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Supporting Information

For

Zwitterionic Shielded Polymeric Prodrug with Folate-Targeting and pH Responsiveness for Drug Delivery

Lei Li[1], Yue Song[1], Jinlin He[1], Mingzu Zhang[1], Jian Liu[2], Peihong Ni[1]*

[1]College of Chemistry, Chemical Engineering and Materials Science, State and Local Joint Engineering Laboratory for Novel Functional Polymeric Materials, Jiangsu Key Laboratory of Advanced Functional Polymer Design and Application, Suzhou Key Laboratory of Macromolecular Design and Precision Synthesis, Soochow University, Suzhou 215123, People's Republic of China

[2]Institute of Functional Nano and Soft Materials (FUNSOM), Soochow University, Suzhou, 215123, P. R. China

To whom correspondence should be addressed. (E-mail: phni@suda.edu.cn)

Experimental section

Materials. The following agents were purchased and used without further purification: 2-methacryloyloxyethyl phosphorylcholine (MPC, 97%, Nanjing Joy-Nature Science &Technology Development Institute), poly(ethylene glycol) methacrylate (PEGMA, $\overline{M_n}$ = 500 g/mol, Aldrich), 2-propynylamine (98%, Shanghai Macklin Biochemical), 4-dimethylamino pyridine (DMAP, 99%, 9 Ding chemistry), *N,N′*-Diisopropylcarbodiimide (DIC, 98%, Aladdin), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC, 99%, Energy Chemical) doxorubicin hydrochloride (DOX·HCl, 99%, Beijing Zhongshuo Pharmaceutical Technology Development), folic acid (FA, 97%, Sinopharm Chemical Reagent), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte- trazolium bromide (MTT, 98%, Sigma-Aldrich). The chain transfer agent, 4-cyano-4-ethylsulfanylthiocarbonylsulfanylentanoic acid (CEP), was synthesized according to previous report.[1,2] Azodiisobutyronitrile (AIBN, A. R., Enox) was purified by recrystallization from ethanol. Milli-Q water (18.2 MΩ cm at 25 °C) was generated using a water purification system (Simplicity UV, Millipore). The other part including solvents such as *N,N*-Dimethylformamide (DMF, A. R., Enox), dimethyl sulfoxide (DMSO, A. R., Enox) and triethylamine (TEA, A.R., Enox) were dried over sodium sulfate anhydrous (Na_2SO_4, 99%, Enox) for 24 h and distilled before use.

Characterizations. ^{1}H NMR and ^{13}C NMR spectra were recorded on the 400 MHz spectrometer (INOVA-400) using deuterated dimethyl sulfoxide-d_6 (DMSO-d_6) as the solvent and tetramethylsilane (TMS) as internal standard. The molecular weights ($\overline{M_n}$) and polydispersity index (PDI) was measured by gel permeation chromatography (GPC) equipped with a Waters 1515 GPC setup equipped with a refractive index detector and a column set (2×PL Aquagel-OH Mixed-M). GPC columns were eluted with degassed buffer that consisted of 0.20 M $NaNO_3$ and 0.01 M Na_2HPO_4 at pH 9.0. Near-monodisperse poly(ethylene oxide) standards (Agilent, 17.8−692.0 kDa) were used for calibration. Free DOX and polymeric prodrug were determined by high performance liquid chromatography (HPLC) (UltiMate 3000, Thermo Fisher Scientific) at 30 °C with acetonitrile/Milli-Q water (50:50, v/v) as the mobile phase at a flow rate of 1.0 mL min^{-1}. The ultraviolet-visible (UV−vis) absorption spectra were recorded at 480 nm on UV−vis spectrophotometer (UV-3150, Shimadzu), and the fluorescence spectra were recorded on a fluorescence spectrophotometer (Cary Eclipse, Agilent Technologies). The morphologies of

polymeric prodrug nanoparticles were observed by TEM instrument (HT7700, Hitachi) operated at an accelerating voltage of 120 kV.

