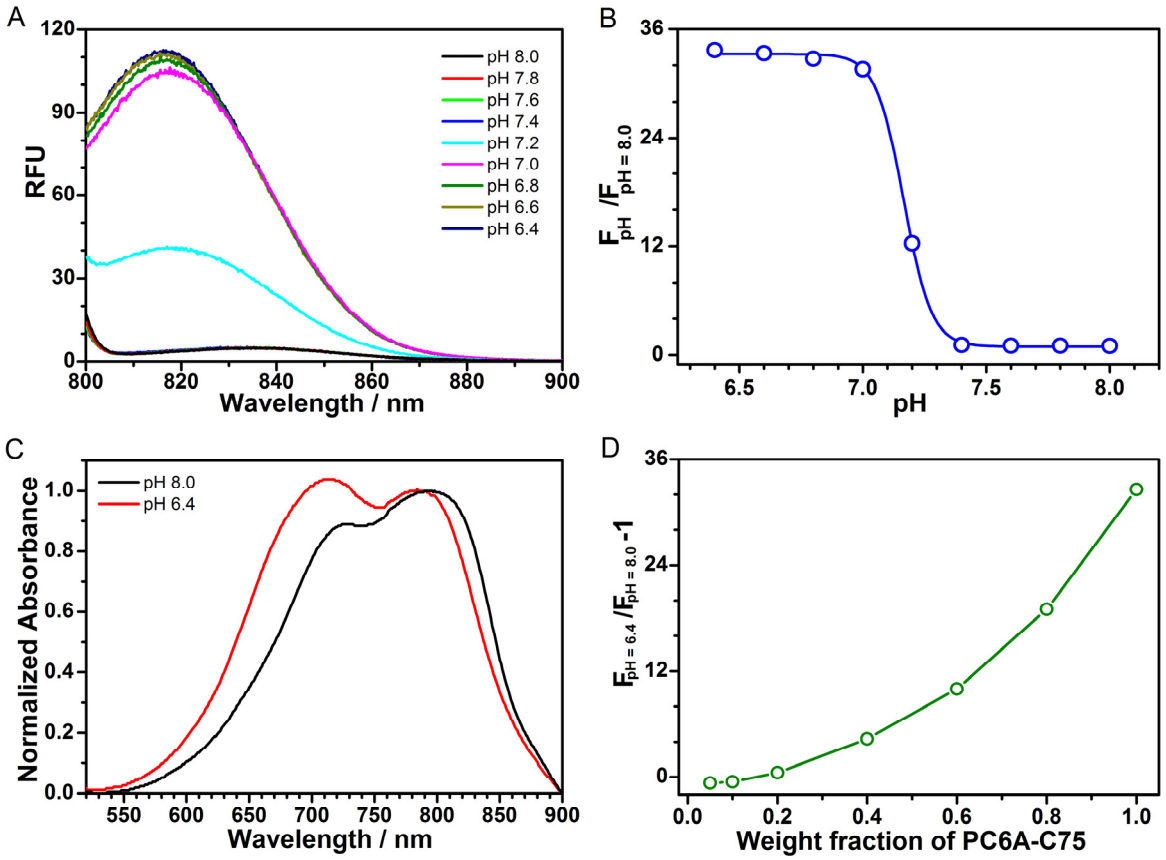

Figure S10. (A) pH dependent fluorescence emission spectra and (B) fluorescence intensity ratio of PC6A-C75 as a function of pH in aqueous solution (copolymer concentrations are at 200 μg/mL). The samples were excited at 790 nm, and the emission spectra were collected from 800 to 900 nm. (C) The absorption spectra with normalization to the monomer peak intensity of PC6A-C75 in aqueous solution at pH 8.0 and 6.4. (D) Change of the fluorescence intensity ratio as a function of weight percentage of PC6A-C75 in the molecular mixture of PC6A-C75 and its corresponding dye-free synthetic precursor.

