Dual-Responsive Polymer Micelles for Target-Cell-Specific Anticancer Drug Delivery

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Supporting Information (SI)

Synthesis of mPEG-PLA Copolymer (PELA). PELA was synthesized through ring-opening polymerization (ROP) of D, L-lactide initiated by mPEG-OH in the presence of stannous octoate ($Sn(Oct)_2$) as the catalyst.[1] Briefly, preweighed mPEG-OH (1.875 g, 2.5 mmol), D, L-lactide (12.549 g, 87.148 mmol) and $Sn(Oct)_2$ (0.178 g, 0.436 mmol) were quickly added to a 150 mL round-bottom flask with a stopcock. The flask was then degassed under vacuum for 5 h with continuous stirring, and heated at 50 °C in the last 30 min to remove the moisture completely. Then the ring-opening polymerization reaction was carried out at 150 °C for 4 h. After the product cooled down, 5 mL of dichloromethane was added to dissolve it, and precipitated in excess cold ethanol. The copolymer PELA was obtained as light yellow viscous material after dried under vacuum (yield: 72.1 %).

Synthesis of Dithiodipropionic Anhydride (DTDPA). DTDPA was synthesized as following: DTDP (1.051 g, 5 mmol) was refluxing with 5 mL of acetyl chloride at 65 °C for 2 h. Then the

reaction solution was evaporated to remove the excess acetyl chloride and precipitated into excess cold ethyl ether. The product was dried under vacuum to give DTDPA (yield: 82.6 %).

Synthesis of Disulfide-Functionalized PELA (PELA-ss-COOH). PELA (8.55 g, 1.5 mmol) and N,N-dimethyl-4-aminopyridine (DMAP) (0.183 g, 1.5 mmol) were dissolved in 30 mL of dried dimethylformamide (DMF). After the two chemicals were completely dissolved, 0.209 mL (1 equiv.) of triethylamine (TEA) was added. Then DTDPA (0.432 g, 2.25 mmol) was dissolved in 10 mL of DMF, and added dropwise into the previous solution. The mixture was kept stirring at 35 °C under nitrogen atmosphere for 24 h. Afterwards, the solution was distilled to remove the excess DMF, precipitated into cold ethyl ether and vacuum dried to give PELA-ss-COOH (yield: 85.8 %).

Synthesis of mPEG-PLA-ss-PEI Terpolymer (PELE). PELA-ss-COOH (5.892 g, 1 mmol), NHS (0.115 g, 1 mmol) and EDC·HCl (0.211 g, 1.1 mmol) were dissolved in 30 mL of dried dichloromethane (DCM). The mixture was stirred at 25 °C under nitrogen atmosphere for 24 h. Afterwards, PEI (9 g, 5 mmol) and TEA (0.139 mL, 1 mmol) were dissolved in 30 mL of DMF, then the solution of PELA-ss-COO-NHS was added dropwise into it. The mixture was reacted in the same condition for another 24 h. The solution was then washed three times with 100 mL of deionized water. The organic phase was collected, evaporated to remove DCM, precipitated in excess cold diethyl ether and dried under vacuum at 40 °C for 6 h to give PELE (yield: 71.4 %).

Synthesis of DMMA-Decorated PELE (PELE-DA). PELE (1.919 g, 0.25 mmol, equivalent to 7.875 mmol of NH_2 and NH) and DMMA (0.331 g, 2.625 mmol) were dissolved in 10 mL of DMSO. The mixture was reacted at 25 °C under nitrogen protection for 48 h. DMSO and unreacted DMMA were removed by dialysis against deionized water. PELE-DA was obtained by lyophilization (yield: 74.9%).

Synthesis of SA-Decorated PELE (PELE-SA). PELE (1.919 g, 0.25 mmol, equivalent to 7.875 mmol of NH_2 and NH) and SA (0.263 g, 2.625 mmol) were dissolved in 10 mL of DMSO. The mixture was reacted at 25 °C under nitrogen protection for 48 h. DMSO and unreacted SA were removed by dialysis against deionized water. PELE-SA was obtained by lyophilization (yield: 74.9%).

Synthesis of Folate-Attached PELE (PELE/FA). Folate (0.221 g, 0.5 mmol), NHS (0.058 g, 0.5 mmol) and EDC·HCl (0.105 g, 0.55 mmol) were dissolved in 30 mL of dried DMSO. The mixture was stirred at RT under nitrogen protection in the dark for 24 h. Then, 3.837 g of PELE (1 equiv.) and 0.069 mL of TEA (1 equiv.) were added, and the mixture was reacted for another 24 h in the same condition. The solution was dialyzed against deionized water to completely remove DMSO and unreated folate. PELE/FA was collected by lyophilization (yield: 85.1%).

Synthesis of Folate-Attached and DMMA-Decorated PELE (PELE/FA-DA). PELE/FA (2.429 g, 0.3 mmol, equivalent to 9.45 mmol of NH_2 and NH) and DMMA (0.397 g, 3.15 mmol) were dissolved in 20 mL of DMSO. The mixture was reacted at 25 °C under nitrogen protection for 48 h. DMSO and unreacted DMMA were removed by dialysis against deionized water. PELE/FA-DA was obtained by lyophilization (yield: 72.8%).

Supplementary Reference

1. Zhou, S. B.; Deng, X. M..; Yang, H. Biodegradable poly (ε-caprolactone)-poly (ethylene glycol) block copolymers: characterization and their use as drug carriers for a controlled delivery system. *Biomaterials* **2003**, *24*, 3563-3570.

Supplementary Figure captions:

Figure S1. ^{1}H NMR spectrum of mPEG-PLA (PELA) in $CDCl_3$.

Figure S2. ^{1}H NMR spectrum of Dithiodipropionic anhydride (DTDPA) in DMSO-d_6.

Figure S3. ^{1}H NMR spectrum of mPEG-PLA-ss-COOH (PELA-ss-COOH) in DMSO-d_6.

Figure S4. ^{1}H NMR spectrum of mPEG-PLA-ss-PEI (PELE) in $CDCl_3$.

Figure S5. ^{1}H NMR spectrum of mPEG-PLA-ss-PEI-DMMA (PELE-DA) in $CDCl_3$.

Figure S6. ^{1}H NMR spectrum of mPEG-PLA-ss-PEI-SA (PELE-SA) in DMSO-d_6.

Figure S7. ^{1}H NMR spectrum of mPEG-PLA-ss-PEI/FA (PELE/FA) in DMSO-d_6.

Figure S8. ^{1}H NMR spectrum of mPEG-PLA-ss-PEI/FA-DMMA (PELE/FA-DA) in DMSO-d_6 (A) and in D_2O (B).

Figure S9. Critical micelle concentration (CMC) of PELE/FA-DA micelles, derived from the plot of I_{339}/I_{333} ratio versus logarithm of micellar concentrations. After calculation, CMC = 3.048×10^{-3} mg/mL.

Figure S10. Size change in PELE, PELE-SA and PELE/FA-DA micelles following incubation with 0.25 mg/mL bovine serum albumin (BSA).

Figure S11. ^{1}H NMR spectra of PELE-SA after incubation at pD 6.8 in D_2O/DCl (25 °C) for different time periods.

Figure S12. (A) Zeta-potential and (B) size change in PELE-SA micelles as a function of incubation time at different pH values determined by DLS measurement.

Figure S13. Fluorescence emission spectra of DOX-loaded PELE/FA-DA micelles under pH 7.4 (A), pH 5.0 (B), different concentrations of GSH (C) for 2 h and in response to different pH values or different concentrations of GSH (D) versus time.

Figure S14. Flow cytometric analyses of folate receptor surface expression of non-teated 4T1 cells, folate-treated 4T1 cells and HeLa cells. The cells were trypsinized and labeled with anti-folate binding protein antibody followed by Alexa Fluor 488 conjugated antirabbit secondary antibody (colored line). Nonspecific fluorescence was measured using the secondary antibody only (shaded area).

Figure S15. Cell viability of 3T3 cells (A), HeLa cells (B) and 4T1 cells (C) with different concentrations of PELE, PELE-DA and PELE/FA-DA micelles at pH 7.4 after incubation for 24 h (n = 4).

Figure S16. Cell viability of 3T3 cells (A), HeLa cells (B) and 4T1 cells (C) with different concentrations of PELE, PELE-DA and PELE/FA-DA micelles at pH 6.8 after incubation for 24 h (n = 4).

