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Electronic Supplementary Information for:

Nanocarriers with dual pH-sensitivity for enhanced tumor cell uptake and rapid intracellular drug release

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Experimental section

Materials

Poly(2-diisopropylaminoethyl methacrylate)-*block*-poly(2-aminoethyl methacrylate hydrochloride) (PDPA-*b*-PAMA, MW 9800, PDI 1.37, i.e., having 24 repeated units of DPA and 21 repeated units of AMA) was synthesized according to our previous report through atom transition radical polymerization (ATRP) method.[1] 3-(4,5-Dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma-Aldrich (USA). 2,3-dimethykmaleic anhydride (DMMA) was procured from TCI (China). Doxorubicin hydrochloride (DOX·HCl) was obtained from Dalian Melone biotechnology Co., Ltd., (China) and desalted with triethylamine.[2] Pyridine, triethylamine (TEA) and dimethyl sulfoxide (DMSO) were purchased from Tianjin Bodi Chemical Holding Co., Ltd. (China) and used after dried with CaH_2. All of the others reagents without further instruction were used as received. Ultrapure water with a resistivity of 18.2 MΩ was used throughout.

Synthesis of DMMA modified PDPA-b-PAMA (PDPA-b-PAMA/DMMA)

As shown in Fig. S1, PDPA-*b*-PAMA (400 mg), excessive DMMA (160 mg), pyridine (0.48 mL) and TEA (0.48 mL) were dissolved in DMSO (5 mL). The mixture was stirred at 40 ℃ for 24 h. After dilution with 20 mL of phosphate buffer solution (PBS, 0.01 M, pH = 8.0) to prevent acid-labile linkage from breakage, the resulting solution was dialyzed against ultrapure water (molecular weight cut-off 3500 Da). PDPA-*b*-PAMA/DMMA was obtained as white powder through lyophilizing from residual solution (Yield: 426 mg). ^{1}H NMR spectrum of PDPA-*b*-PAMA/DMMA (400 MHZ, DMSO-d_6, δ): 0.24-0.83 ppm (-C(C***H***$_3$)CH_2-), 0.961 ppm

(-(CH(C***H***$_3$)$_2$)$_2$), 1.89 ppm (-C(C***H***$_3$)=C(C***H***$_3$)-), 1.91 ppm (-C(CH$_3$)C***H***$_2$-), 2.90-3.85 ppm (-C(O)OCH$_2$CH$_2$NHC(O)-(C***H***(CH$_3$)$_2$)$_2$; -C(O)OCH$_2$C***H***$_2$NHC(O)-(CH(CH$_3$)$_2$)$_2$; -C(O)OCH$_2$C***H***$_2$NHC(O)-C(CH$_3$)=C(CH$_3$)-; overlap with H_2O of DMSO-d_6), 4.06-4.29 ppm (-C(O)OC***H***$_2$CH$_2$NHC(O)-C(CH$_3$)=C(CH$_3$)-; -C(O)OC***H***$_2$CH$_2$NHC(O)-(CH(CH$_3$)$_2$)$_2$)

^{1}H NMR spectroscopy

The ^{1}H NMR spectra were recorded with a Bruker Avance spectrometer (AVII-400; Bruker, Karlsruche, Germany) at 400 MHz in DMSO-d_6 using tetramethylsilane (TMS) as an internal standard or in D_2O with/without DCl (NaOD) for pH adjustion.

Preparation of PDPA-*b*-PAMA/DMMA micelles

The PDPA-*b*-PAMA/DMMA micelles were prepared by dialysis method. After PDPA-*b*-PAMA/DMMA (100 mg) absolutely dissolved into DMSO (2 mL), PBS (8 mL, 0.01M, pH 8.0) was dropwise added. The mixture was continuously stirring for additional 3 h. Finally, the final solution was dialysed against ultrapure water to remove DMSO. The resulting micelle solution was passed through a 0.45 μm filter and stored at 4 ℃ for further measurements.

