Electronic Supplementary Material (ESI) for Chemical Communications
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Electronic Supporting Information

# Morpholino-decorated long circulating polymeric micelles with the function of surface charge transition triggered by pH changes

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## Experimental details

***Materials:***

D,L-lactide(D,L-LA) (Alfa Aesar) was recrystallized in ethyl acetate and vacuum-dried for 24 h at 40°C before use. Morpholin-4-yl-acetic acid hydrochloride and 3-(morpholin-4-yl)-propionic acid hydrochloride were bought from J&K Scientific LTD. Poly(ethylene glycol) (HO-PEG-OH, 2000 Da, Fluke) and methoxy poly(ethylene glycol) (MeO-PEG-OH, 2000 Da, Aldrich) were dehydrated by azeotropic distillation of water in toluene, and vacuum-dried at 80°C for 24 h. Triethylamine, toluene and dichloromethane (Tianjin Chemical Company) were distilled before use. Stannous octanoate ($Sn(Oct)_2$), fluorescein (Aladin Reagent), diisopropylcarbodiimide (DIC), 4-dimethylaminopyridine (DMAP) (Beijing Bomaijie Technology Co., Ltd.) and paclitaxel (Beijing Huafeng United Technology Company) were used without further purification.

***Synthesis of morpholin-4-yl-acetyl-poly(ethylene glycol) (MA-PEG-OH) and 3-(morpholin-4-yl)-propionyl-poly(ethylene glycol) (MP-PEG-OH)***

Poly(ethylene glycol) (2.0 g, 1 mmol), morpholin-4-yl-acetic acid hydrochloride

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(0.1815 g, 1 mmol), triethylamine (0.1210 g, 1mmol) and DMAP (catalytic amount) were dissolved in dichloromethane (15 mL) and the solution was stirred for 30 min. To this solution was added dropwise a solution of DIC (0.23 mL, 1.5 mmol) in dichloromethane (5 mL). The resulting solution was heated to 40°C and stirred for 24 h. The mixture was concentrated under vacuum using a rotary evaporator to remove most of the solvent. The residue was precipitated in cold ethyl ether and the precipitate was collect by filtration. The product was redissolved in dichloromethane and the solution was washed with 20% NaCl solution. After drying with anhydrous $MgSO_4$ for 4 h, the organic phase was reprecipitated in cold ether and the product (MA-PEG-OH) was vacuum-dried for 24 h at 35°C. MP-PEG-OH was synthesized similarly. Fig. S1 shows the $^1$H NMR spectra of MA-PEG-OH and MP-PEG-OH.

***Synthesis of MAP, MPP and MeP***

MA-PEG-OH (0.30 g, 0.15 mmol) and D,L-lactide (0.998 g, 7 mmol) were added to a 25-mL round-bottom flask and the system was vacuumized at 80°C for 2 h. After filling with nitrogen and cooling to room temperature, toluene (4 mL) was added to the flask. The system was evacuated and back-filled with nitrogen four cycles at room temperature under stirring and then was heated to 120°C. To the flask a solution of stannous octanoate (10 mg) in toluene (1 mL) was added. The system was heated to 150°C and maintained at the temperature for 12 h under stirring. After cooling to room temperature, dichloromethane (5 mL) was added to the flask to dissolve the product. The product, MAP, was precipitated in cold ethyl ether, redissolve in dichloromethane and reprecipitated in cold ethyl ether twice to remove impurities, and vacuum-dried. MPP and MeP were synthesized similarly using MP-PEG-OH and MeO-PEG-OH, respectively, instead of MA-PEG-OH. Fig. S1 shows the $^1$H NMR spectra of the three block copolymers.

***Preparation of blank micelles***

A block copolymer sample (50 mg) was dissolved in acetonitrile (5 mL) and stirred for 12 h. To this solution double distilled water (15 mL) was added dropwise and the mixture was stirred for another 12 h. The resulting solution was dialyzed (Mw cut-off:

3500 Da) against water for two days with exchange of water every 4 h, followed by lyophilizing to give blank micelles.

***Preparation of fluorescein-loaded micelles***

A block copolymer sample (30 mg) and fluorescein (15 μg) were dissolved in acetone (2 mL) and the mixture was stirred for 2 h. To this solution double distilled water (4 mL) was added dropwise and the mixture was stirred for another 12 h. The resulting solution was dialyzed (Mw cut-off: 3500 Da) against acetone/water (5% v/v) for 4 h with exchange of medium every 2 h and water 9 h with exchange of water every 3 h, followed by lyophilizing to give fluorescein-loaded micelles.

