Supporting Information

Inspired by Nonenveloped Viruses Escaping from Endo-lysosomes: pH-Sensitive Polyurethane Micelle for Effective Intracellular Trafficking

Nijia Song[a], Lijuan Zhou[a], Jiehua Li[a,*], Zhicheng Pan[a], Xueling He[a, b], Hong Tan[a, **], Xinyuan Wan[a], Jianshu Li[a], Rong Ran[a], Qiang Fu[a]

[a]College of Polymer Science and Engineering, State Key Laboratory of Polymer Materials Engineering, Sichuan University, Chengdu 610065, China.

[b]Laboratory Animal Center of Sichuan University, Huaxi Clinical College, Sichuan University, Chengdu, 610040, China.

* Corresponding author, Fax: 0086-28-85405402, Email: jiehua_li@scu.edu.cn

** Corresponding author, Fax: 0086-28-85405402, Email: hongtan@scu.edu.cn

Supporting Materials and Methods

pH-sensitive polyurethane (pHPU)

Herein, oligomers bearing pH-sensitive hydrazone bonds including (PCLH, MW 3380.7, Chart S1 (B)) and hydrazone-ended methoxyl-poly(ethylene glycol) (m-PEGH, MW 1956.1, Chart S1 (D)) as well as a novel tripetide chain extender (TCE, Chart S1 (F)) containing active carboxyl groups for preparation of these pH-sensitive polyurethanes (pHPU) were synthesized in our laboratory according to previous reports[1-4].

The pHPU have been synthesized using LDI, BDO and TCE as hard segment, PCL, PCLH and m-PEGH as soft segment[2]. Briefly, LDI and PCL/PCLH (molar ratio 1:1) were prepolymerized for 1 h at 60 °C under a dry nitrogen atmosphere and kept for another 1.5 h after 0.1% stannous octoate as catalyst was added. Then chain extension was carried out in chronological order: the first one was BDO at 60 °C for 1 h and the followed was tripeptide at 40 °C for 4 h. Finally, the polymers were terminated by m-PEGH at 60 °C for 6 h. After that, the products were precipitated in anhydrous ethyl ether and dried under vacuum at 60 °C for 3 days. Moreover, it has been demonstrated that these pH-sensitive polyurethanes showed good biocompatibility in vivo[2]. The structure of pHPU (Mn: 21149) and its monomers are shown in Chart S1. As a comparison, polyurethanes possessing similar structure without hydrazone bonds (Mn: 18636) were used to prepare pH-insensitive polyurethane micelles (PM).

Supporting Experimental

Detection Critical micelle concentrations

To determine the critical micelle concentration (CMC), fluorescence spectra of micelle solutions containing pyrene as a probe were recorded on a fluorescence spectrometer (F-7000 FL, Hitachi, Japan) at room temperature. Pyrene dissolved in acetone was added to blank vials to evaporate solvent before different concentrations of sample solution were added. The final pyrene concentration was 5.0×10^{-7} M. All samples were immersed in a sonicator for 4 h at room temperature. Steady-state fluorescence spectra were recorded with bandwidths of 5.0 nm for both excitation and 5.0 nm for emission, respectively. With the excitation wavelength set at 372.0 nm, the intensity ratio of the peak at 337.0 nm to that at 334.0 nm was varied as a function of concentration of micelle solution from emission spectroscopy, and CMCs are calculated accordingly[5].

Preparation of actively targeted pHPM

To generate actively targeted pHPM-Tra, the human epidermal growth factor receptor-2 (HER2) monoclonal antibody Trastuzumab (Tra) was coupled to micelles via the pendant carboxyl groups of TCE. Similar to the method previously reported[1, 6], 9 mL of the micelle solution was mixed with 1 mL of HEPES-buffered saline (pH 7.0) for 30 min; 6.2 μL of 0.25 M EDC·HCl and 9.3 μL of 0.25 M HOSu were added, and the mixture was incubated for 1 h at room temperature. After the pH was adjusted to 7.5 by 1 M NaOH, 25 μL Tra (1 mg mL^{-1}) was added into the mixture and the reaction was kept for another 8 h at room temperature with gentle stirring. The resultant solution was dialyzed

against phosphate buffered saline (PBS, pH 7.4) and the passed through a 0.45-μm pore size syringe filter. The amount of grafted antibody was confirmed by enzyme-linked immunosorbent assay.