Figure S17. Fluorescence images showing the viability of 3T3 cells following treatment with different concentrations of PELE, PELE-DA and PELE/FA-DA micelles at pH 7.4 for 24 h. The live cells were stained green while the dead cells were stained red.

Figure S18. Fluorescence images showing the viability of 3T3 cells following treatment with different concentrations of PELE, PELE-DA and PELE/FA-DA micelles at pH 6.8 for 24 h. The live cells were stained green while the dead cells were stained red.

Figure S19. Cellular uptake of free DOX, PECL, PELE, PELE-DA and PELE/FA-DA micelles at pH 7.4 (A) or pH 6.8 (B) after incubation with A549 cells for 3 h. DAPI (4', 6-diamidino-2-phenylindole, blue) was used to stain cell nuclei.

Figure S20. Flow cytometry analysis of HeLa cells treated with free DOX and DOX-loaded micelles (DOX-equivalent dose: 5 μg/mL), or medium alone (control) at pH 7.4 or pH 6.8 for 3 h at 37 °C.

Figure S21. (A) CLSM images and (B) Flow cytometry analysis of DOX-loaded PELE-SA at pH 7.4 or pH 6.8 after incubation with 4T1 cells for 1 h (DOX-equivalent dose: 5 μg/mL). Lysosomes were stained with Lysotracker (green).

Figure S22. Comparison of intracellular DOX release from free DOX, PELE/FA-DA, PELE-DA and PECL micelles by observing intracellular DOX distribution in HeLa cells with confocal laser scanning microscopy (CLSM). Nuclei and lysosomes were stained with Hoechst 33342 (blue) and Lysotracker (green) respectively. Cells were incubated at pH 6.8 for 3 h at 37 °C (DOX-equivalent dose: 5 μg/mL). Pink color shows localization of DOX (red) in nucleus (blue), while yellow color is an indication for localization of DOX (red) in lysosomes (green).

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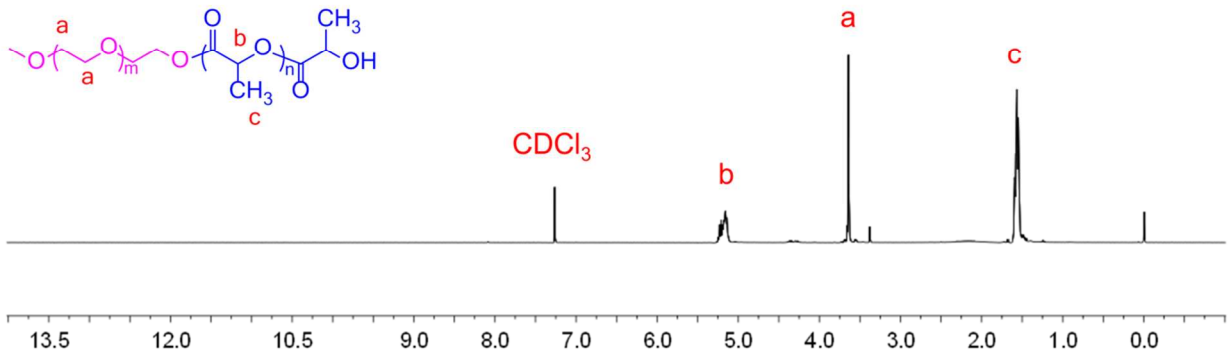
Figure S24. Inhibition rate of tumor growth calculated by tumor volume at day 21.

Table S1. Characterizations of polymers.

Table S2. Characterizations of micelles.



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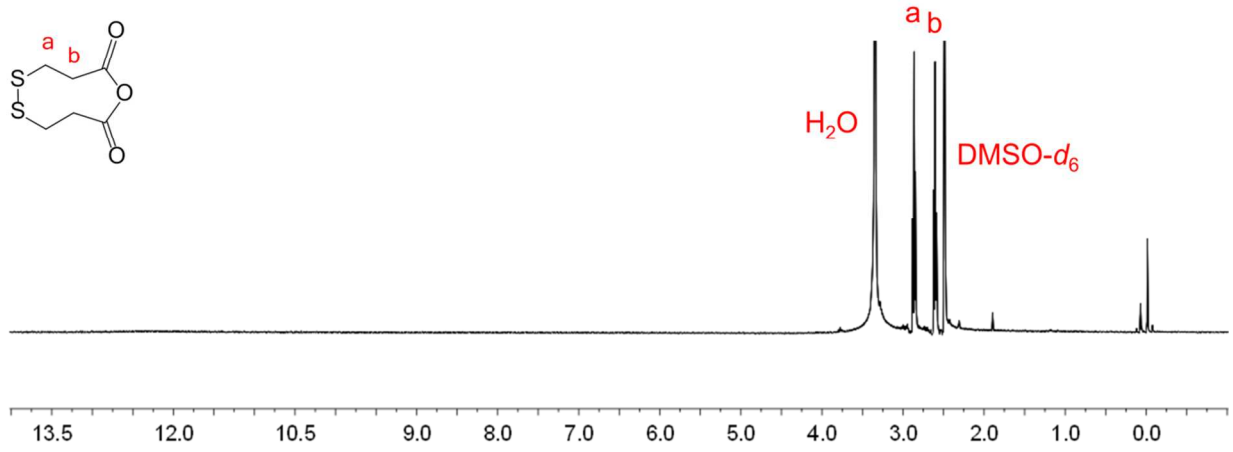


Figure S2. ^{1}H NMR spectrum of Dithiodipropionic anhydride (DTDPA) in DMSO-d_6.

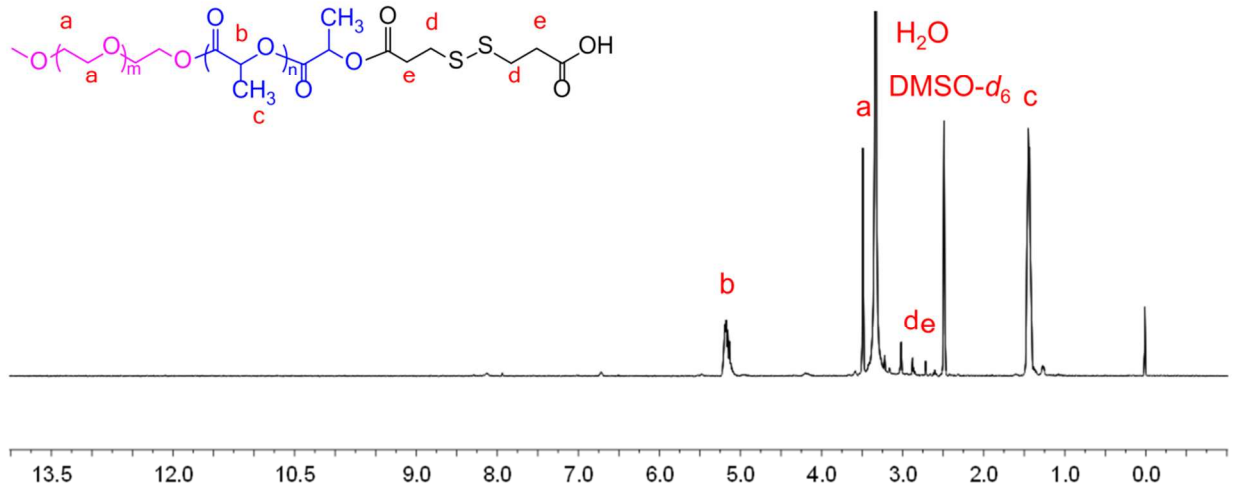


Figure S3. ^{1}H NMR spectrum of mPEG-PLA-ss-COOH (PELA-ss-COOH) in DMSO-d_6.