Determination of critical micelle concentration (CMC)

PDPA-*b*-PAMA/DMMA block copolymer can self-assemble into micelles with PDPA block as the hydrophobic inner core and PAMA/DMMA block as the hydrophilic outer corona in aqueous at a concentration value higher than its CMC. The CMC value was determined by

fluorescence spectroscopy using pyrene as fluorescence probe at pH 7.4 condition. Steady-state fluorescent spectra were recorded on a Shimadzu RF-5301PC luminescence spectrometer at room temperature. An aliquot of 1 mL acetone solution of pyrene was transferred into 20 mL vials and the acetone evaporated to dryness. Then a series of micellar solution ranging from 1×10^{-5} to 1.0 mg mL^{-1} were added into the vials to give a final pyrene concentration 6×10^{-7} mol L^{-1}. The solution was incubated at room temperature overnight under shaking. The measured emission wavelength was set at 395 nm and band width of emission was set to 3 nm. The ratios of the excitation spectra's fluorescent intensities at 337.6 nm and 335.2 nm ($I_{337.6}/I_{335.2}$) were calculated and plotted against the logarithm of polymer mass concentration.

Dynamic light scattering (DLS) and zeta potential

The zeta potential, hydrodynamic diameter (D_h) and size distribution of polymeric self-assemblies were measured at different pH conditions by DLS instrument (Zetasizer Nano ZS 90, Malvern, UK) with a He-Ne laser (λ = 633 nm) at a scattering angle of 90°. To investigate the zeta potential, D_h and size distribution of micelles at different pH conditions, polymeric micelles were dissolved in pre-prepared buffer solution (0.2 mol L^{-1} Na_2HPO_4 and 0.1 mol L^{-1} citric acid) with corresponding pH values. The final concentration of polymeric was 0.5 mg mL^{-1}. Before being transferred into sample cuvette, the polymer solution was subjected to ultrasonic treatment. Each sample was measured three times at 37 ℃ and the D_h were obtained from the average of three measurements (n = 3).

Transmission electron microscopy (TEM)

The morphologies of micelles were observed by TEM (Hitachi H-600 transmission electron microscope, JOEL Ltd., Japan) at an accelerating voltage of 75 kV. Samples were dissolved in predetermined buffer solution with different pH values. After that, a drop of pre-prepared copolymer solution (0.5 mg mL^{-1}) was deposited on a carbon-coated copper grid (200 meshes). The films on the grid were negatively stained with 1 wt/v% phosphotungstic acid for 60 s. Finally, after a thorough air-drying, the stained samples were photographed.

Protein adsorption of the micelles and quantification of the adsorbed bovine serum albumin (BSA)

BSA was employed as model protein to determine the effect of different pH on protein adsorption on micelles. 2 mg of PDPA-*b*-PAMA/DMMA or PDPA-*b*-PAMA micelles were co-incubated with 400 μg of BSA buffer solutions (4 mL, 0.01M, pH = 7.4, 6.5 and 5.0), respectively. After incubation at 37 ℃ for 1 h, the mixture was centrifuged at 16000 rmp for 15 min to precipitate the BSA adsorbed micelle. Then, the supernatant was collected for BSA quantification.

The content of BSA in was analyzed with commercial BCA Protein Assay Kits (Beyotime, Shanghai, China). 20 μL of polymeric micellar supernatant (n = 6) and 200 μL of BCA reagent were charged into 96-well plate and subsequently co-incubated at 37 ℃ for 30 min. According to the pre-prepared calibration curve, the residual concentration of BSA was calculated by a micro plate reader (Spectra Plus, Tecan, Zurich, Switzerland) recorded the adsorption intensity at 570 nm.

Preparation of DOX-loaded micelles and quantification of loaded DOX

Drug-loaded PDPA-*b*-PAMA/DMMA was prepared by step-wise dialysis technique. 50 mg of PDPA-*b*-PAMA/DMMA sample and 10 mg of desalted DOX were mixed in 4 mL DMSO. After stirring overnight in a sealed vial at room temperature, the organic solution was dialyzed against distilled water (molecular weight cut-off 3500 Da). The ultrapure water was replaced every 2 h for the first 10 h and then every 4 h for 1 day. The DOX-loaded micelles were passed through 0.45 μm filters and collected for further determinations. All operations were performed in photophobic condition.