Self-assembly Behavior of Prodrug. The critical aggregation concentration (CAC) values were determined by the fluorescence probe method using pyrene as the hydrophobic probe. Typically, a predetermined pyrene solution in acetone was respectively added into a series of ampoules. Acetone was evaporated and replaced with prodrug nanoparticles at different concentrations in the range of 500 to 2×10^{-4} mg L^{-1}. The final concentration of pyrene in each ampoule was 6×10^{-6} mol L^{-1}. The samples were sonicated for 20 min, stirred at room temperature for 48 h, and analyzed on fluorescence spectrophotometer (Cary Eclipse, Agilent Technologies) at the excitation wavelength of 335 nm and an emission wave length of 350 to 550 nm, with both bandwidths set at 2.5 nm. From the pyrene emission spectra, the intensity ratio (I_3/I_1) of the third band (382 nm, I_3) to the first band (371 nm, I_1) was analyzed as a function of polymer concentration. The CAC value was defined as the point of intersection of the two lines in the plot of fluorescence versus polymer concentration. [3,4]

The average particle size ($\overline{D}_z$) and size polydispersity index (size PDI) of the prodrug nanoparticles were determined using DLS instrument (Zetasizer Nano ZS, Malvern), while the morphology was observed on a TEM instrument (HT7700, Hitachi) operated at 120 kV. Briefly, P(MPC-*co*-PEGMA-BZ)-*g*-DOX (PMPD1) and FA-P(MPC-*co*-PEGMA-BZ)-*g*-DOX (FA-PMPD1) (25 mg) prodrug were dissolved in 25 mL phosphate buffer solutions (PBS) by ultrasound and further stirred for 24 h before use, respectively. The concentration of prodrug nanoparticles was 1 mg mL^{-1}. Normally, using a freeze-drying method to gain the TEM sample, 10 μL of the solution was dripped onto the carbon-coated copper grid and the solvent in its frozen solid state was directly removed without melting in a freeze-drier. The morphology of the prodrug nanoparticle was then imaged on a normal TEM at room temperature.

***In Vitro* Drug Release from PMPD1 and FA-PMPD1 Prodrug Nanoparticles.** PMPD1 and FA-PMPD1 polymeric prodrug (25 mg) were dissolved 30 mL PBS solution (10 mM, pH 7.4) by ultrasound and further stirred for 24 h before use, respectively. The solution were then filtered through a Φ 0.45 mm Millipore filter and some of which were finally lyophilized for calculating the DOX content. Then, each 5 mL of the prodrug nanoparticles was transferred into a dialysis

membrane (MWCO 7000), and the dialysis membranes were placed into a series of centrifuge tubes with 30 mL of phosphate buffer solutions (pH 7.4) or acetate buffer solution (pH 5.0). All the centrifuge tubes were kept constantly shaking with a speed of 160 rpm at 37.5 °C. At predetermined intervals, 5 mL of the solution was taken out and replenished with an equal volume of the corresponding fresh buffer solution. The fluorescence spectrophotometer was employed to determine the content of releasing DOX. The excitation wavelength was set at 480 nm while emission spectra were recorded with a 5 nm slit width over a wavelength from 500 to 650 nm. The DOX and FA content (*wt*%) were calculated according to the eqn (S1) and (S2), respectively:

$$C_{\mathrm{DOX}}(wt\%) = \frac{C_{\mathrm{UV-vis}}}{C_{\mathrm{FA\text{-}PMPD}}} \times 100 \qquad \text{(S1)}$$

where $C_{\mathrm{UV-vis}}$ represents the concentration of DOX measured by UV−vis, while $C_{\mathrm{FA\text{-}PMPD}}$ is the concentration of prodrug nanoparticles.

$$C_{\mathrm{FA}}(wt\%) = \frac{C_{\mathrm{UV-vis}}}{C_{\mathrm{FA\text{-}PMPD}}} \times 100 \qquad \text{(S2)}$$

where $C_{\mathrm{UV\text{-}vis}}$ represents the concentration of FA measured by UV−vis, while $C_{\mathrm{FA\text{-}PMPD}}$ is the concentration of prodrug nanoparticles.

