

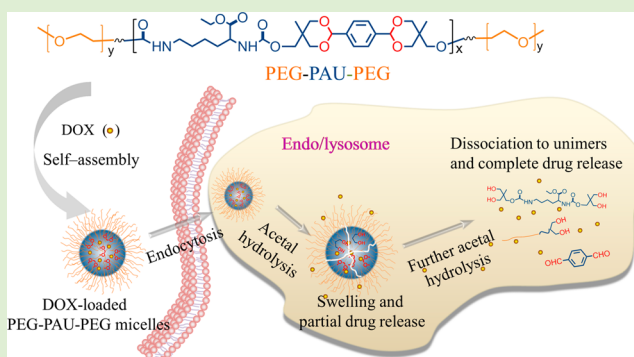
# Micelles Based on Acid Degradable Poly(acetal urethane): Preparation, pH-Sensitivity, and Triggered Intracellular Drug Release

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## Supporting Information

**ABSTRACT:** Polyurethanes are a unique class of biomaterials that are widely used in medical devices. In spite of their easy synthesis and excellent biocompatibility, polyurethanes are less explored for controlled drug delivery due to their slow or lack of degradation. In this paper, we report the design and development of novel acid degradable poly(acetal urethane) (PAU) and corresponding triblock copolymer micelles for pH-triggered intracellular delivery of a model lipophilic anticancer drug, doxorubicin (DOX). PAU with  $M_n$  ranging from 4.3 to 12.3 kg/mol was conveniently prepared from polycondensation reaction of lysine diisocyanate (LDI) and a novel diacetal-containing diol, terephthalilidene-bis(trimethylolethane) (TPABTME) using dibutyltin dilaurate (DBTDL) as a catalyst in *N,N*-dimethylformamide (DMF). The thiol-ene click reaction of Allyl-PAU-Allyl with thiolated PEG ( $M_n = 5.0$  kg/mol) afforded PEG-PAU-PEG triblock copolymers that readily formed micelles with average sizes of about 90–120 nm in water. The dynamic light scattering (DLS) measurements revealed fast swelling and disruption of micelles under acidic pH. UV/vis spectroscopy corroborated that acetal degradation was accelerated at pH 4.0 and 5.0. The *in vitro* release studies showed that doxorubicin (DOX) was released in a controlled and pH-dependent manner, in which ca. 96%, 73%, and 30% of drug was released within 48 h at pH 4.0, 5.0, and 7.4, respectively. Notably, MTT assays displayed that DOX-loaded PEG-PAU-PEG micelles had a high *in vitro* antitumor activity in both RAW 264.7 and drug-resistant MCF-7/ADR cells. The confocal microscopy and flow cytometry experiments demonstrated that PEG-PAU-PEG micelles mediated efficient cytoplasmic delivery of DOX. Importantly, blank PEG-PAU-PEG micelles were shown to be nontoxic to RAW 264.7 and MCF-7/ADR cells even at a high concentration of 1.5 mg/mL. Hence, micelles based on poly(acetal urethane) have appeared as a new class of biocompatible and acid-degradable nanocarriers for efficient intracellular drug delivery.



## INTRODUCTION

Polyurethanes are one of the most important synthetic biomaterials that are widely used in various medical devices including heart valves, catheters, vascular grafts, and prostheses.<sup>1–3</sup> Taking advantage of their excellent biocompatibility and physical properties, polyurethanes in particular degradable polyurethanes have been explored for different biomedical applications such as controlled drug release and tissue engineering.<sup>4–8</sup> In order to attain degradability, polyurethanes are typically prepared by polycondensation reaction of degradable polyester or polycarbonate diols with diisocyanate.<sup>9–11</sup> It should be noted, however, that these degradable polyurethanes generally exhibit a slow degradation rate, which does not match controlled drug release applications.