***Preparation of paclitaxel-loaded micelles***

A block copolymer sample (100 mg) and paclitaxel (5 mg) were dissolved in acetonitrile (5 mL) and the mixture was stirred for 12 h. To this solution double distilled water (15 mL) was added dropwise and the mixture was stirred for another 12 h. The resulting solution was dialyzed (Mw cut-off: 3500 Da) against water for two days with exchange of water every 4 h, followed by lyophilizing to give paclitaxel-loaded micelles. The paclitaxel-loading capacity was determined by dissolving the paclitaxel-loaded micelles in acetonitrile followed by HPLC analysis (column: WondaSil$^{TM}$ C18, 5μm, 4.6 × 150 mm; eluent: acetonitrile/water 45/35, v/v; flow rate: 0.8 mL min$^{-1}$; detector: UV detector at 227 nm).

All the lyophilized powders were re-dispersible in water and the sizes of the re-dispersed micelles determined by DLS were close to those of the original micelles. It has been reported that lyophilization of polymer micelles containing a hydrophilic long chain would not significantly affect the structures of the micelles.[1-3]

***In vitro release***

A solution of paclitaxel-loaded micelles (5 mL, 6 mg mL$^{-1}$) was dialyzed (Mw cut-off 3500) against 10 mL of phosphate buffer (10 mM phosphate, 150 mM NaCl, 0.5% Tween 80, pH 7.4). At preset time points, 1 mL of the extra fluid was taken for paclitaxel analysis by HPLC and 1 mL of fresh phosphate buffer was added.

***Flow cytometry study***

HeLa cells were cultured in DMEM (Dulbecco's modified Eagles Medium) medium with 5% penicillin-streptomycin and 10% fetal bovine serum (FBS) at 37°C with 5% $CO_2$. After gathering cells by using a solution of 0.02% (w/v) EDTA and 0.25% (w/v) trypsin, a 200-μL sample of cells ($5\times10^4$ cells $mL^{-1}$) was seeded onto a 12-well plate with 1 mL of DMEM medium. After incubation at 37°C for 24 h, the medium was removed and the cells were washed twice with phosphate buffered saline (PBS, pH 7.4). Then 1 mL of PBS at pH of 7.4, 7.0, 6.8 or 6.5 was added to each well (each pH in three wells), then 60 μL of fluorescein-loaded micelles (0.5 μg fluorescein/mg polymer) was added to each well. After 40 min of incubation, the medium was discarded and the cells were washed twice with 1 mL of PBS (pH 7.4), detached by 0.02% (w/v) EDTA and 0.25% (w/v) trypsin solution, and then dispersed in 0.25 mL of PBS for flow cytometric measurement. Cells were analyzed on a Facs Calibur (Becton Dickinson, Franklin Lakes, NJ, USA). The excitation wavelength was 488 nm and the fluorescence was measured with the FL1 channel. A total of 10,000 cells per sample were analyzed. The number of cells with fluorescence intensity higher than that of unlabeled cells was used to determine the fraction of labeled cells. The baseline was obtained by analyzing unlabeled control cells.

***Confocal image analysis***

Hela cells were seeded onto 22 mm glass covers placed in a 6-well plate with the cell density is $5\times10^4$ cells $mL^{-1}$. After incubattion for 24 h, the medium was removed and washed twice with PBS (pH 7.4). Then 0.9 mL of PBS at pH of 7.4, 7.0, 6.8 or 6.5 was added to each well, followed by addition of 60 μL of fluorescein-loaded micelles (0.5 μg fluorescein/mg polymer) to each well. After incubation for 30 min, the medium was discarded and the cells were washed twice with 1 mL of PBS (pH 7.4). The cells were fixed in PBS (pH 7.4) – 4% formaldehyde for 15 min at room temperature. The medium was discarded and the cells were washed twice with PBS (pH 7.4) and then examined using a Carl Zeiss LSM 510 meta fluorescence microscope.