***In Vitro* Cytotoxicity Test.** A standard MTT assay was employed to evaluate the cytotoxicity of P(MPC-*co*-PEGMA-BZ), P(MPC-*co*-PEGMA-BZ)-*g*-DOX, and FA-P(MPC-*co*-PEGMA- BZ)-*g*-DOX. L929 cells, HeLa cells, and HepG2 cells were seeded in 96-well plates at a density of about 5×10^4 cells per well and cultured in DMEM culture medium with 10% serum and 1% penicillin/streptomycin in an incubator at 37 °C under a 5% CO_2 atmosphere for 24 h. Then, free DOX, P(MPC-*co*-PEGMA-BZ) coploymer solutions, and FA-P(MPC-*co*-PEGMA-BZ)-*g*-DOX prorug nanoparticles with different concentrations were separately added into each well and incubated with the cells for another 48 h, repectively.

Afterwards, 25 μL of the MTT stock solution (5 mg mL^{-1} in PBS) was added to each well. After incubation for another 4 h, the DMEM medium was removed and 150 μL of DMSO was added to each well. The optical density (OD) at 570 nm of each well was measured on a microplate reader (Bio-Rad 680). The cell viability was calculated according to the following eqn (S3):

$$\text{Cell viability (\%)} = \frac{\text{OD}_{\text{treated}}}{\text{OD}_{\text{control}}} \times 100 \qquad \text{(S3)}$$

where $OD_{treated}$ and $OD_{control}$ stand for the OD values of the wells treated with samples and the control wells without sample, respectively. The data are gathered and processed as the average values with standard deviations. The IC_{50} value of PMPD1 and FA-PMPD1 prodrug nanoparticles were calculated by GraphPad Prism 5 software.

Cellular Uptake and Intracellular Release of DOX. Cellular uptake and intracellular drug release studies of P(MPC-*co*-PEGMA-BZ)-*g*-DOX and FA-P(MPC-*co*-PEGMA-BZ)-*g*-DOX prodrug in HeLa cells were real-time monitored using the live cell imaging system (Cell'R, Olympus, Japan). HeLa cells were seeded in Φ 35 mm glass Petri dish at 1.0×10^5 cells and cultured in high-glucose DMEM culture medium at 37 °C under a 5% CO_2 atmosphere for different times. Subsequently, the culture medium was removed by washing three times using phosphate buffer saline (PBS). The cell nucleus was stained with H 33342 for 15 min. Afterwards, the culture medium was replaced with a DMEM medium containing free DOX, P(MPC-*co*-PEGMA-BZ)-*g*-DOX and FA-P(MPC-*co*-PEGMA-BZ)-*g*-DOX (4 mg L^{-1} of DOX). The images were captured at excitation wavelengths of 480 nm (red) and 340 nm (blue) for 6 h.

Flow Cytometry Analysis. The cellular uptake of free DOX, P(MPC-*co*-PEGMA-BZ)-*g*-DOX, and FA-P(MPC-*co*-PEGMA-BZ)-*g*-DOX against HeLa cells was measured using a BD FACS Verse (BD Biosciences, San Jose, CA) flow cytometry. Typically, HeLa cells were cultured in Φ 35 mm culture dish with the density of 5×10^4 cells cm^{-2} and adhered for 12 h. After that, 1 mL of fresh medium was prepared to replace the culture medium, which was either P(MPC-*co*-PEGMA-BZ)-*g*-DOX and FA-P(MPC-*co*-PEGMA-BZ)-*g*-DOX, and the final DOX concentration was kept at 4 mg L^{-1}. At the designed time, the culture medium was removed and the cells were washed three times with PBS. Subsequently, the cells were digested with trypsin, and each culture dish was added with 2 mL of new culture medium. The solutions were centrifuged at 1200 rpm for 5 min. At last, the residual cells were dispersed by adding with 0.5 mL of PBS, and the data were analyzed with Cell Quest software.