After re-dissolving of the above prepared DOX-loaded lyophilized samples in DMSO, the content of DOX loaded in polymeric micelles were detected by fluorescence correlate spectrophotometer (Shimadzu RF-5301PC) with the excitation wavelength, emission wavelength and slit at 480, 555 and 3 nm, respectively. Subsequently, according to the pre-established calibration curve, the DOX loading efficiency (LE) and entrapment efficiency (EE) were calculated using following Equations (1) and Equations (2), respectively:

$$\text{Loading efficiency (\%)} = \frac{M_{DOX}}{M_{Polymer} + M_{DOX}} \times 100 \qquad (1)$$

$$\text{Encapsulation efficiency (\%)} = \frac{M_{DOX}}{M_{added}} \times 100 \qquad (2)$$

In which M_{DOX} is the mass of DOX loaded in micelles, $M_{Polymer}$ is the mass of copolymers in formulations and M_{added} is the mass of added DOX.

In vitro release profiles of DOX from micelles

The drug release behaviors of DOX-loaded micelles were handled in buffer solutions (0.01 M, pH = 7.4, 6.5 and 5.0). 10 mL of DOX-loaded micellar solution (at a concentration of 0.5 mg mL^{-1})

was added into dialysis tubes (MWCO 3500). Subsequently, it was immerged into 90 mL of buffer solution to incubate at 37 ℃ in an incubator shaker (100 rpm) for drug release investigation. At pre-determined time intervals, the external drug release buffers of dialysis bags were collected and replaced by fresh buffers. The concentration of released drug were detected using fluorescence correlate spectrophotometer (Shimadzu RF-5301PC) with the excitation wavelength, emission wavelength and slit at 480, 555 and 3 nm, respectively. The cumulative amount of the released DOX was calculated according to the pre-established calibration curve. Finally, the percentage of DOX released from micelles was plotted against time.

Intracellular DOX release

The intracellular release behaviors of DOX-loaded micelles were determined by a TCRS SP5 confocal laser scanning microscopy (CLSM, Leica, Germany) using HeLa cells. In brief, HeLa cells in culture media (artificially modulating the pH to 6.5) were incubated for 1 day in 6-well cell culture plates with a clean cover slip placed into each well to ~70% confluency (~2×10^5 cells/well). Then the culture media was replaced by fresh media containing DOX-loaded PDPA-*b*-PAMA/DMMA (filtered through 0.45 μm syringe driven filters before used) at a concentration of 0.5 mg mL^{-1}. After 6 h, the culture media was removed and the cells were washed three times with PBS (pH 7.4). Then, cell lysosomes were stained with Lysotracker Green DND-26 (250 nM) for 20 min and the cells were fixed with 4 w/v% formaldehyde at 4 ℃ for 20 min. After that, cell nuclei were stained with Hoechst 33342 (5 μg mL^{-1}) for additional 10 min. Finally, the cover slips were placed on a glass microscope slide and the prepared samples were subjected for fluorescence imaging by CLSM. DOX was excited at 480 nm with emission at 555

nm, and 504/511 nm, 352/504 nm for Lysotracker Green DND-26 and Hoechst 33342, respectively.

Cytotoxicity assay (MTT)

The *in vitro* cytotoxic behavior of PDPA-PAMA/DMMA blank micelles was evaluated by standard MTT assay against HeLa cells. HeLa cells without polymers were used as control. In brief, the cells were cultured with 10% heat-inactivated fetal bovine serum (FBS), 100 units mL^{-1} of penicillin and 100 μg mL^{-1} of streptomycin in Dulbecco's modified eagle medium (DMEM) at 37 ℃ with an atmosphere of 5% CO_2 and 95% relative humidity. Growing cells (10^4 cells/well) were seeded on 96-well micro plate (Nunc Co., Wiesbaden, Germany) and incubated in 100 μL of DMEM/well for 24 h. Then fresh culture media containing serial dilutions of polymeric micelles was used to treat the cells for 24 h. After that, cells were incubated with 10 μL of MTT stock solution in PBS (5 mg mL^{-1}) each well for 4 h. Then the medium was removed and the produced formazan crystals were solubilized in DMSO (100 μL/well). The absorbance was measured at 570 nm by a micro plate reader (Spectra Plus, Tecan, Zurich, Switzerland). The relative cell viability (%) was calculated using the equation (3):

$$\text{Cell viability} = \frac{A_{test}}{A_{control}} \times 100\% \qquad (3)$$

where A_{test} and $A_{control}$ are the mean absorbance value of treatment group and the mean absorbance value of control group (without the polymers), respectively.