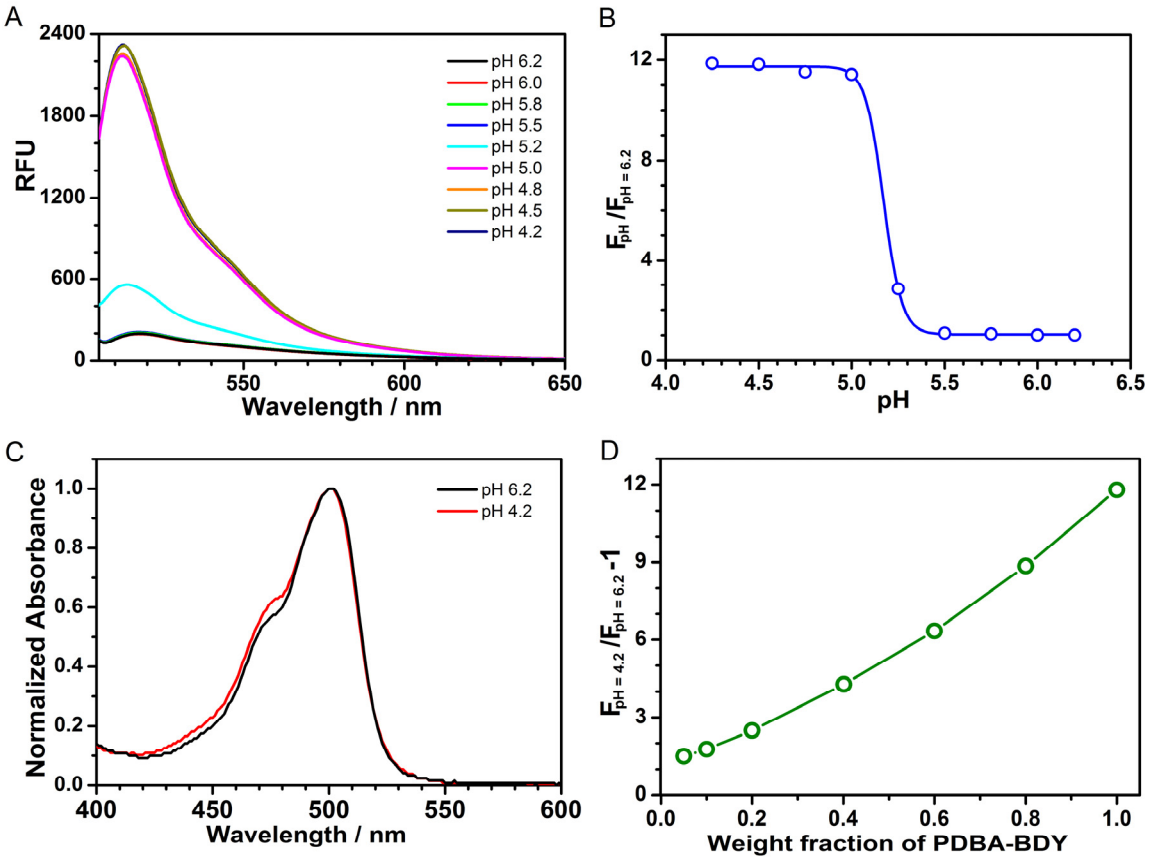

Figure S11. (A) pH dependent fluorescence emission spectra and (B) fluorescence maximum intensity ratio of PDBA-BDY in aqueous solution (copolymer concentrations are at 200 μg/mL). The samples were excited at 498 nm, and the emission spectra were collected from 505 to 650 nm. (C) The absorption spectra with normalization to the monomer peak intensity of PDBA-BDY in aqueous solution at pH 6.2 and 4.2. (D) Change of the fluorescence maximum intensity ratio as a function of weight percentage of PDBA-BDY in the molecular mixture of PDBA-BDY and its corresponding dye-free synthetic precursor.