Reference

(1) Xu, X. W.; Smith, A. E.; Kirkland, S. E.; McCormick, C. L. Aqueous RAFT Synthesis of pH-Responsive Triblock Copolymer mPEO−PAPMA−PDPAEMA and Formation of Shell Cross-Linked Nanoparticles. *Macromolecules* **2008**, *41*, 8429-8435.

(2) Convertine, A. J.; Benoit, D. S. W.; Duvall, C. L.; Hoffman, A. S.; Stayton, P. S. Development of a Novel Endosomolytic Diblock Copolymer for siRNA Delivery. *J. Controlled Release* **2009**, *133*, 221-229.

(3) Wilhelm, M.; Zhao, C. L.; Wang, Y. C.; Xu, R. L.; Winnik, M. A.; Mura, J. L.; Riess, G.; Croucher, M. D. Poly (styrene-ethylene oxide) Block Copolymer Micelle Formation in Water: A Fluorescence Probe Study. *Macromolecules* **1991**, *24*, 1033-1040.

(4) Schacher, F.; Walther, A.; Ruppel, M.; Drechsler, M.; Müller, A. H. E. Multicompartment Core Nanoparticles of Triblock Terpolymers in Organic Media. *Macromolecules* **2009**, *42*, 3540-3548.

Supplementary Figures

1. List of Supplementary Figure Captions:

Figure S1. ^{1}H NMR spectrum of PEGMA-BZ monomer.

Figure S2. ^{13}C NMR spectrum of PEGMA-BZ monomer.

Figure S3. ^{1}H NMR spectrum of 4-cyano-4-ethylsulfanylthiocarbonylsulfanylentanoic acid (CEP).

Figure S4. ^{31}P NMR spectrum of P(MPC-*co*-PEGMA-BZ) random copolymer.

Figure S5. ^{1}H NMR spectrum of propargyl folic acid.

Figure S6. UV−vis spectra of (A) PMPD1 prodrug, (B) PMP1, and (C) the reaction product of PMP1 with triethylamine.

Figure S7. UV−vis spectra of free folate and FA-PMPD1 prodrug in ethanol.

Figure S8. Intensity ratios (I_3/I_1) as a function of Log C for (A) PMPD1 and (B) FA-PMPD1 prodrug nanoparticles in pH 7.4 buffer solution.

Figure S9. TEM image of PMPD1 prodrug nanoparticles (A) and the particle size distribution curve (B) corresponding to the TEM sample (scale bar 200 nm). The concentration was 0.5 mg mL^{-1}.

Figure S10. Variation of particle size for the FA-PMPD1 prodrug nanoparticles in pH 7.4 buffer

solution at different times.