An enzyme-linked immunosorbent assay (ELISA) was performed to confirm the successful conjugation of Tra to pHPM and measure the amount of grafted antibody, as described below. The solution of lyophilized samples re-dissolved in Na_2CO_3 buffer (0.05 M) was added into 96-well plates (100 μL/well) and kept at 4 °C overnight, then washed with PBS containing 0.1% Tween-20 and 0.05% NaN_3 (PBS/Tween/azide), followed by a single wash with PBS/azide. Afterwards, the plates were incubated with a blocking buffer containing 2% bovine serum albumin (BSA) in PBS/Tween/azide for 2 h and washed again as above process. Thereafter, a horseradish peroxidase (HRP)-conjugated anti-Tra (secondary antibody, 5 μg mL^{-1}) was added into each well. The plates were incubated again at 37 °C for 1 h and washed as above. Coloration was initiated by treating with o-phenylenediamine (OPD, Sigma, St. Louis, MO) for 30 min at 37 °C in the dark and stopped by 50 μL of 2 M H_2SO_4. The optical density (OD) was measured at 492 nm by a microplate reader (Model 680, Bio Rad Corp.). The spectrophotometer was calibrated with standard 0.5 to 800 ng mL^{-1} Tra solutions. Solutions of unconjugated micelles were served as the blank. The concentration of Tra in the micelles was determined by optical density against the standard curve, and calculated as the weight of antibody (micrograms antibody) associated with unit weight (milligrams) of lyophilized pHPM. Standard equation extrapolated from the standard curve was list as below:

$$\text{Antibody Concentration} = 15460.04111 \times \mathbf{OD}^2 + 62.84282 \times \mathbf{OD} - 96.33645$$

The grafting rate of monoclonal antibody trastuzumab conjugated to the micelles is around 12% –

15%. The antibody content on the micelles is approximately 4 μg antibody/mg micelles.

Preparation of superparamagnetic iron oxide nanoparticles (SPIONs)

The preparation of SPIONs is from an existing method of chemical coprecipitation. $FeCl_3 \cdot 6H_2O$ (5.41 g, 0.02 mol) and $FeCl_2 \cdot 4H_2O$ (1.99 g, 0.01 mol) were added into 100 mL of deoxygenated deionized water under nitrogen, and the solution was heated to 80 °C in 30 min. Then 25 mL of aqueous ammonium hydroxide was dropwise added at the rate of 3 drops per second with the stirring speed to 800 rpm. After the solution color changing from brown to black, 1 mL of oleic acid was added. The dispersion was continuously stirred for another 1 h under a nitrogen flow to complete the reaction. The mixture was cooled to room temperature naturally and dialyzed against deionized water for two days to remove ammonia and impurities. The achieved product was stored at 4 °C.

SPIONs loading

PH-sensitive polyurethane (pHPU) solution of DMAc with the concentration of 5 mg mL^{-1} was added into SPION solution dropwise under an ultrasound environment. The micelle solution was dialyzed against deionized water in a dialysis tube (MWCO 3500) for about 3 days to remove the DMAc at room temperature. The whole mixture was centrifugalized at 3000 rpm for 20 min and passed through a 0.45 μm pore-sized syringe filter (Milipore, Carrigtwohill, Co. Cork, Ireland). The concentration of SPIONs wrapped in pHPM was approximately 1mg mL^{-1}, and the free SPIONs was diluted to the same magnitude.