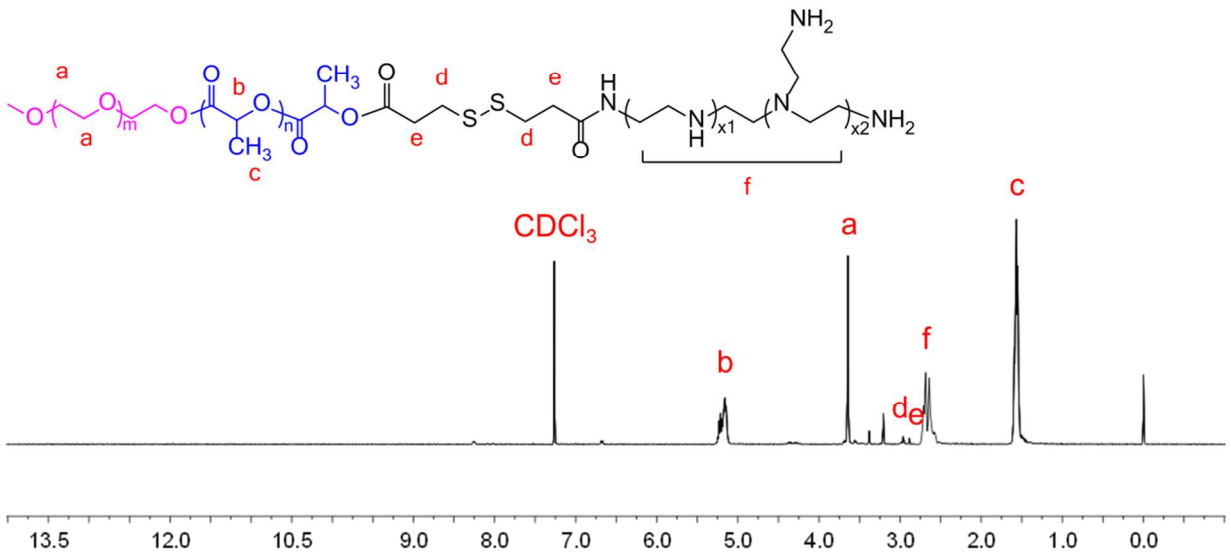


Figure S4. ^{1}H NMR spectrum of mPEG-PLA-ss-PEI (PELE) in $CDCl_3$.

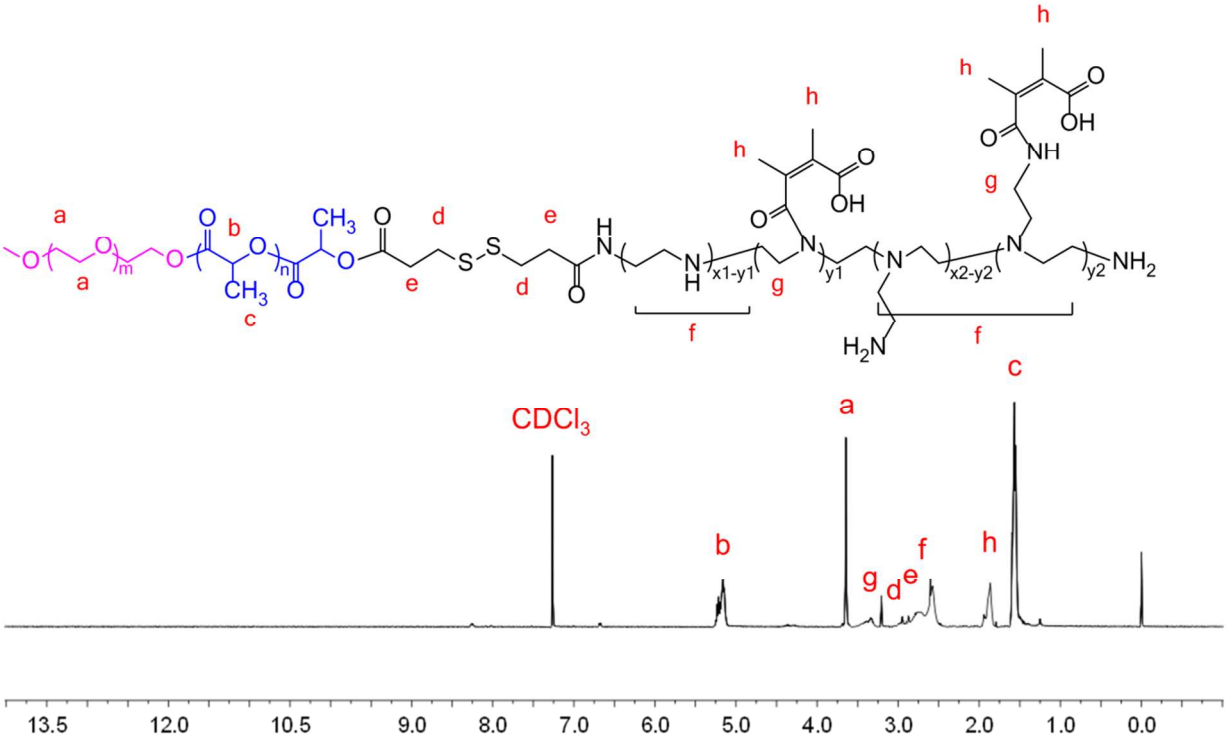


Figure S5. ^{1}H NMR spectrum of mPEG-PLA-ss-PEI-DMMA (PELE-DA) in $CDCl_3$.

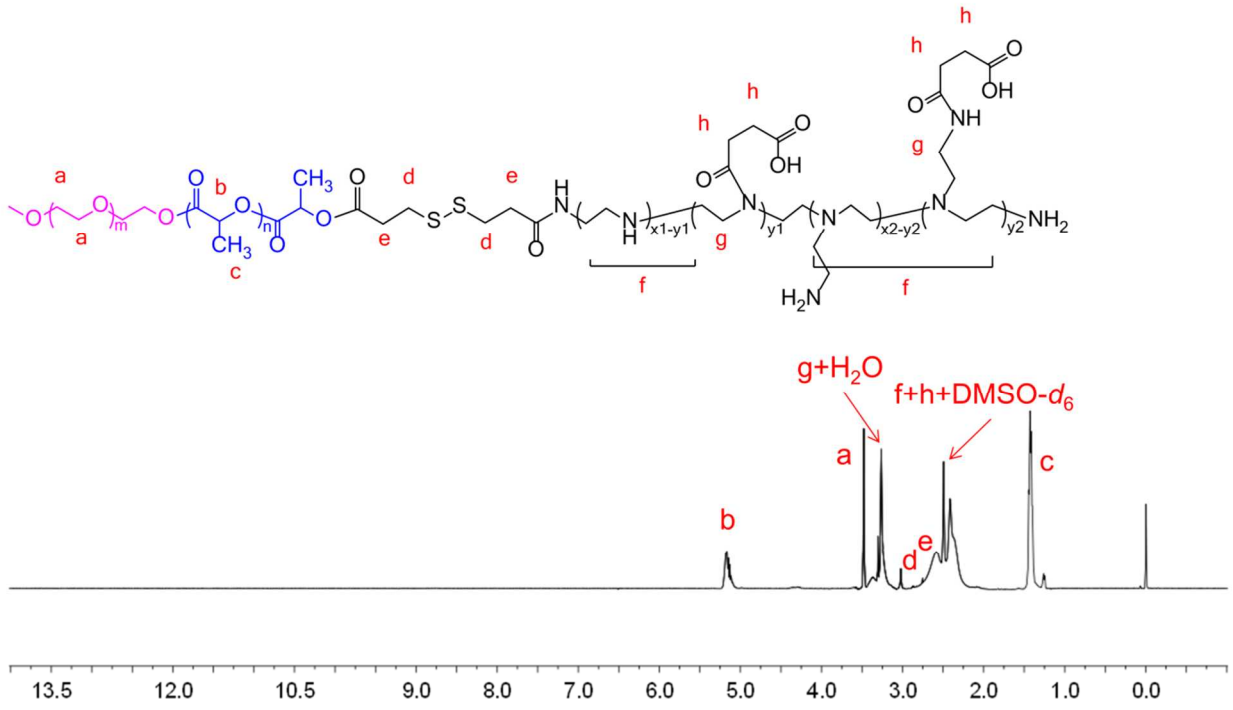


Figure S6. ^{1}H NMR spectrum of mPEG-PLA-ss-PEI-SA (PELE-SA) in DMSO-d_6.