Figures for Various Measurements

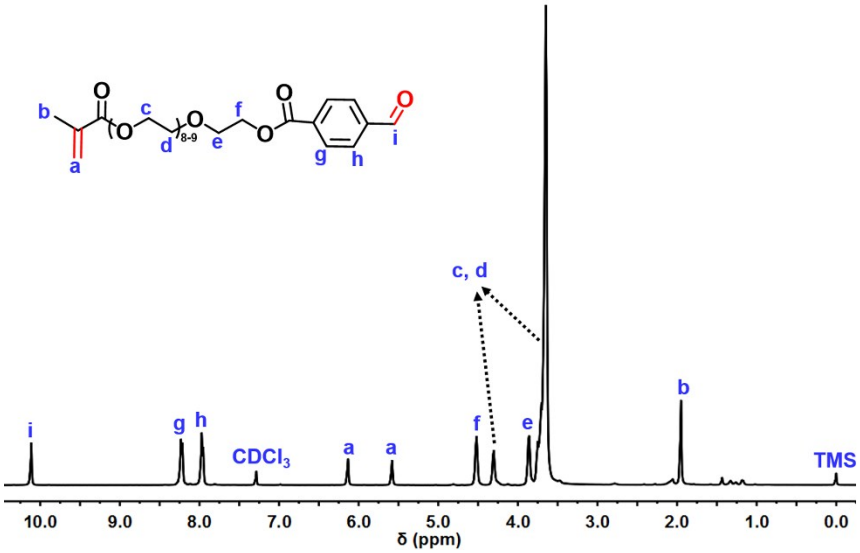


Figure S1. ^{1}H NMR spectrum of PEGMA-BZ monomer.

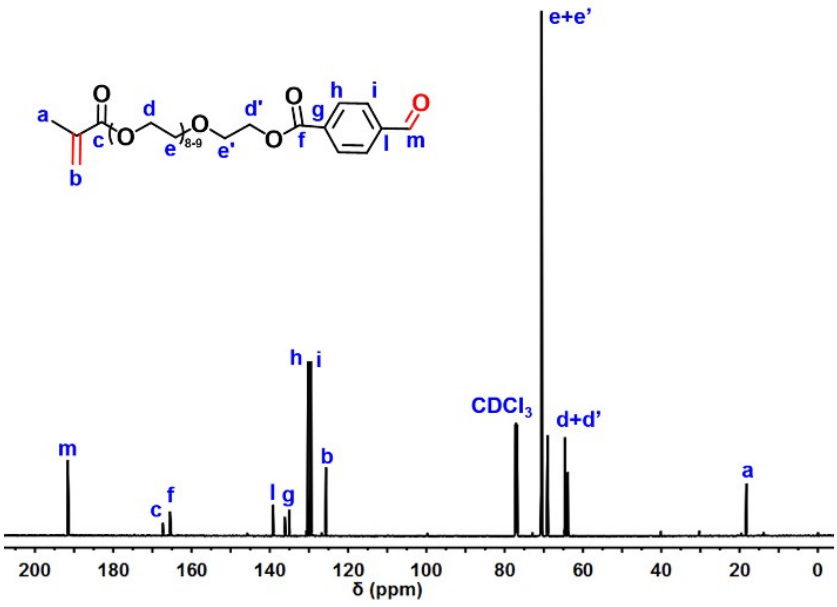


Figure S2. ^{13}C NMR spectrum of PEGMA-BZ monomer.

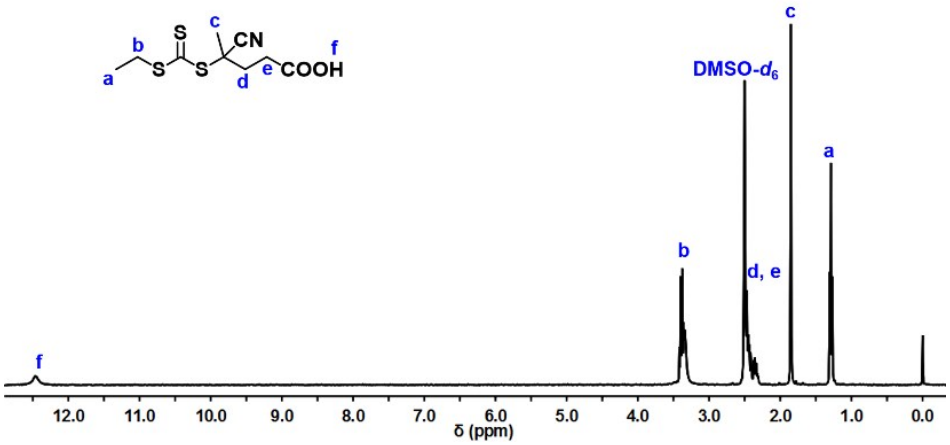


Figure S3. ^{1}H NMR spectrum of 4-cyano-4-ethylsulfanylthiocarbonylsulfanylentanoic acid (CEP).