***On-time scanning confocal microscope***

Hela cells were seeded onto a Lab-Tek® chamber slide system with 8-wells (Thermo Fisher Scientific Inc.) with a cell density of $1.6\times10^4$ cells/mL. After incubated for 24 h, the medium was removed and the cells were washed with PBS twice. Cell membranes were stained in Alexa Fluro® 594 conjugate of wheat germ agglutinin (Invitrogen) solution (5 mg/L). After the solution was discarded and the cells were washed with PBS for three times, 130 μL of PBS with the preset pH value was added in each well for imaging. The imaging process was recorded on a Perkin Elmer Confoncal System Ultra VIEW VOX with excitation at 561 nm (Alexa Fluro® 594 conjugate) and 488 nm ( fluorescein). Five sites were chosen in each well, and the pictures were taken in every two minutes. After the first picture was taken, 200 μL of the fluorescein-loaded micelles in PBS with the same pH value (0.4 mg/g) was added into each well.

***Cytotoxicity assay***

*H*ela cells were seeded in a 96-well plate at a density of $1 \times 10^4$ cells/well in DMEM medium. After culture for 24 h, the medium was replaced with 200 μL of fresh PBS at pH of 7.4, 6.8, or 6.5 containing paclitaxel-loaded micelles or black micelles at preset concentrations. After incubation for 1 h, the medium was replaced with 200 μL fresh DMEM. After incubation for another 23 h, the medium was removed and the cells were washed three times with PBS (pH 7.4). To each well 20 μL of MTS/PMS solution (20/1, v/v, Cell-Titer 96 AQueous kit) and 70 μL of PBS (pH 7.4) were added. After incubation for 3 h, the absorbance at 490 nm in each well was recorded using a SpectraMax M5 microplate reader. The spectrophotometer was calibrated to zero absorbance using PBS solution without cells. The relative cell viability compared to control wells containing PBS solution without micelles was calculated by $[Abs]/[Abs]_0$， where [Abs] and $[Abs]_0$ are the average absorbance of the test and the control samples, respectively.

***Protein adsorption***

Bovine serum albumin (BSA) was used as a model protein to determine the effect of pH on protein adsorption on micelles. The micelles were incubated with BSA solution

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in PBS (0.01 M) of different pHs, with the final concentration of micelles and BSA was 0.75 and 1.0 mg/mL. After incubation at 37°C for 12 h, the solution was centrifuged at 15,000 rpm for 20 min to precipitate the protein adsorbed micelles. The BSA concentration in the supernatant was determined using UV-Vis spectroscopy by measuring the maximal absorbance at 240 nm. Then, the adsorbed BSA on the micelles was calculated against a standard calibration curve of BSA.

***In vivo blood clearance kinetics assay***

Male KM mice (body weight 20 ± 2 g) were used for blood clearance kinetics assay**.** The sample of fluorescein-loaded micelles in physiological saline (8 mg/mL, 0.15 mL) was administered to the mice by intravenous injection into the tail vein under gaseous isoflurane anesthesia. At the preset time points, blood samples were collected into heparinized tubes. At each time point, blood sampling was performed on six mice and the blood samples were centrifuged at 4000 rpm for 10 min to isolate the plasma fraction. Then 100 μL of supernatant was taken and analyzed by a fluorescence spectrometer (excitation and emission wavelengths were 460 and 512 nm, respectively). Blank samples from mice with the administration of physiological saline in the same way were analyzed as the back-ground fluorescence of the plasma.