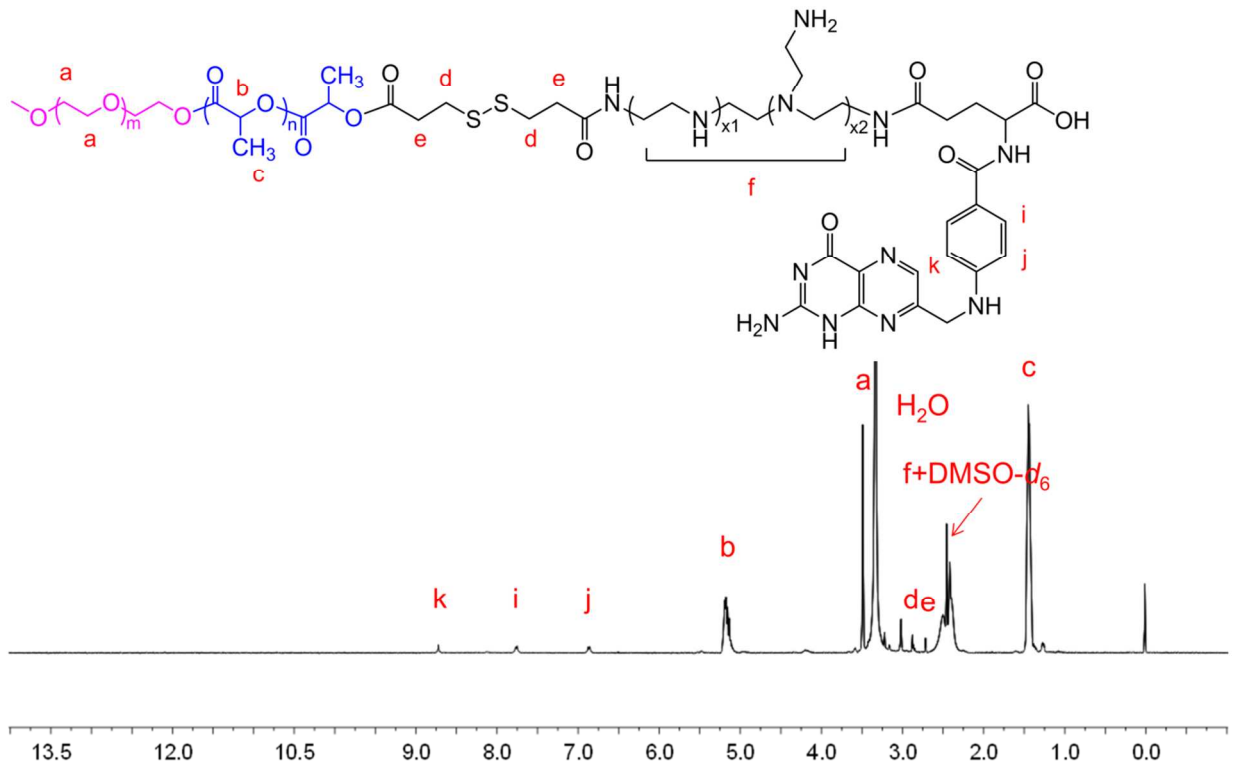


Figure S7. ^{1}H NMR spectrum of mPEG-PLA-ss-PEI/FA (PELE/FA) in DMSO-d_6.

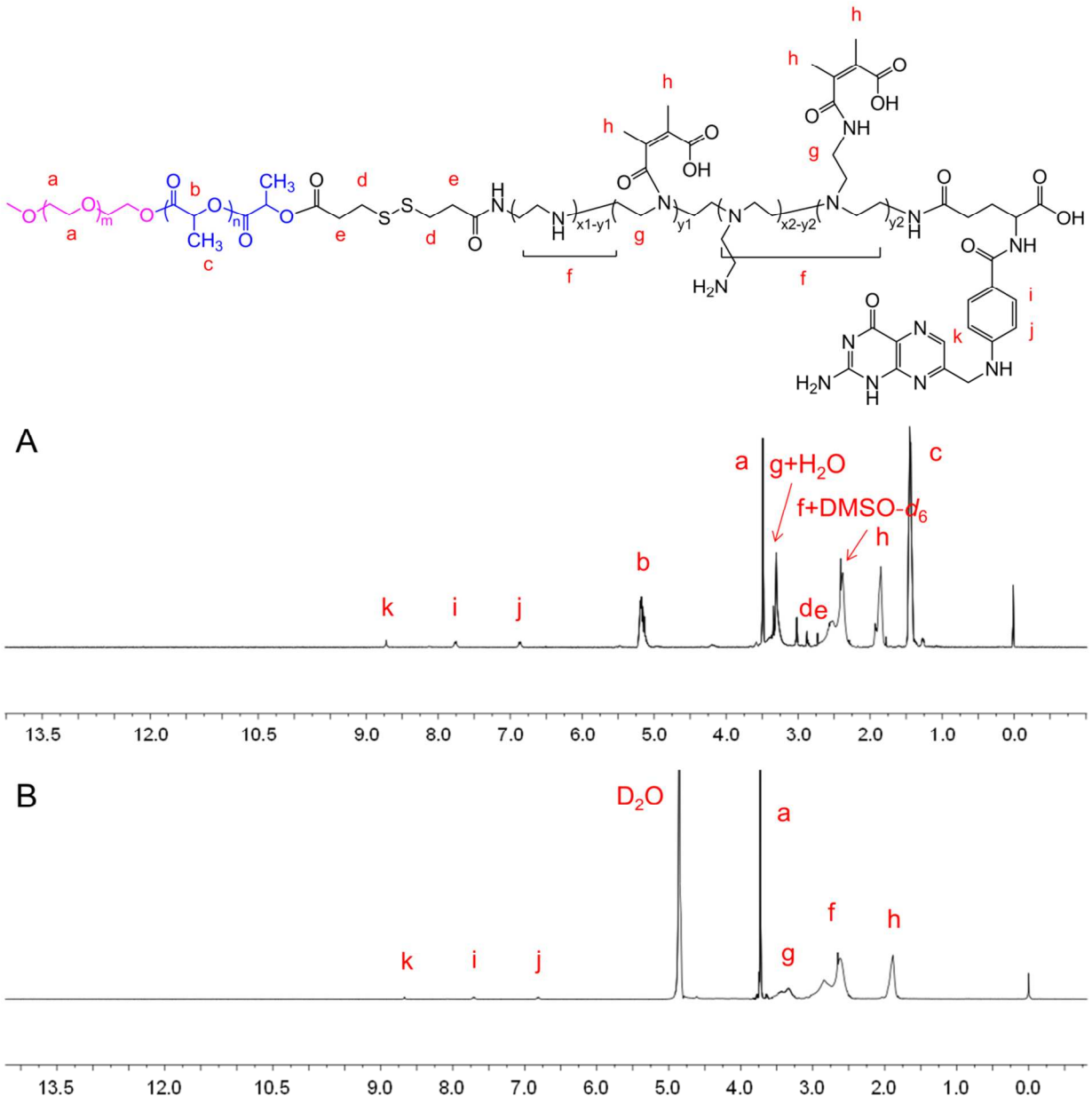
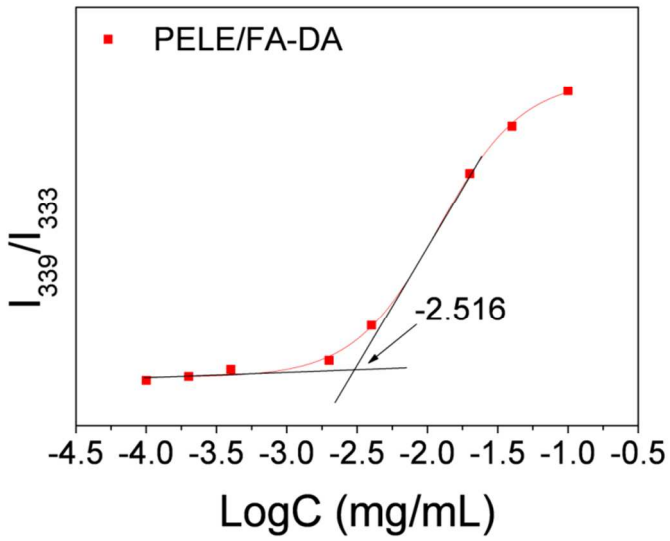


Figure S8. ^{1}H NMR spectrum of mPEG-PLA-ss-PEI/FA-DMMA (PELE/FA-DA) in DMSO-d_6 (A) and in D_2O (B).