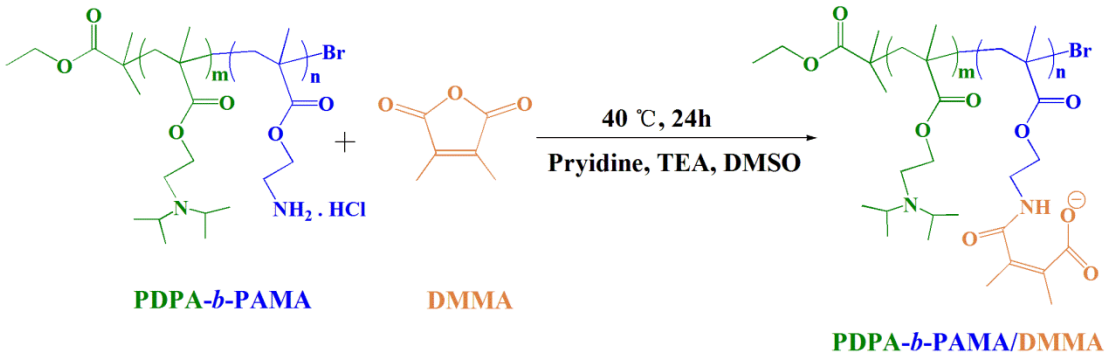

Fig. S1. Synthetic route of PDPA-*b*-PAMA/DMMA.

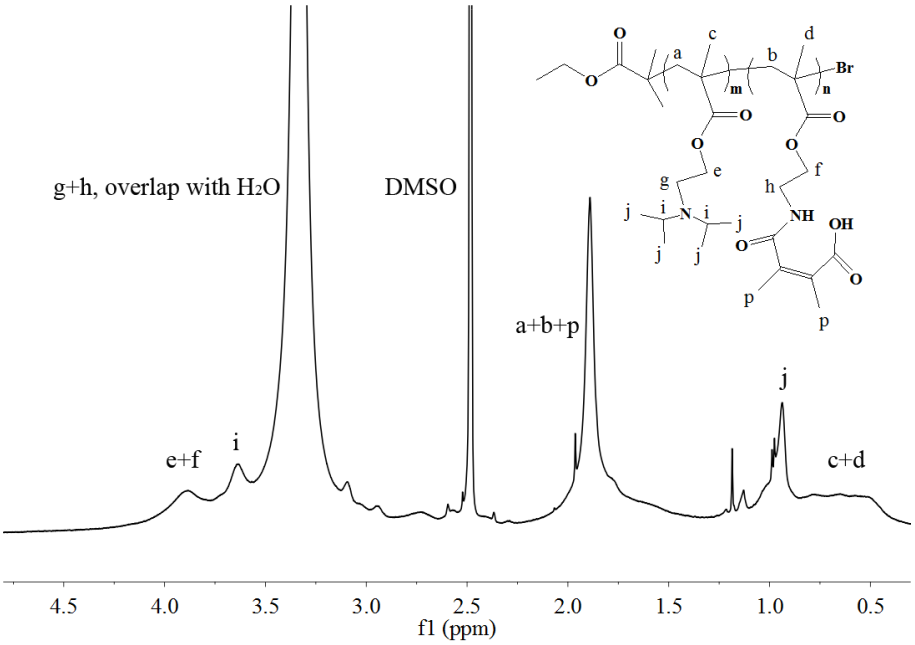

Fig. S2. ^{1}H NMR spectrum of PDPA-*b*-PAMA/DMMA in DMSO-d_6.