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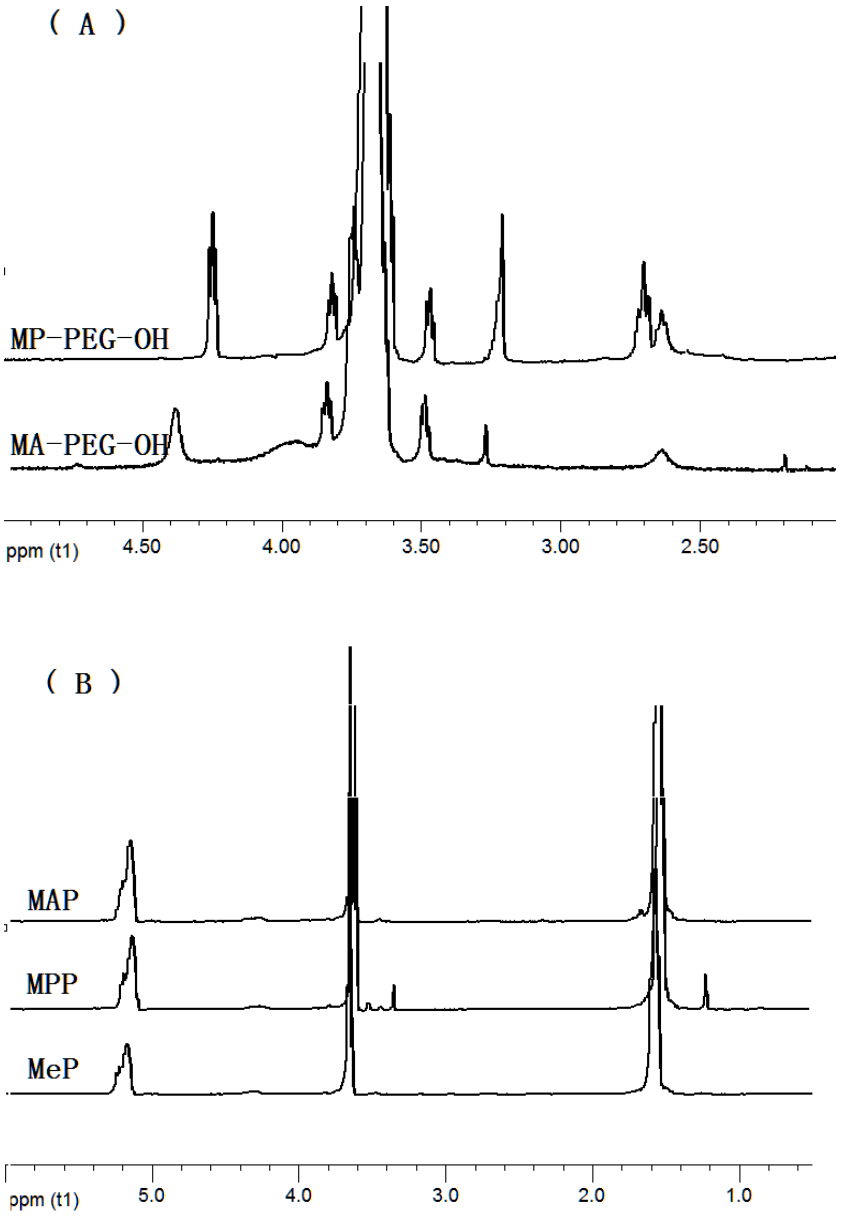