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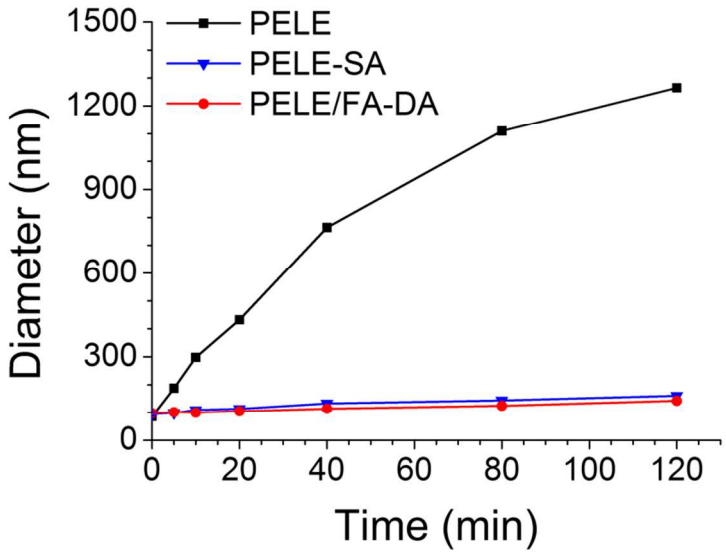

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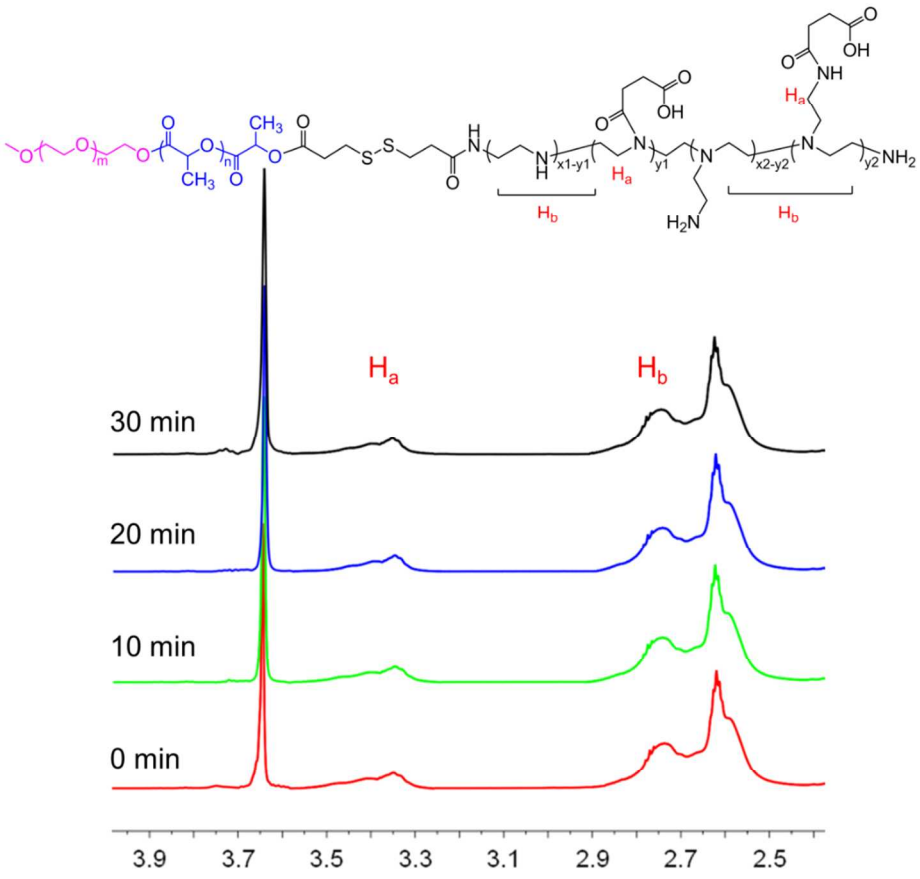

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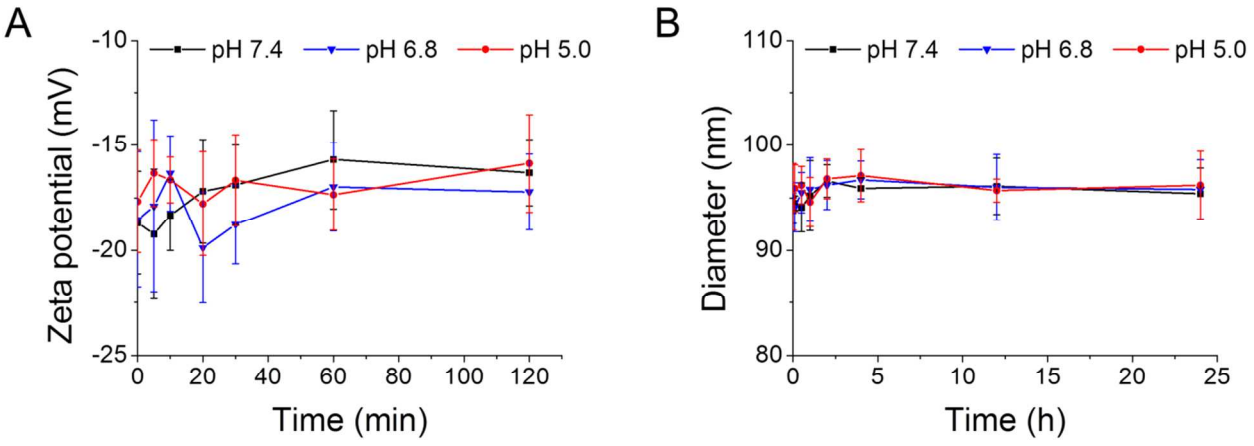

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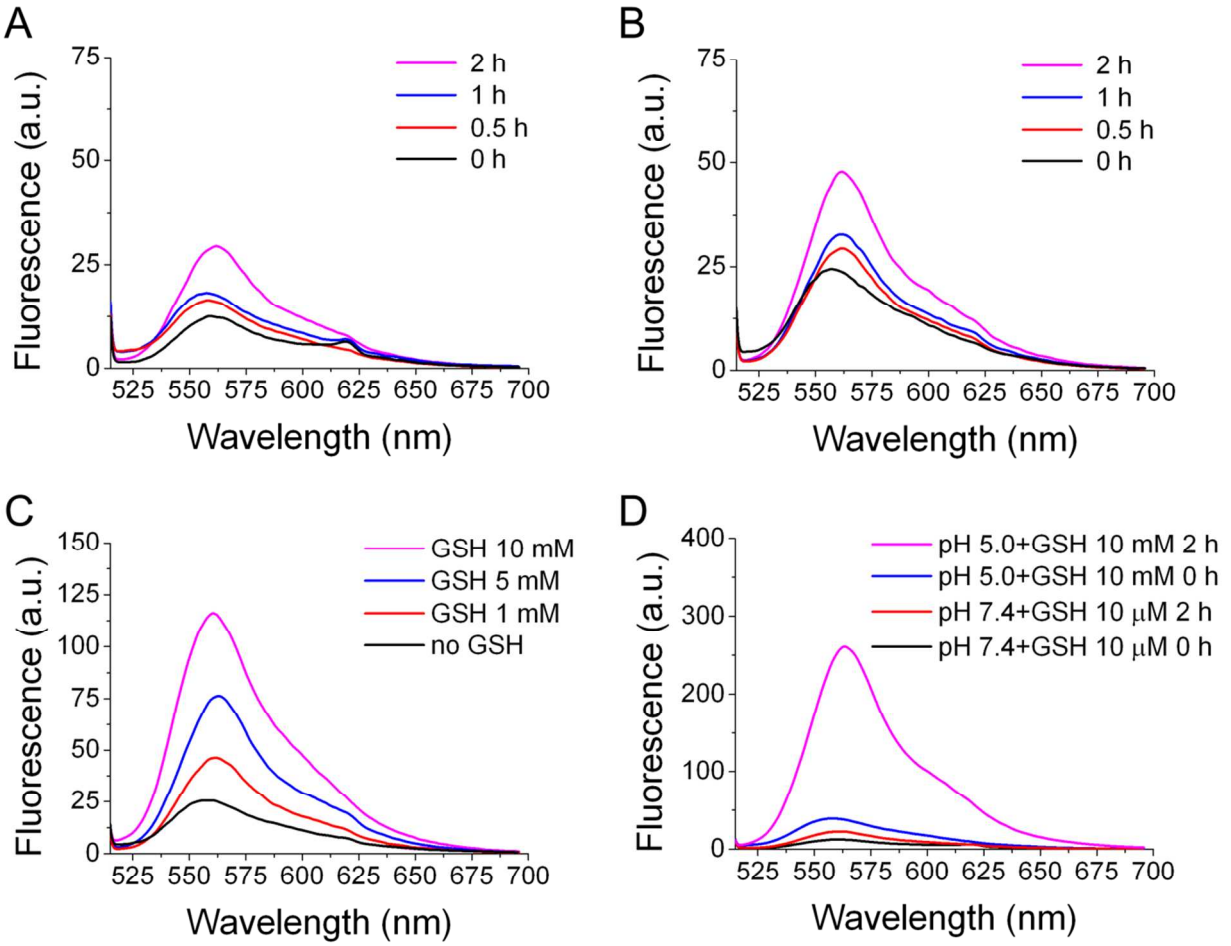