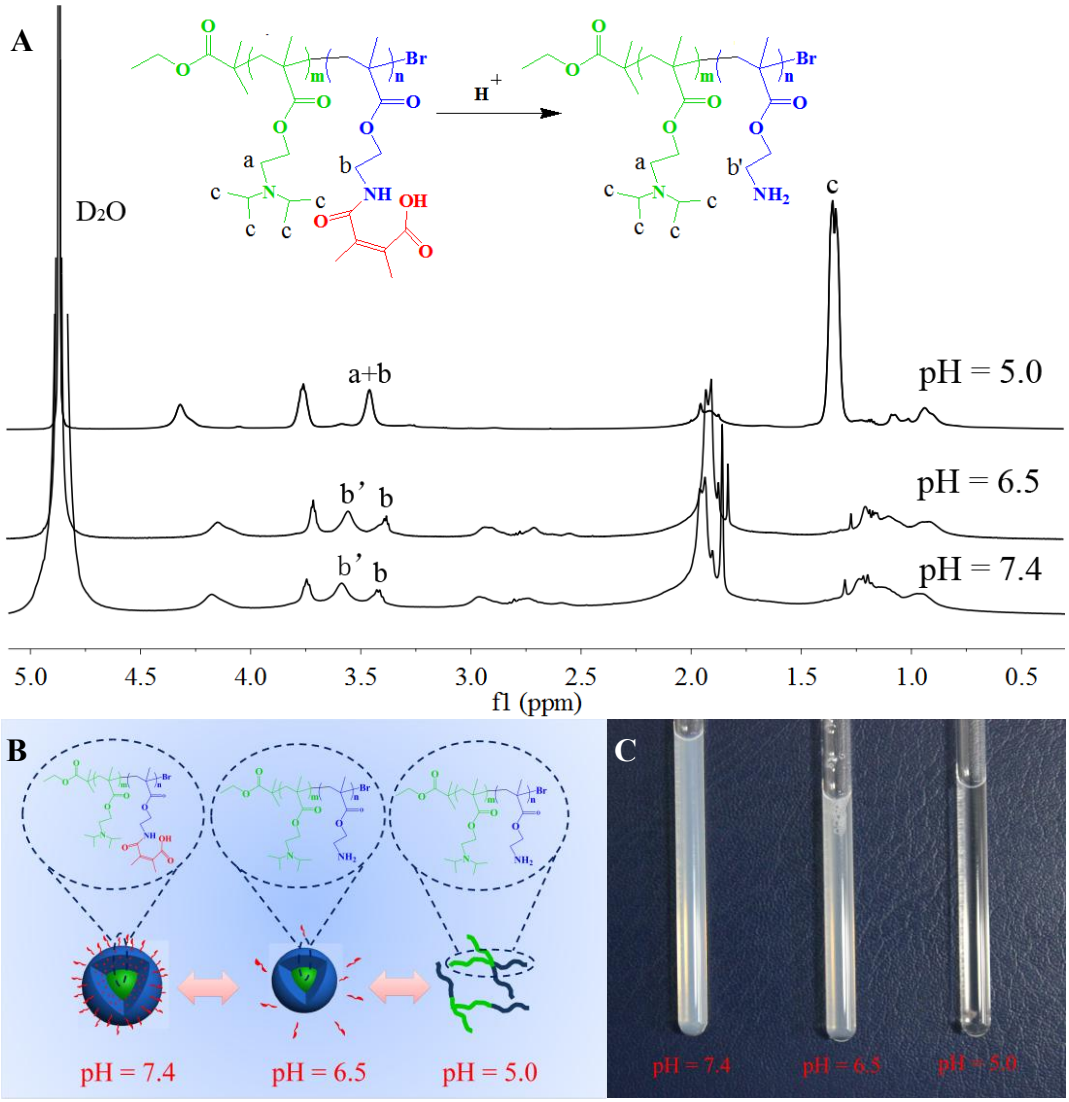

Fig. S3. ^{1}H NMR spectra (A), illustration (B) and photographs (C) of PDPA-*b*-PAMA/DMMA self-assemblies at different pHs (7.4, 6.5 and 5.0).