In the past several years, stimuli-sensitive degradable polyurethanes, e.g., containing reductively cleavable disulfide bonds<sup>12–14</sup> and acid labile hydrazone bonds,<sup>15–17</sup> have been designed and developed for triggered drug release. Tan et al. reported that acid-sensitive degradable micelles based on hydrazone-containing multiblock polyurethane doubled pacli-

taxel (PTX) release at pH 5.0 with regard to pH 7.4, exhibiting an enhanced anticancer effect against A431 squamous carcinoma tumor cells *in vitro*.<sup>15–17</sup> PTX-loaded reduction-responsive multiblock polyurethane micelles based on *L*-lysine ethyl ester diisocyanate (LDI), poly( $\epsilon$ -caprolactone) (PCL), butanediol, bis(2-hydroxyethyl)disulfide and poly(ethylene glycol) (PEG) showed effective growth inhibition of HepG2 cells.<sup>12</sup> Notably, sophisticated pH and reduction dual-responsive degradable multiblock polyurethane nanoparticles loaded with triptolide showed improved therapeutic efficacy *in vivo* in nude mice bearing the A431 tumor model.<sup>18</sup>

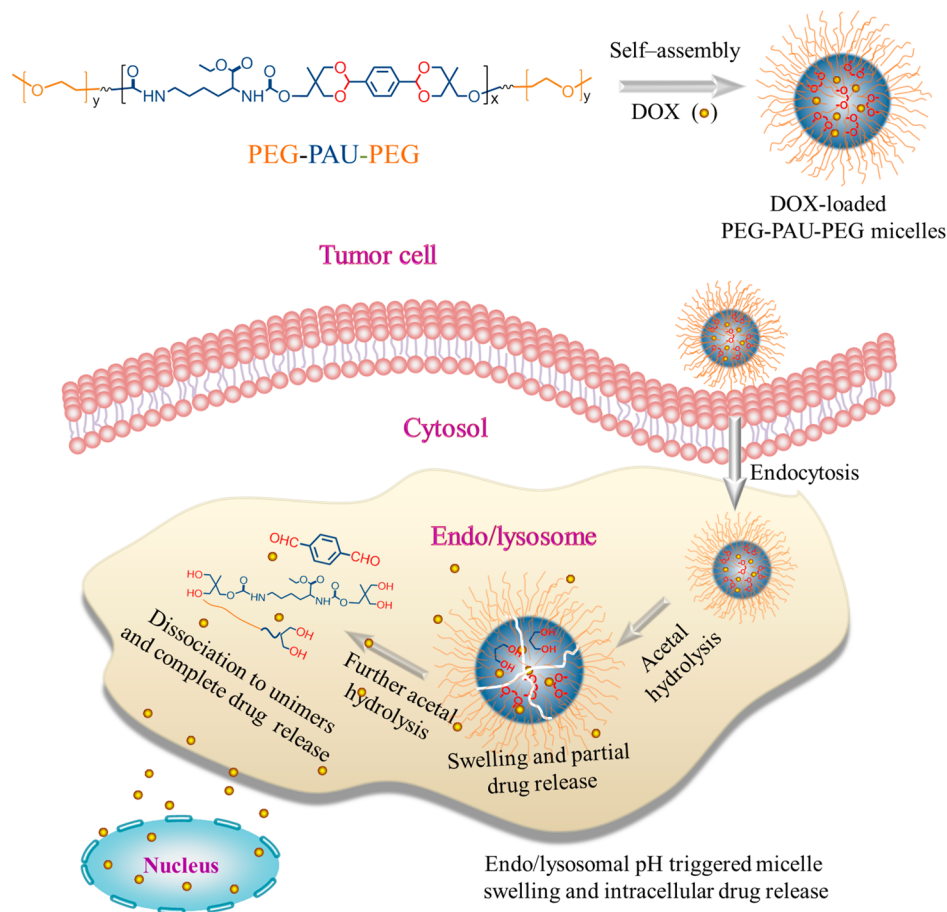
pH-sensitive degradable micelles have attracted great interest for tumor-targeted drug delivery because there exists a natural pH gradient in the tumor microenvironment as well as inside the tumor cells (e.g., in the endo/lysosomal compartments).<sup>19,20</sup> Various pH-sensitive degradable micelles have