Supporting Chart and Figures

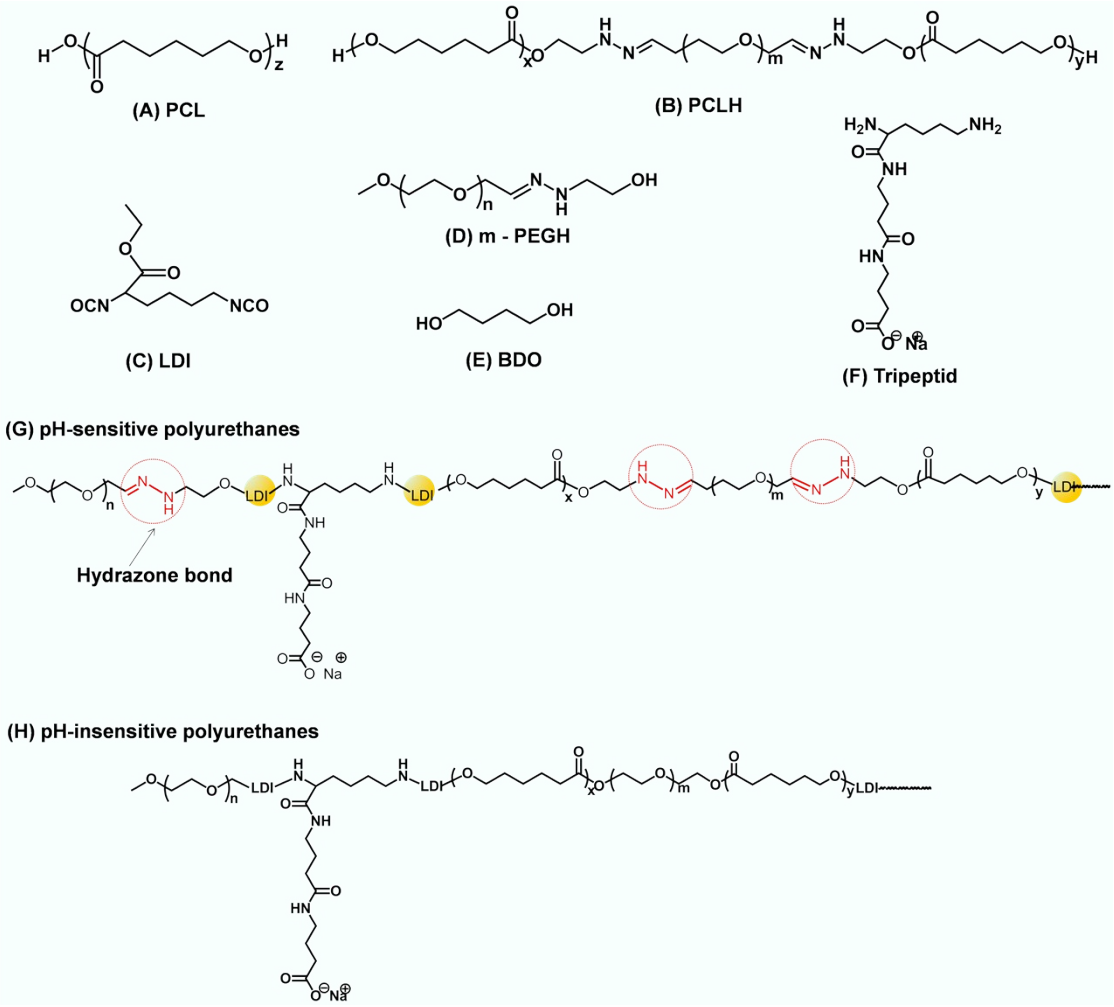

Chart S1. Schematic molecular structures of polyurethanes and monomers. (A) PCL, (B) PCLH, (C) LDI, (D) m-PEGH, (E) BDO, (F) Tripeptid and typical schematic structure of pHPU with (G) or PU without hydrazone bond (H).

pH-sensitive biodegradable polyurethanes (pHPU) have been prepared in our previous work using L-lysine ethyl ester diisocyanate (LDI), 1, 4-butanediol (BDO) and tripeptide chain extender (TCE) as hard segment, poly(ε-caprolactone)-hydrazone-poly(ethylene glycol)-hydrazone-poly(ε-caprolactone) (PCLH) as the soft segment, and hydrazone-linked methoxyl-poly(ethylene glycol) (m-PEGH) as end-capper. As reported in our previous work[2], characteristic peaks belonged to different segments can be

found in the ^{1}H NMR spectra. Concretely, chemical shifts at 5.77, 6.19 and 6.75 ppm are ascribed to (-NHCONH-), (-NHCONH-), and (-OCONH-) units, respectively. Specifically, the characteristic peak of hydrazone bond in pHPU is at 6.95 ppm. Moreover, the peak of methylene protons of the PCL is at 3.96 ppm (-CH_2O-), and the peaks of methylene (-CH_2OCO-) and methyl (-CH_3) protons in the ethoxyl group of LDI units are at 4.05 and 1.15 ppm, respectively. More structure characterization results can be found in our previous report [2].

Table S1 Micellization characterization of pHPM.

Sample	Size (nm)	PdI	Zeta potential (mV)
pHPM	67.5±0.6	0.166±0.010	-14.50±0.66
PM	65.7±1.4	0.184±0.010	-17.87±0.57
pHPM-Tra	76.0±0.7	0.119±0.010	-6.19±0.46

After antibody conjugation, both the average size and zeta potential were increased, from 67.5 nm and −14.5 mV to 79.3 nm and −6.2 mV, respectively.