As shown in Fig. S3, PDPA-PAMA/DMMA can self-assemble into micelles with the hydrophobic PDPA inner core at pH 7.4 and 6.5. However, when the pH value decreased to 5.0, the hydrophilicity of fully protonated PDPA block from hydrophobic to hydrophilic,[3] i.e., the block copolymer shows double hydrophilicity instead of amphiphilicity. The double hydrophilicity of block copolymer leads to the disassembly of micelle, which has benefit to fast intracellular drug release for efficient tumor killing.

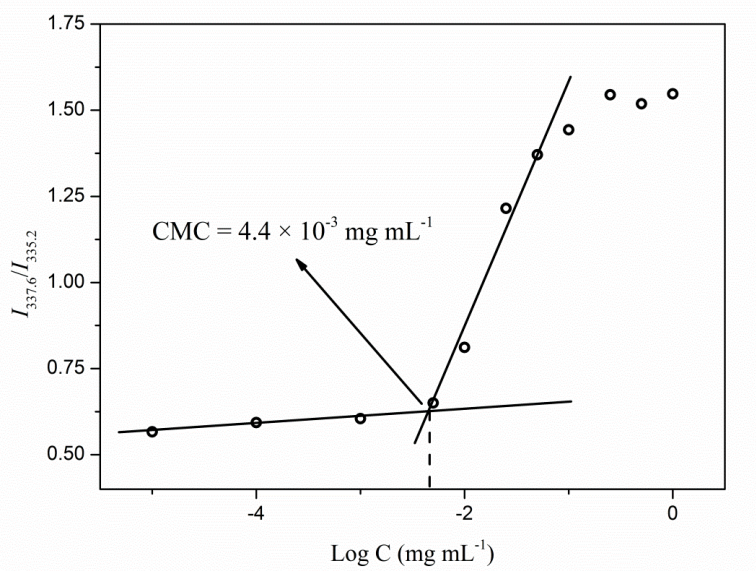

Fig. S4. Plots of fluorescence intensity ratio $I_{337.6}/I_{335.2}$ from pyrene excitation spectra against the concentration of PDPA-*b*-PAMA/DMMA at pH 7.4. The black dash line indicated the CMC value.

CMC is paramount parameter of thermodynamic stability for micelle system under high dilution, especially for micelles used for *in vivo* drug delivery. The CMC value of PDPA-*b*-PAMA/DMMA was measured using pyrene fluorescence method and calculated by plotting the fluorescence intensity ratio of $I_{337.6}/I_{335.2}$ against concentration. As demonstrated in Fig. S3, PDPA-*b*-PAMA/DMMA has low CMC value, exhibiting potential application in drug delivery system.