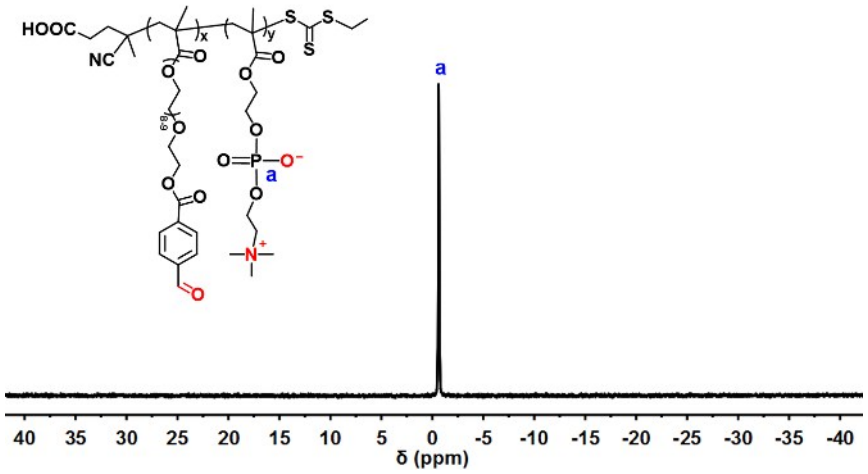


Figure S4. ^{31}P NMR spectrum of P(MPC-*co*-PEGMA-BZ) random copolymer.

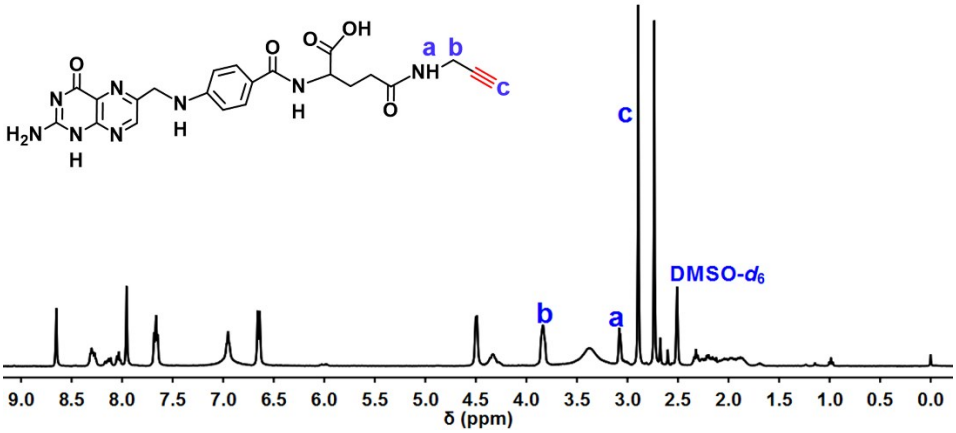
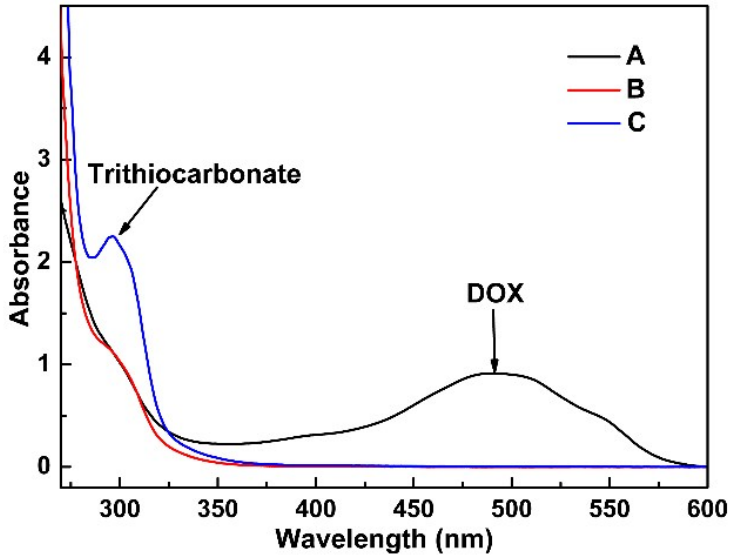


Figure S5. ^{1}H NMR spectrum of propargyl folic acid.