**Fig. S1.** $^{1}$H NMR spectra of copolymers.

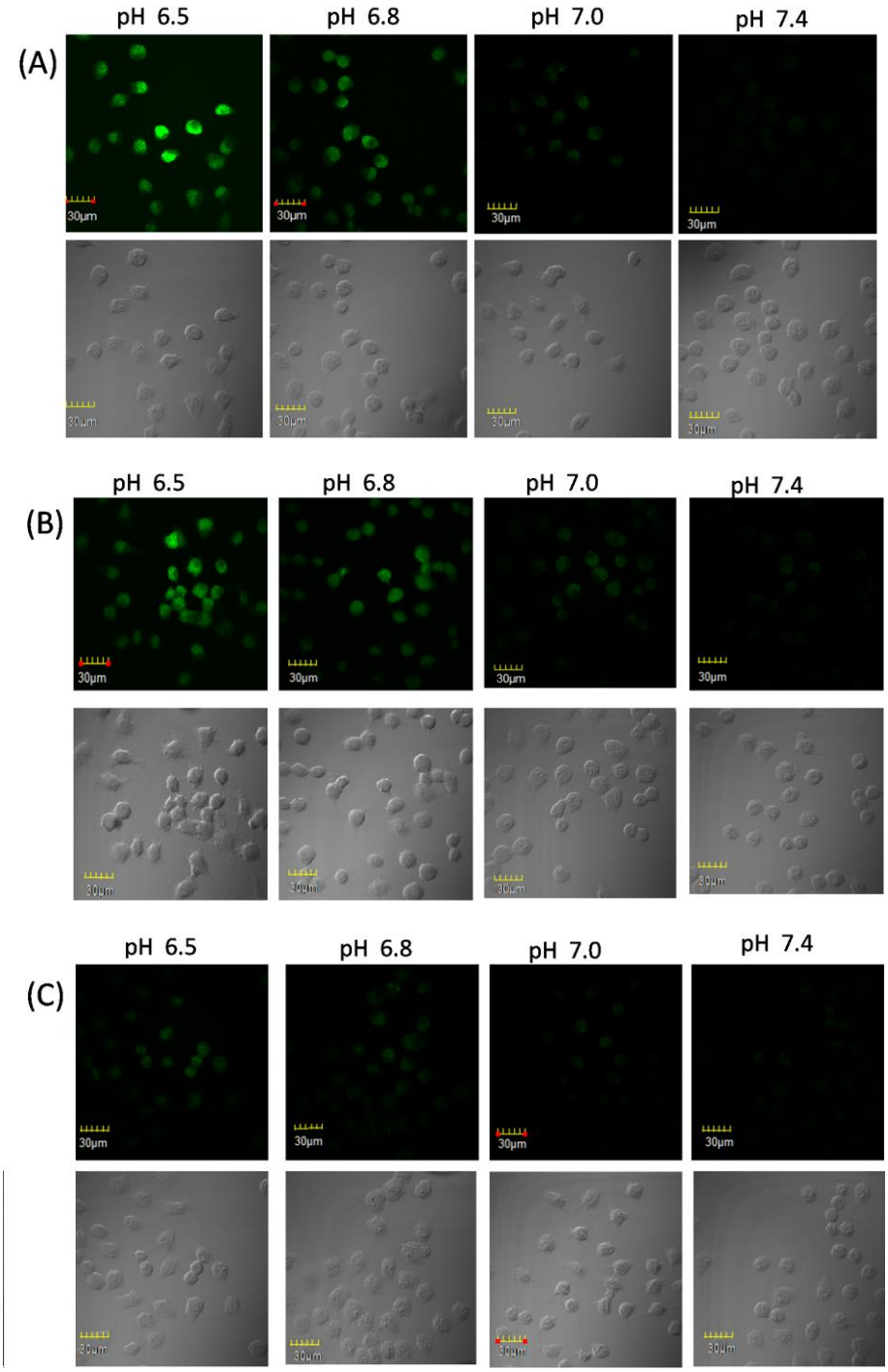


**Fig. S2.** Confocal laser scanning microscopy images of HeLa cells incubated with fluorescein-loaded micelles of (A) MAP, (B) MPP, (C) MeP for 40 min at different pHs