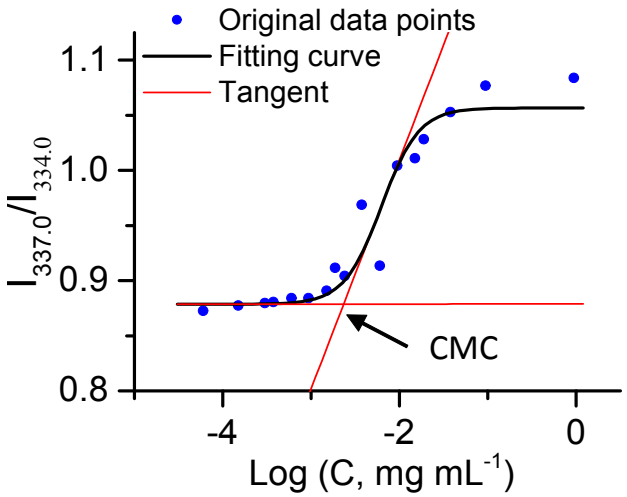

Figure S1 $I_{337.0}/I_{334.0}$ ratios in the excitation spectra as a function of micellar concentrations (log C).

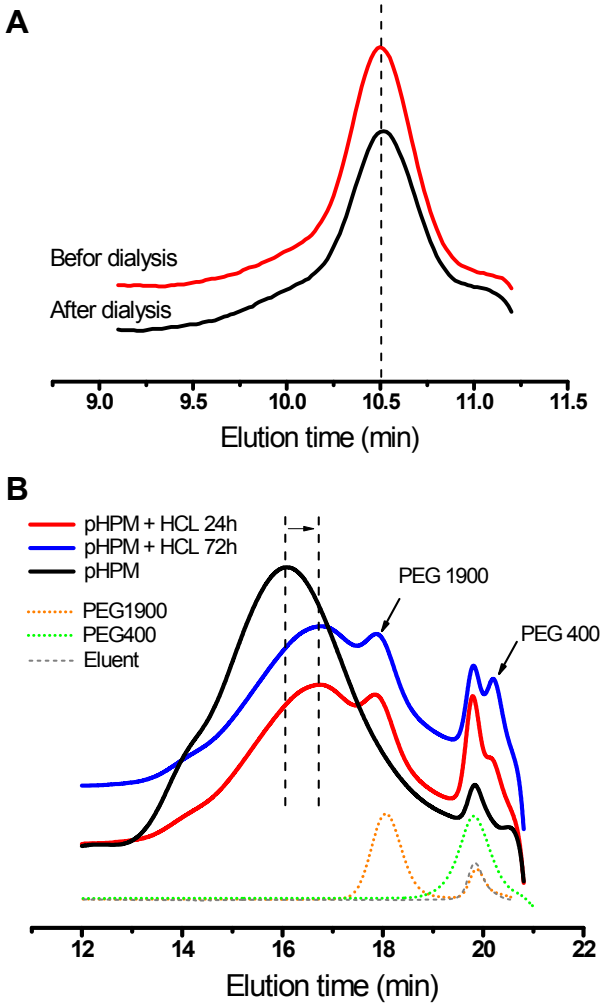

Figure S2 (A) GPC diagram of pHPM before and after dialysis. The eluent is H_2O/ Na_2CO_3/NaN_3. (B) GPC diagram of pHPM before and after degradation for 24 h or 72 h in response to weak acidic condition (pH ~4). PEG 1900 was taken as sample (organic eluent). The eluent is *N, N*-dimethyl-formamide (DMF)/LiBr.

According to the degradation mechanism illustrated in Figure 2A, the peaks of PEG1900 appear in the curves for pHPM after 24 h incubation with HCL, which is caused by the detachment of the long chain PEG corona (m-PEGH). Then the hydrophobic cores are gradually exposed, and the hydrazone bonds from the soft segment (PCLH) start to fracture, leading to the dissociation of short PEG400. The peak of PEG400 is observed after 72 h and has a little offset affected by the solvent peak.