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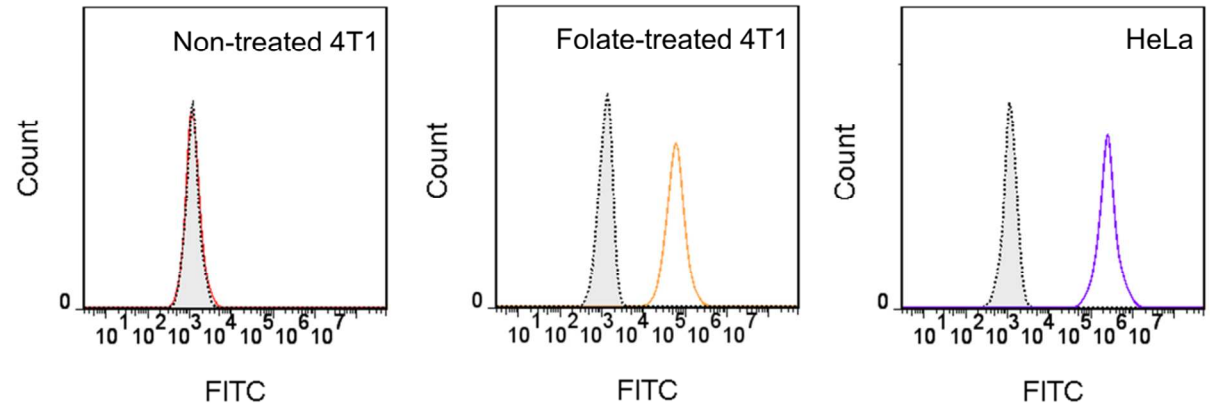

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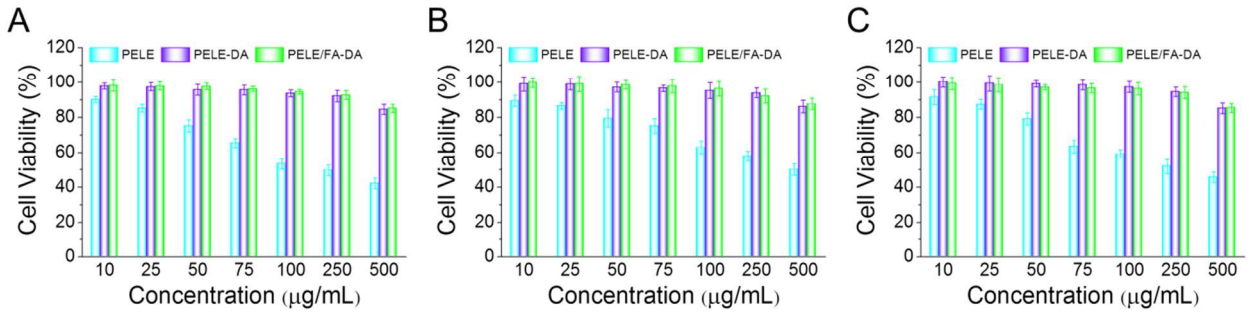


Figure S15. Cell viability of 3T3 cells (A), HeLa cells (B) and 4T1 cells (C) with different concentrations of PELE, PELE-DA and PELE/FA-DA micelles at pH 7.4 after incubation for 24 h (n = 4).

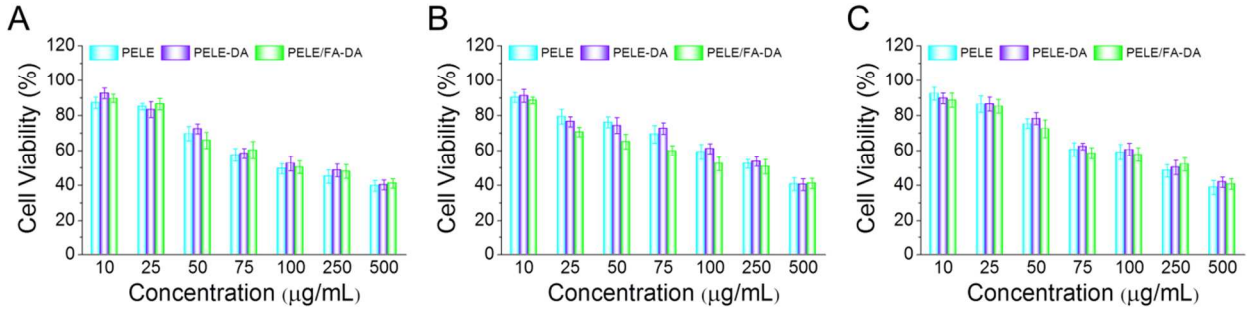
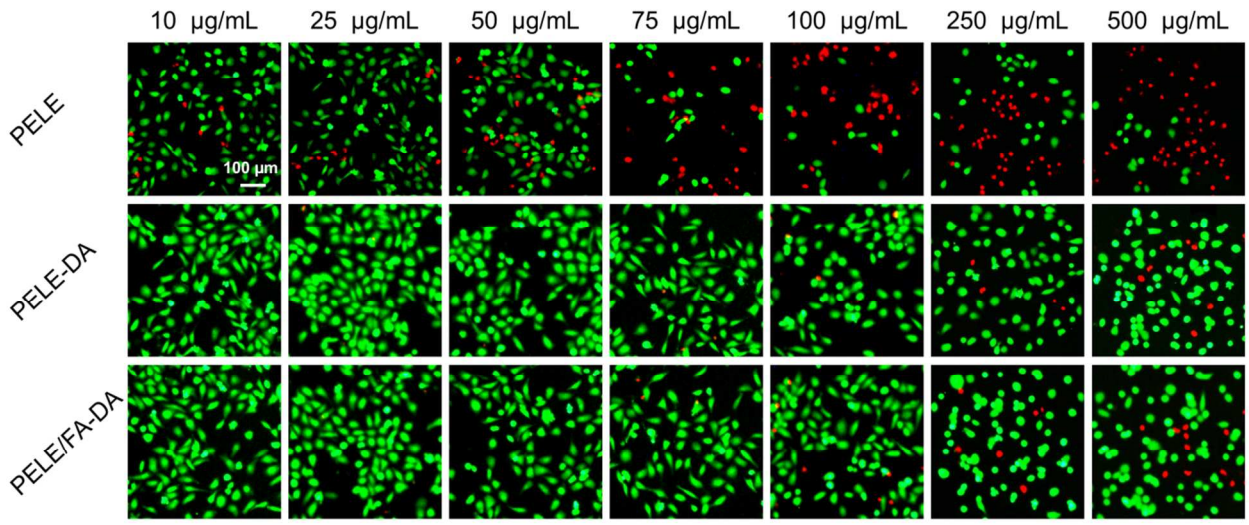


Figure S16. Cell viability of 3T3 cells (A), HeLa cells (B) and 4T1 cells (C) with different concentrations of PELE, PELE-DA and PELE/FA-DA micelles at pH 6.8 after incubation for 24 h (n = 4).



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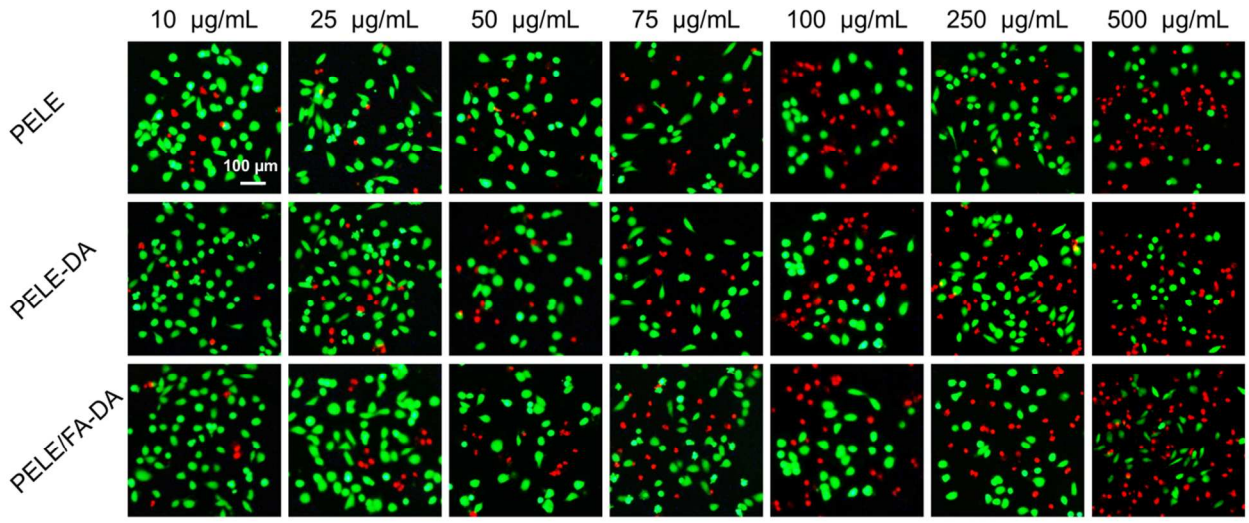