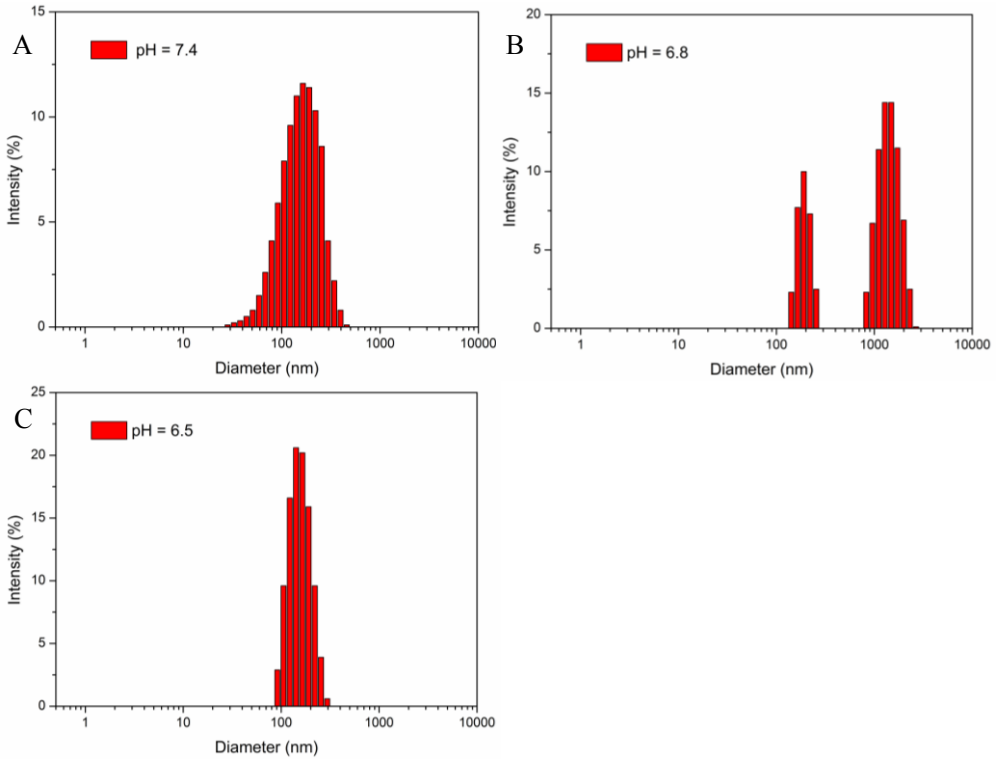

Fig. S5. Size distribution of PDPA-*b*-PAMA/DMMA micelles at different pH (0.5 mg mL^{-1}; 37 ℃).

The D_h size distribution and zeta potential of polymeric self-assemblies at different pH are measured at a final concentration of 0.5 mg mL^{-1} at 37 ℃ . PDPA-*b*-PAMA/DMMA micelles show a narrow D_h size distribution at pH = 7.4 and 5.0 (Fig. S3A, S3B). However, it displays a bimodal size distribution at pH = 6.8, which may be resulted from the secondary aggregation through the zwitterionic surface electric attraction caused by partial DMMA hydrolysis.

Table S1. Characteristic of the drug-loaded micelle.

Sample	Polymer/drug (w/w)	LE (%)	EE (%)
PDPA-*b*-PAMA/DMMA	5:1	15.7	81.1

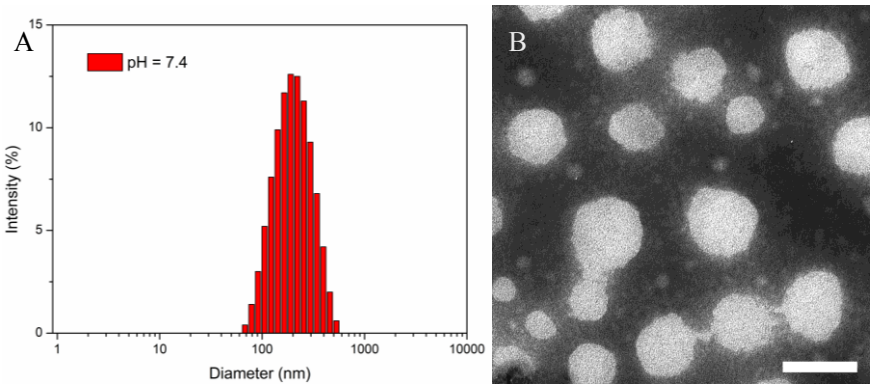

Fig. S6. Size distribution (A) of DOX-loaded PDPA-*b*-PAMA/DMMA micelles at 37 ℃ and their TEM micrograph (B) (0.5 mg mL^{-1}; pH 7.4). The scale bar in TEM micrograph is 50 nm.

After loading of desalted DOX into the hydrophobic PDPA block core structure, the D_h size and morphology were measured by DLS and TEM. The D_h size detected by DLS and the dehydrated size observed by TEM are 187.6 ± 12.4 nm and ~ 50 nm, respectively. Compared with the drug-free micelles, the slight increase of diameter size of DOX-loaded micelle is because that the incorporation of DOX expands the volume of micellar core. Yet, the diameter size of TEM micrographs is smaller than that of DLS measurements. That is because of TEM and DLS measurements at absolutely dried and hydrated status, respectively.

Referance

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