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Scheme 1. Acid-Degradable PEG-PAU-PEG Triblock Copolymer Micelles for pH-Triggered Intracellular DOX Delivery



been developed based on different acid-labile bonds such as ortho ester,<sup>21,22</sup> hydrazone,<sup>23,24</sup> cis-aconitic amide,<sup>25,26</sup> and acetal.<sup>27</sup> In particular, acetal bond is interesting due to its great pH sensitivity.<sup>28–32</sup> We prepared pH-sensitive degradable micelles and polymersomes by incorporating pendant acetal groups at the side chain of polycarbonate or polyacrylate.<sup>33–36</sup> The hydrolysis of the pendant acetal groups would transform the hydrophobic block into hydrophilic, resulting in accelerated drug release.<sup>36</sup> PTX-loaded pH-sensitive degradable nanocarriers based on the acetalated  $\alpha$ -cyclodextrin revealed significantly improved cytotoxic activity against various tumor cells.<sup>37</sup> We found that acetal-linked PTX prodrug micellar nanoparticles exhibited potent growth inhibition of human cancer cells *in vitro*.<sup>38</sup>

In this paper, triblock copolymer micelles based on novel acid degradable poly(acetal urethane) (PAU) were designed and developed for pH-triggered intracellular delivery of DOX. (Scheme 1). PEG-PAU-PEG triblock copolymers could be readily prepared from polycondensation reaction between lysine diisocyanate (LDI) and a diacetal-containing diol, terephthalaldehyde-bis(trimethylolethane) (TPABTME) followed by thiol-ene click conjugation with thiolated PEG at both ends. To the best of our knowledge, this represents the first report on the synthesis of poly(acetal urethane)s that are subject to main chain degradation under an acidic condition. The preparation of micelles, pH-responsivity, and intracellular drug release behaviors were investigated.

## EXPERIMENTAL SECTION

**Materials.** Poly(ethylene glycol) monomethyl ether (PEG,  $M_n = 5.0$  kg/mol, Sigma) was dried by azeotropic distillation from anhydrous toluene. Triphosgene (BTC, Shanxi Jiaocheng Jingxin Chemical Factory) was recrystallized from ethyl acetate. Azobis(isobutyronitrile) (AIBN, 98%, J&K) was recrystallized twice from methanol. Dichloromethane (DCM) and dimethyl sulfoxide (DMSO) were dried over  $\text{CaH}_2$  and distilled prior to use. Dibutyltin dilaurate (DBTDL, 99%, J&K), *N,N*-dimethyl-formamide (DMF, 99.8%, Alfa Aesar), terephthalaldehyde (TPA, 99%, J&K), L-lysine ethyl ester dihydrochloride (LEED, 98%, J&K), trimethylolethane (TME, 99%, J&K), triethylamine (TEA, 99.5%, Sigma-Aldrich) and *p*-toluenesulfonic acid (PTSA, 99%, Adamas-beta) were used as received. Doxorubicin hydrochloride (DOX-HCl, 99%, Beijing Zhongshuo Pharmaceutical Technology Development Co., Ltd.) was desalted with TEA in DMSO prior to use. Thiolated poly(ethylene glycol) (PEG-SH) and LDI were synthesized and purified as previous reported.<sup>39,40</sup>

**Characterization.**  $^1\text{H}$  NMR spectra were recorded on a Unity Inova 400 spectrometer operating at 600 MHz using  $\text{DMSO-}d_6$  as a solvent. The chemical shifts were calibrated against residual solvent signals. Elemental analyses were performed on a PerkinElmer EA 240. Fourier transform infrared spectrometry (Varian 3600 FTIR) was performed on thermo scientific spectrophotometer with Omnic software for data acquisition and analysis. Polymers were grounded into KBr powder and pressed into discs prior to FTIR analysis. The molecular weight and polydispersity of copolymers were determined by a Waters 1515 gel permeation chromatograph (GPC) instrument equipped with MZ-gel SD plus columns (500 Å, 10E3 Å, 10E4 Å) following a differential refractive-index detector (RID 2414). DMF containing 0.05 mol/L LiBr was used as the mobile phase at a flow rate of 0.8 mL/min at 40 °C. A series of narrow polystyrene standards were

used for molecular weight calibration. The dynamic light scattering (DLS) measurements were carried out at 25 °C using Zetasizer Nano-ZS from Malvern Instruments equipped with a 633 nm He–Ne laser using backscattering detection. Transmission electron microscopy (TEM) was performed using a HT7700 TEM operated at an accelerating voltage of 120 kV. The samples were prepared by dropping 10  $\mu$ L of 0.2 mg/mL micelle suspension on the copper grid followed by staining with 1 wt % phosphotungstic acid.