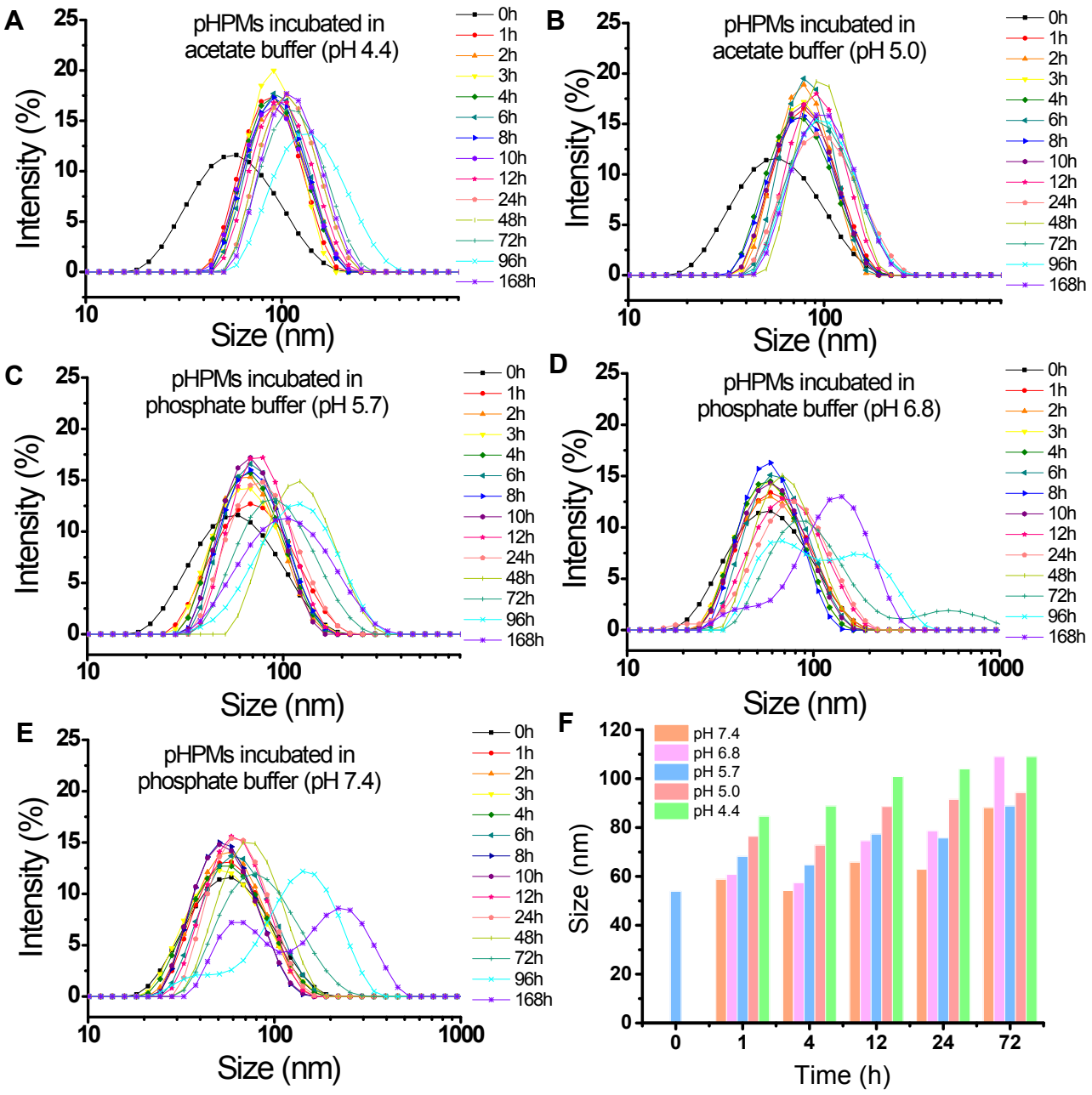

Figure S3 pH-responsive behaviors of pH-sensitive polyurethane micelles (pHPM) in an acidic environment in size distributions. pHPM was exposed to buffer media under various pH conditions for different times. Both size distributions by intensity and average particle size changing over time were recorded. (A) pH = 4.4, (B) pH = 5.0, (C) pH = 5.7, (D) pH = 6.8, (E) pH = 7.4 and (F) diagram of size change trend in a pH-dependent manner.