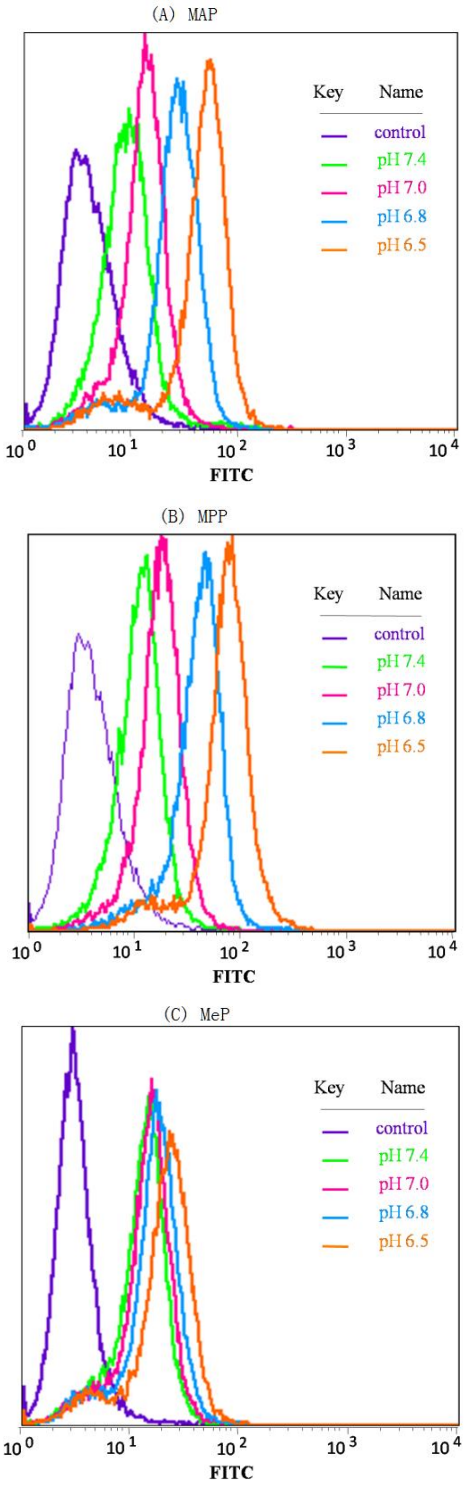


**Fig. S3.** Flow cytometry results of HeLa cells incubated with fluorescein-loaded micelles of (A) MAP, (B) MPP, or (C) MeP for 40 min at different pHs.

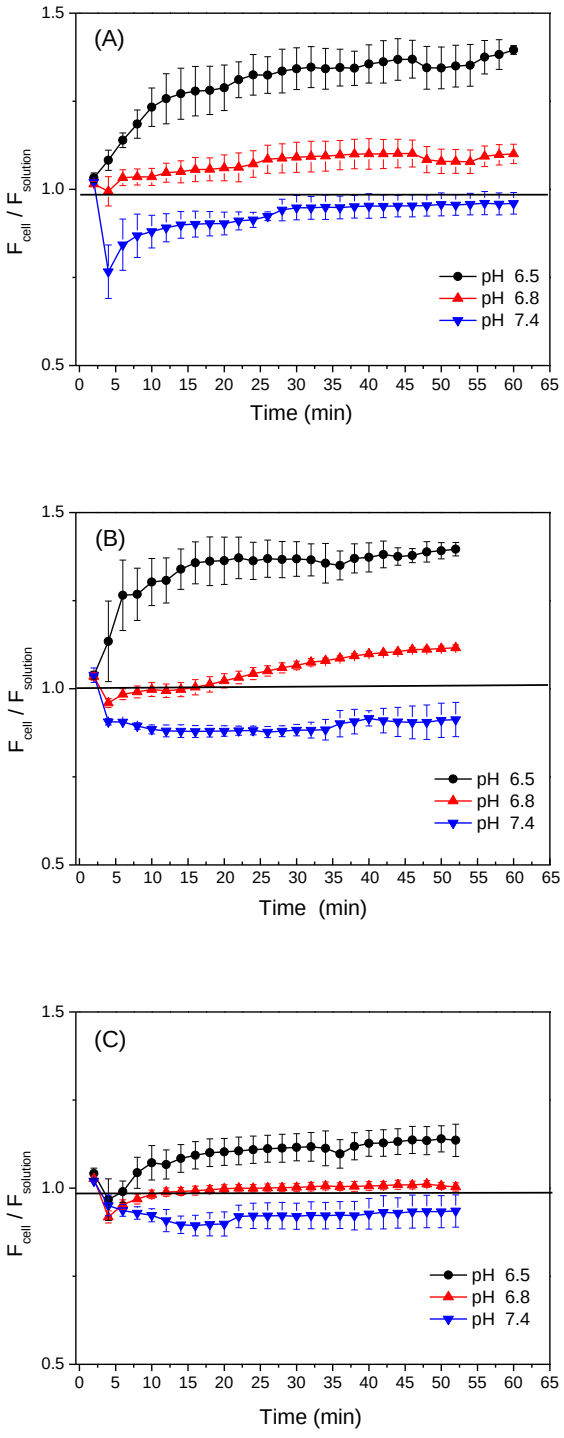


**Fig. S4.** Plot of the ratio of the fluorescence intensities in cells and in solution ($F_{cell}/F_{solution}$) determined by on-time scanning confocal microscope. HeLa cells were incubated with fluorescein-loaded micelles of (A) MAP, (B) MPP, and (C) MeP at different pHs.