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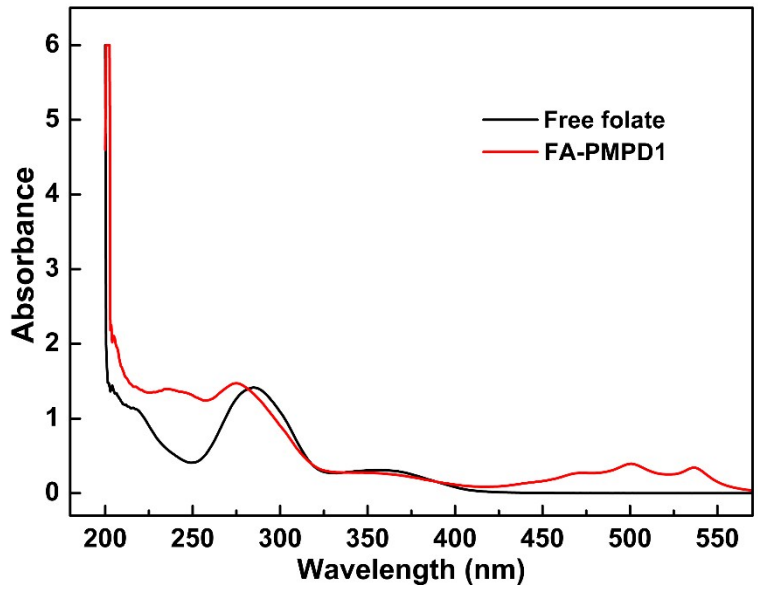

Figure S7. UV−vis spectra of free folate and FA-PMPD1 in ethanol.