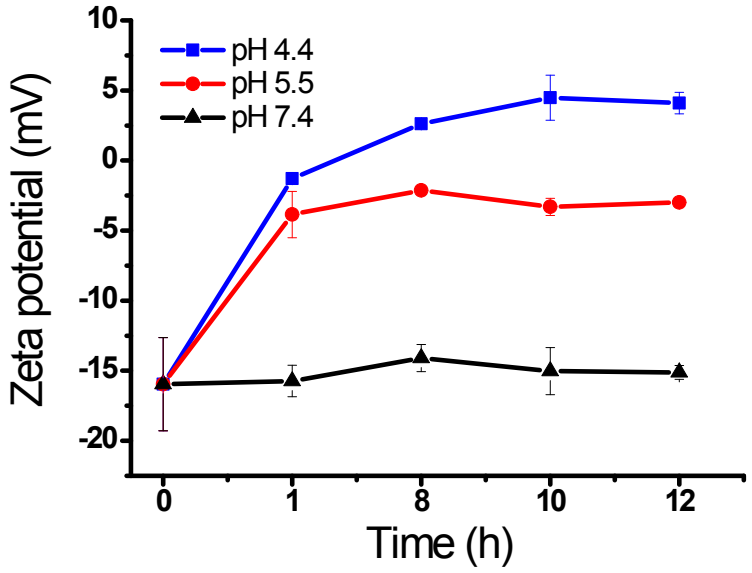

Figure S4 Zeta potential data at each period of pHPM after incubated in medium with different pH.

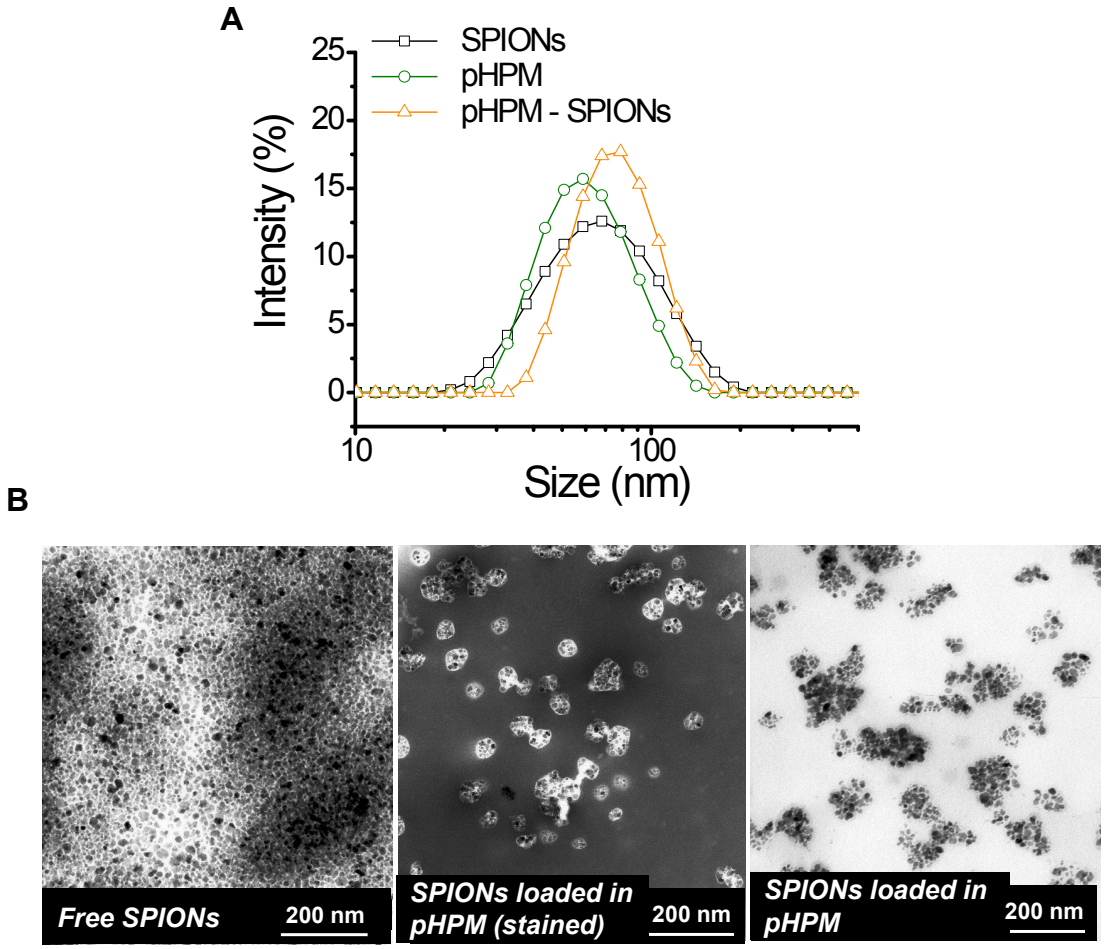

Figure S5 (A) Size distributions of free superparamagnetic iron oxide nanoparticles (SPIONs), pH-sensitive polyurethane micelles (pHPM), and SPIONs loaded pHPM, (B) TEM images of free SPIONs and SPIONs loaded pHPM with or without dye.