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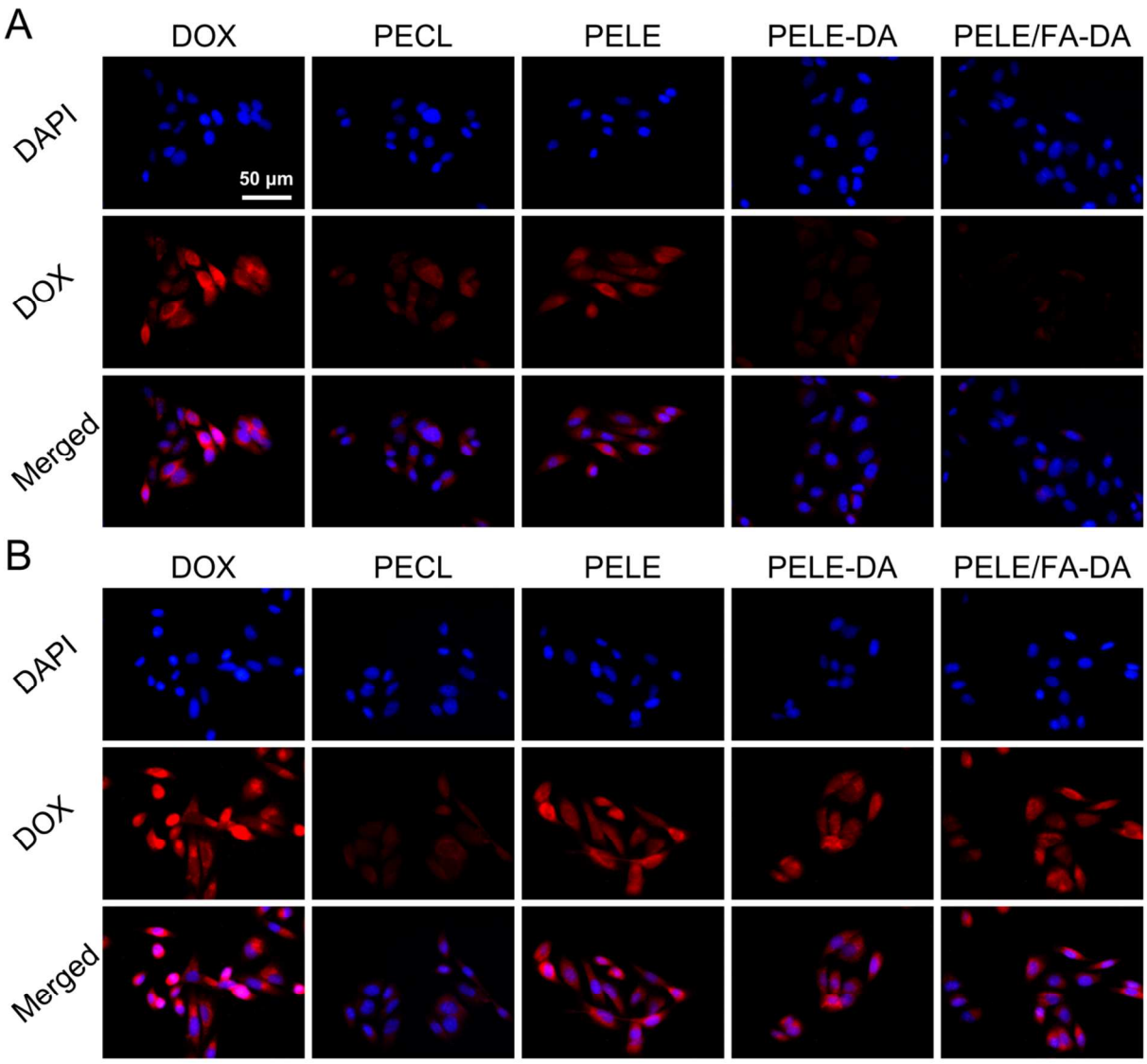

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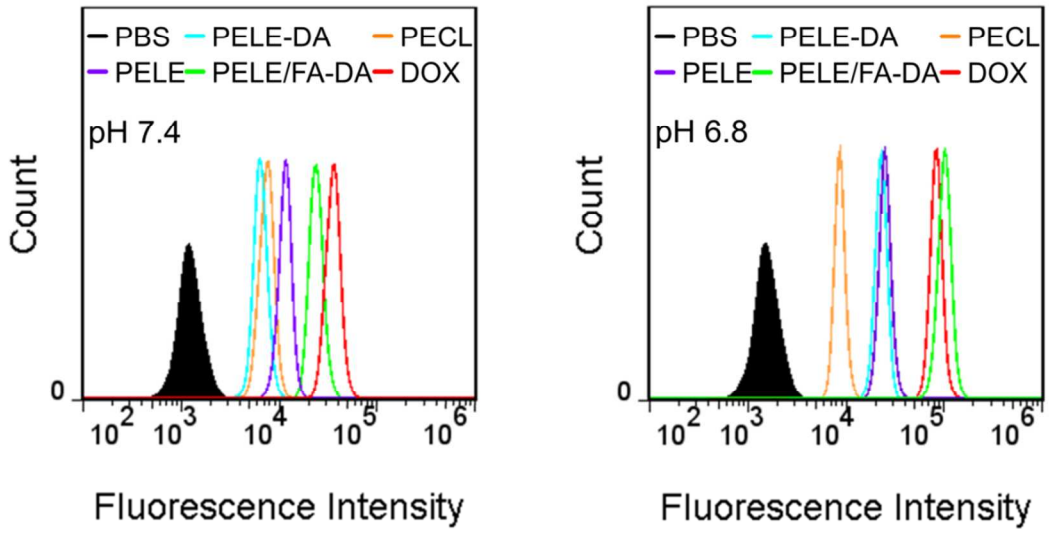