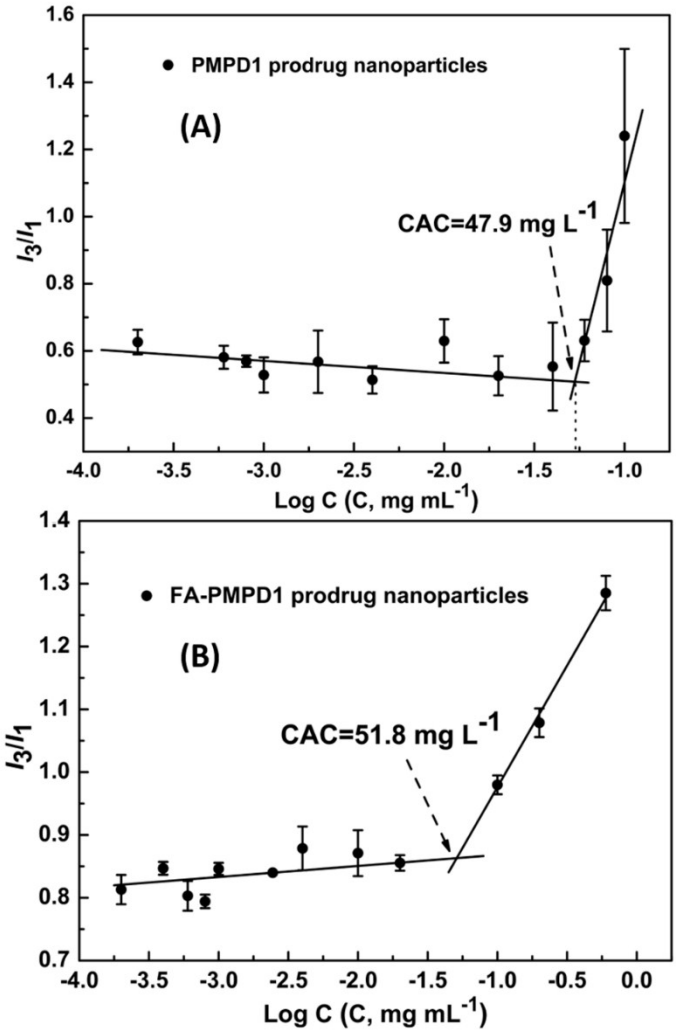

Figure S8. Intensity ratios (I_3/I_1) as a function of Log C for (A) PMPD1 and (B) FA-PMPD1 prodrug nanoparticles in pH 7.4 buffer solution.

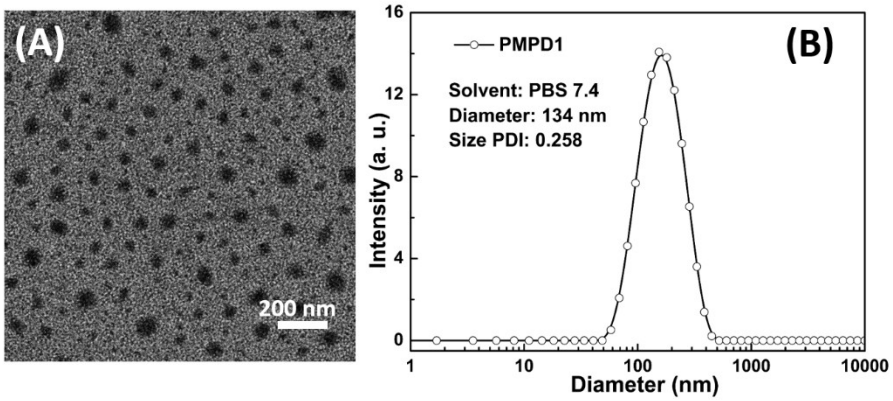

Figure S9. TEM image of PMPD1 prodrug nanoparticles (A) and the particle size distribution curve (B) corresponding to the TEM sample (scale bar 200 nm). The concentration was 0.5 mg mL^{-1}.

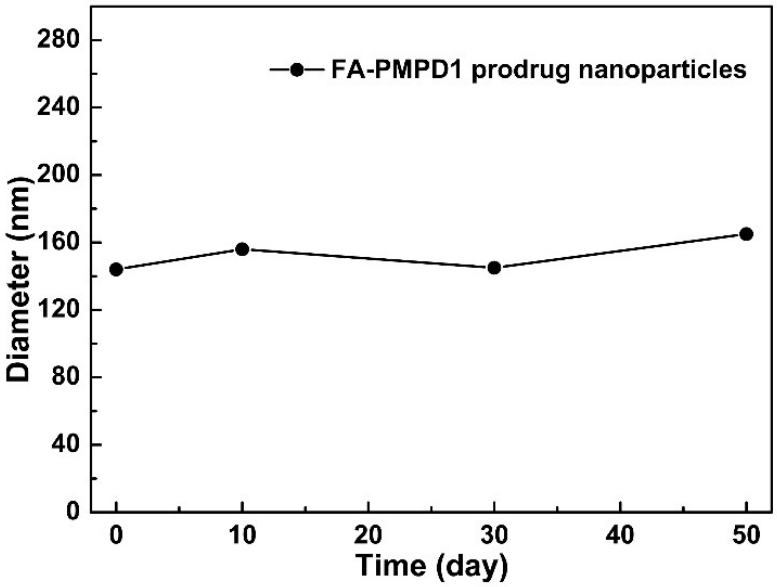

Figure S10. Variation of particle size for the FA-PMPD1 prodrug nanoparticles in pH 7.4 buffer solution at different times.