The micelle diameter increases slightly after SPIONs wrapped. In addition, as shown in the TEM images, apparently, the aggregation morphology of free SPIONs and wrapped SPIONs is different. After the micelle loading SPION samples were stained, the morphology of wrapped SPIONs by pHPU can be clearly identified due to the density difference between SPIONs and pHPU.

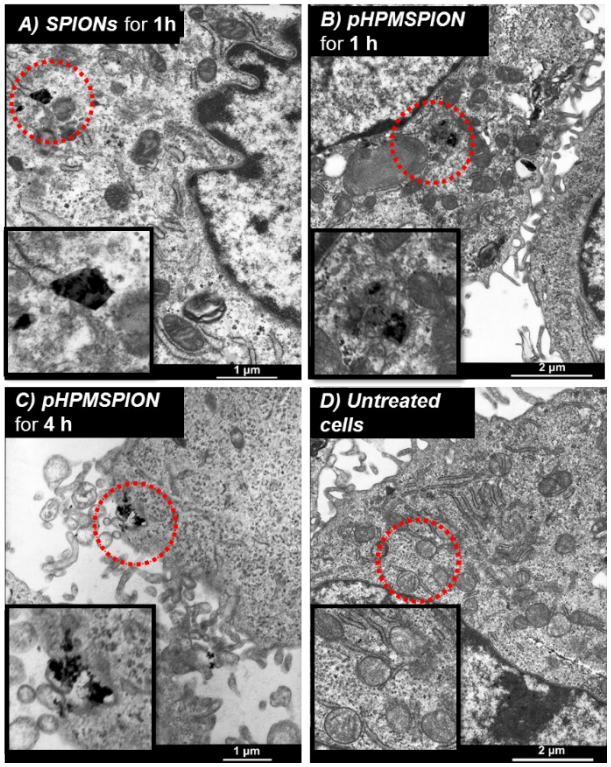

Figure S6 TEM images of (A) free superparamagnetic iron oxide nanoparticales (SPIONs) and (B) SPIONs loaded pH-sensitive polyurethane micelles (pHPMSPION) in SKOV-3 cells for 1 h. (C) TEM image of the course that micelles interact with cell membranes when the cells were cultured with pHPM loading SPIONs for 4 h. As can be seen, the membrane concaved and trapped the pHPM loading SPIONs to form phagocytic vesicles. (D) Cells without any treatment were set as blank control, of which organelles, such as lysosome, mitochondria and endoplasmic reticulum, were clear to be observed. There is an enlarged view of each figure at the lower left corner. Pictures above were the ones which didn't shown in the manuscript (**Figure 4B)**