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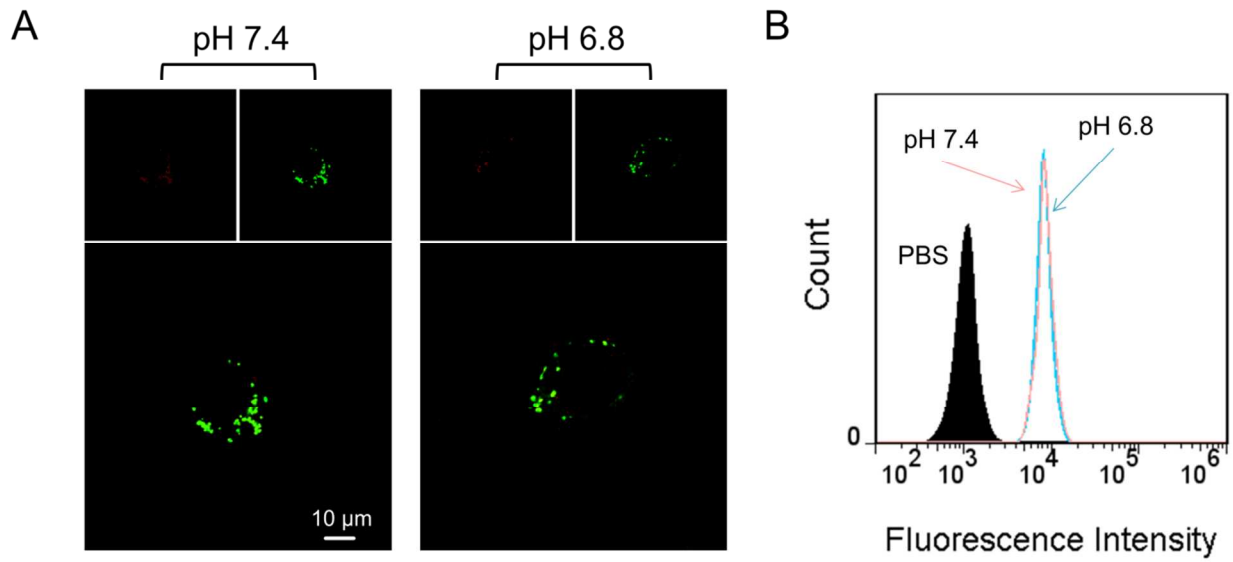
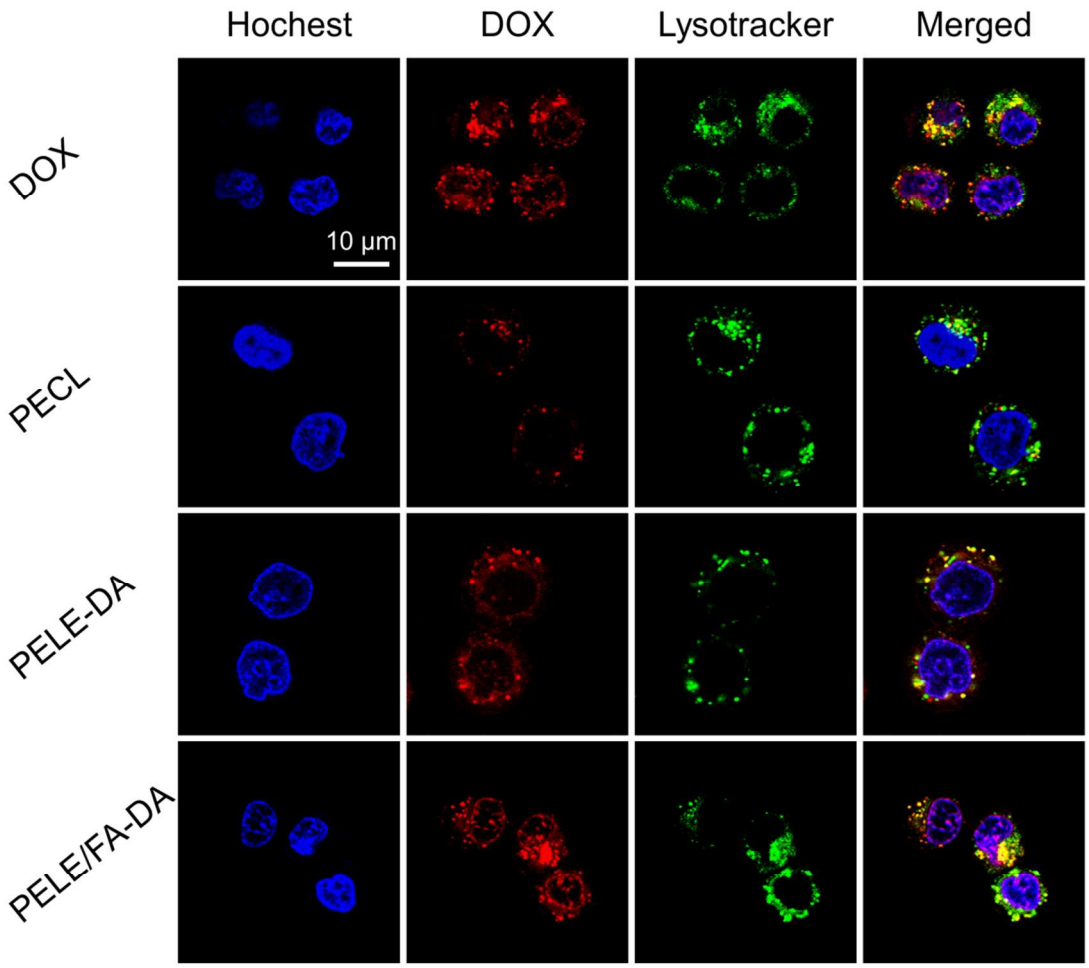


Figure S21. (A) CLSM images and (B) Flow cytometry analysis of DOX-loaded PELE-SA at pH 7.4 or pH 6.8 after incubation with 4T1 cells for 1 h (DOX-equivalent dose: 5 μg/mL). Lysosomes were stained with Lysotracker (green).



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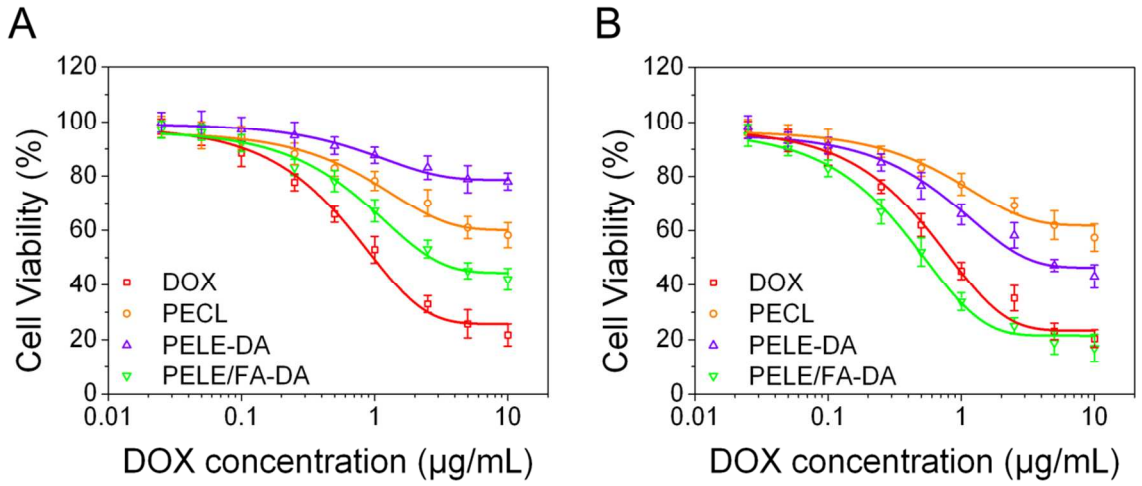

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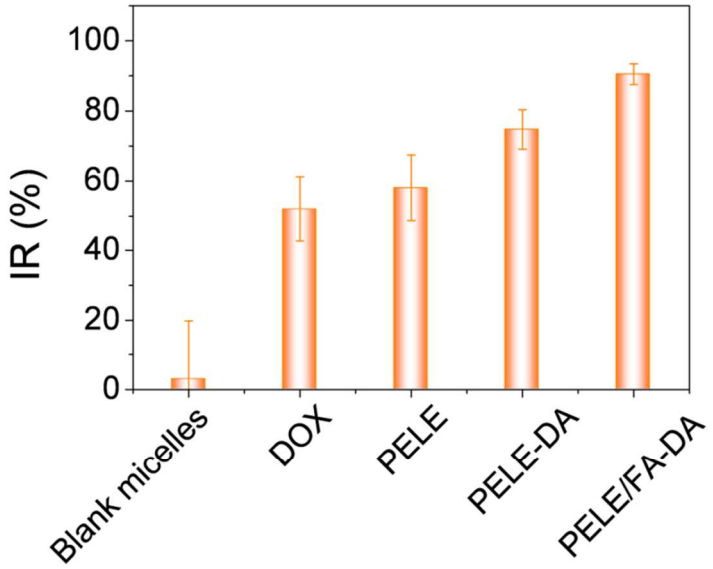

Figure S24. Inhibition rate of tumor growth calculated by tumor volume at day 21.

Table S1. Characterizations of polymers.

Sample	M_n (theory)	M_n[a]	M_n[b]	PDI[b]
PELE	7700	7800	9200	1.33
PELE-SA	8700	8600	10300	1.46
PELE-DA	9000	8900	11000	1.48
PELE/FA-DA	9400	9100	11500	1.39

[a] Calculated by ^{1}H NMR.
[b] Determined by GPC.

Table S2. Characterizations of micelles.

Sample	Z-average (nm)[a]	PDI[a]	LC(%)[b]	EE(%)[b]
PELE	85.2 ± 5.06	0.19 ± 0.08	2.46 ± 0.26	27.06 ± 2.88
PELE-SA	93.8 ± 8.16	0.17 ± 0.11	5.79 ± 0.18	63.73 ± 1.96
PELE-DA	91.6 ± 5.77	0.20 + 0.06	5.61 ± 0.14	61.67 ± 1.52
PELE/FA-DA	97.0 ± 6.35	0.15 ± 0.09	5.68 ± 0.33	62.48 ± 3.63

[a] Determined by DLS.
[b] Measured by UV-vis spectrophotometer. Feed weight ratio (drug/polymer = 1: 10).