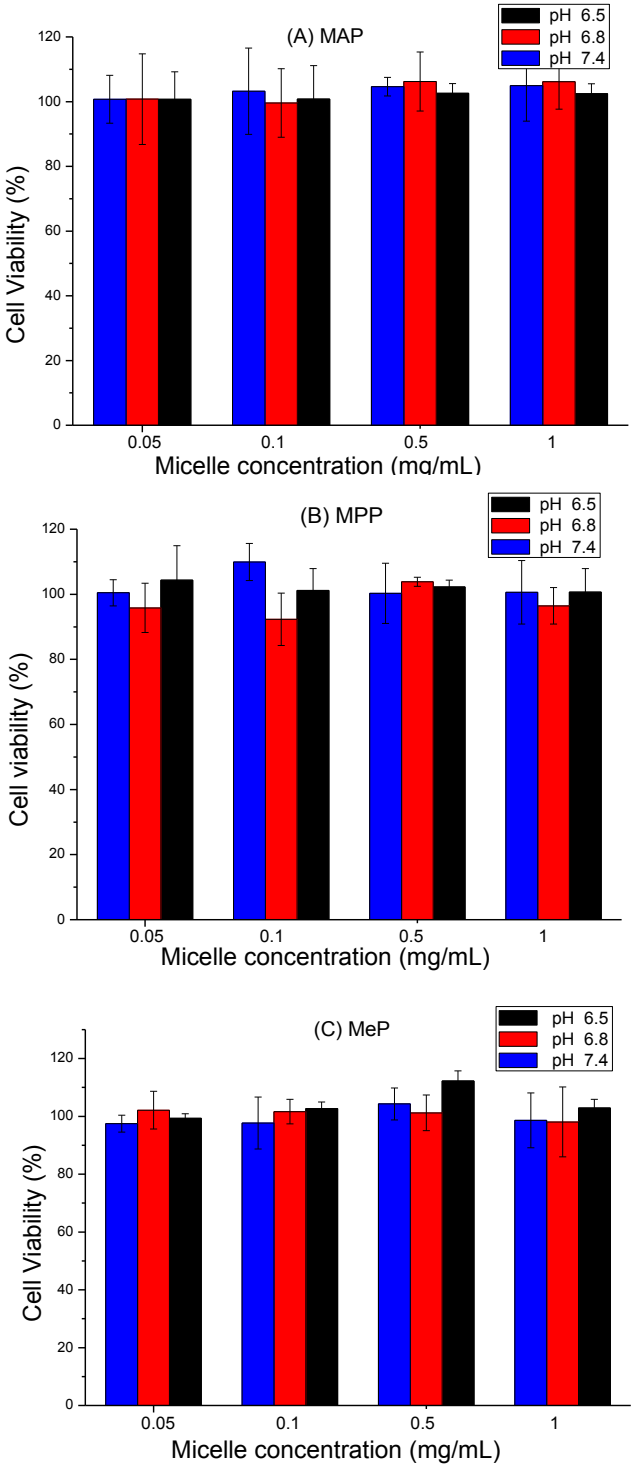


**Fig. S5**. Cytotoxicity of the blank micelles (viability of HeLa cells was determined after incubation with each kind of micelles at 37°C for 24 h).

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**Table S1**. Block compositions of diblock copolymers.

| Copolymer | Polymerization degree of PEG[a] | Polymerization degree of PLA[b] | $M_n$[c] | $M_w/M_n$[c] |
|---|---|---|---|---|
| MAP | 44 | 75 | 7586 | 1.40 |
| MPP | 44 | 76 | 7756 | 1.47 |
| MeP | 44 | 59 | 6664 | 1.54 |

[a] Polymerization degree of PEG was calculated from the average molecular weight provided by the supplier of PEG or MeO-PEG. [b] Polymerization degree of PLA was calculated from $^1$H NMR of the copolymer by using PEG segment as a reference. [c] $M_n$ and $M_w/M_n$ were determined by GPC.

**Table S2**. Properties of paclitaxel-loaded micelles.

| Copolymer | MAP | MPP | MeP |
|---|---|---|---|
| Loading capacity[a] (%) | 2.51 ± 0.09 | 3.13 ± 0.10 | 1.76 ± 0.06 |
| Diameter[b] (nm) | 44.6 ± 2.1 | 44.2 ± 4.1 | 39.3 ± 17.6 |

[a] Paclitaxel loading capacity ($W_{drug}/W_{copolymer} \times 100\%$). [b] Average diameter determined by dynamic light scattering.