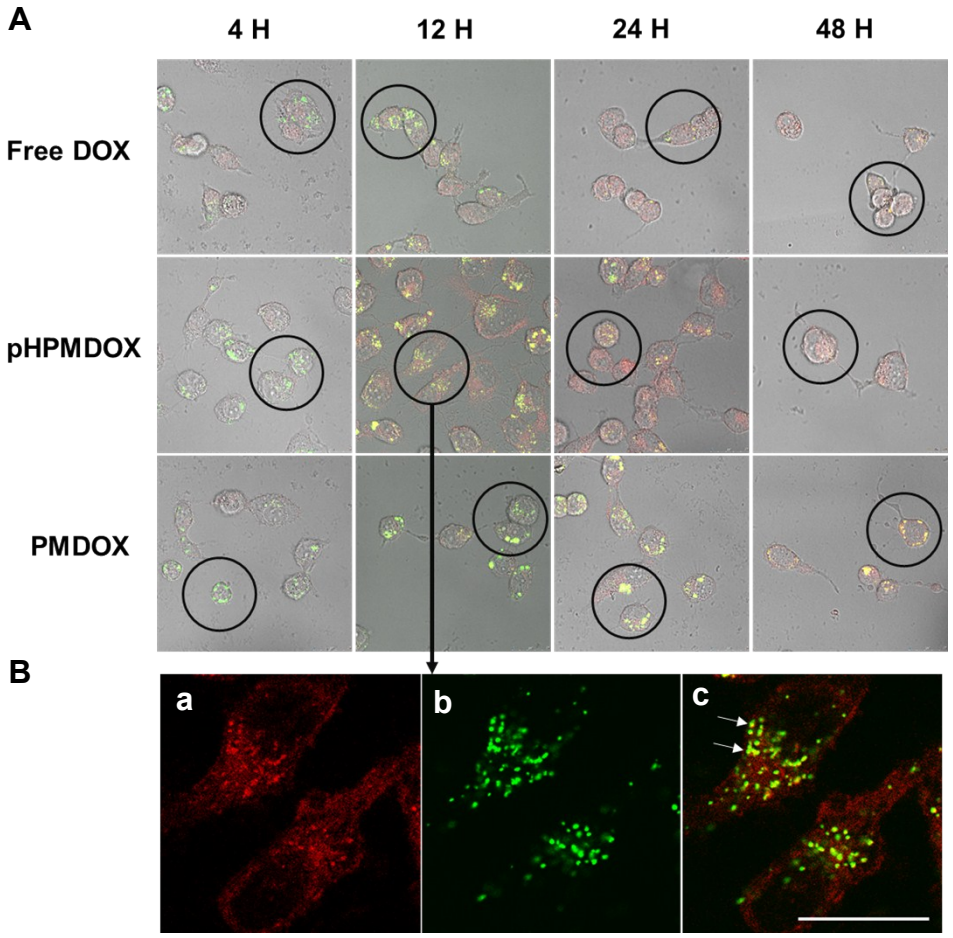

Figure S7 (A) CLSM images superposed with bright field**.** SKOV-3 cells were stained with Lysotracker (green channel), and red channel fluorescence emission originated from doxorubicin (DOX). Scale bar is 25 μm. (B) Enlarged view for the circle marked region of pHPMDOX with 12 h incubation, a) pHPMDOX, b) Lysotracker, c) merged.

As shown in Figure S7B, when pHPMDOX was trapped in endo-lysosome, it appears intensive and bright dot, and can be superimposed with the green fluorescence from endo-lysosome. The overlapped area was yellow in the merged image (white arrows).

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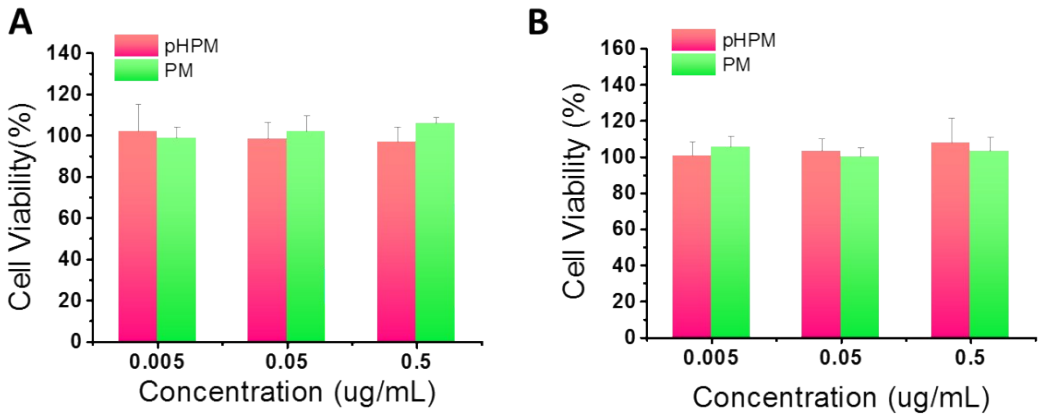

Figure S8 Cytotoxicity of pH-sensitive polyurethane micelles in SKOV-3 tumor cells. Cell viability was determined by the MTT assay after (A) 24 h and (B) 72 h of incubation with various concentrations of pHPM and micelles without hydrazone backbone (PM).

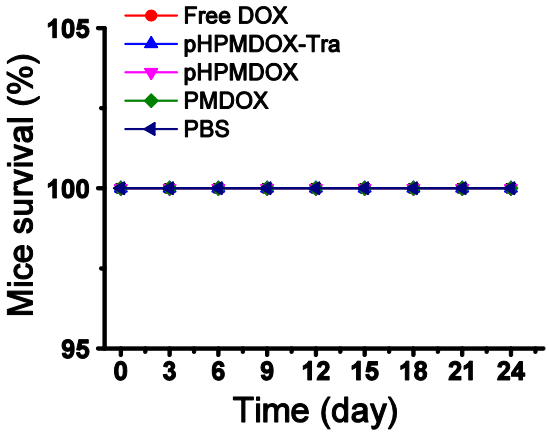

Figure S9 Survival curve of tumor-bearing mice receiving different treatments. It should be noticed that all points are overlap at the same time for different samples.

References

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