**Synthesis of Terephthalilidene-bis(trimethylolethane) (TPABTME).** TPABTME was synthesized via aldolization reaction between TPA and TME. In brief, TPA (6.7 g, 50 mmol), PTSA (0.65 g, 3.42 mmol), and TME (30 g, 250 mmol) were dispersed in toluene (200 mL) and stirred at 120 °C for 8 h. The reaction mixture was concentrated by evaporation, the residue was washed with phosphate buffer (PB, 10 mM, pH 7.4), and the product was collected by filtration and dried *in vacuo* for 2 days. Yield: 94%. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  (ppm): 1.14, 0.682 (6H, CH<sub>3</sub>–C–(CH<sub>2</sub>)<sub>3</sub>–), 3.17, 3.67 (d, 2H, HO–CH<sub>2</sub>–C–), 3.57, 3.80, 3.87 (d, 8H, –C–CH<sub>2</sub>–O), 4.7 (t, 2H, HO–CH<sub>2</sub>–), 5.40 (d, 2H, –O–CH–O–(C)–), 7.40 (m, 4H, aromatic protons). Elemental analysis for C<sub>18</sub>H<sub>26</sub>O<sub>6</sub>: C: 63.89; H 7.74. Found: C: 64.00; H: 7.18.

**Synthesis of pH-Sensitive Degradable Allyl-PAU-Allyl.** Allyl-PAU-Allyl was prepared by polycondensation reaction between TPABTME and LDI at varying LDI/TPABTME molar ratios from 1/0.97, 1/0.96 to 1/0.93 followed by termination with allyl alcohol. Briefly, under a N<sub>2</sub> atmosphere, to a solution of LDI (0.226 g, 1 mmol) and TPABTME (0.314 g, 0.93 mmol) in DMF (2.7 mL) was added a catalytic amount of DBTDL (5 mg) under stirring. The reaction was allowed to proceed at 65 °C for 48 h. Fifty milligrams of allyl alcohol was added dropwise, and the reaction was continued at 65 °C for another 12 h. The resulting polymer was isolated by twice precipitation in diethyl ether, filtration, and drying *in vacuo*. Yield: 90%. <sup>1</sup>H NMR (600 MHz, DMSO-*d*<sub>6</sub>): LDI:  $\delta$  1.16, 1.31, 1.38, 1.60, 1.65, 2.96, 3.64, 4.07; TPABTME:  $\delta$  0.73, 1.16, 3.65, 3.74, 3.82, 3.92, 5.40, 5.46 7.40; vinyl:  $\delta$  4.45, 5.16, 5.27, 5.87; –NH–COO–:  $\delta$  7.12, 7.18, 7.56, 7.63.

**Synthesis of pH-Sensitive Degradable PEG-PAU-PEG Triblock Copolymer.** PEG-PAU-PEG triblock copolymer was synthesized via click reaction between PEG-SH and Allyl-PAU-Allyl. Briefly, under a N<sub>2</sub> atmosphere, to a stirred solution of Allyl-PAU-Allyl (0.1 g, 8.3  $\mu$ mol) and AIBN (0.04 g) in 1,4-dioxane (5 mL) was added PEG-SH (0.165 g, 32.4  $\mu$ mol). The reaction was allowed to proceed at 90 °C for 48 h. The resulting copolymer was isolated by precipitation in methanol/diethyl ether for more than three times (in order to remove PEG-SH homopolymer), filtration and drying *in vacuo*. Yield: 65%. <sup>1</sup>H NMR (600 MHz, DMSO-*d*<sub>6</sub>): PEG:  $\delta$  3.24, 3.52; LDI:  $\delta$  1.16, 1.32, 1.38, 1.61, 1.65, 2.96, 3.64, 4.09; TPABTME:  $\delta$  0.73, 1.16, 3.65, 3.74, 3.82, 3.92, 4.2, 5.40, 5.46, 7.40; –NH–COO–:  $\delta$  7.12, 7.18, 7.56, 7.63.

**Micelle Formation and Critical Micelle Concentration.** Micelles were prepared by dropwise addition of 0.9 mL of PB (10 mM, pH 7.4) into 1.0 mL of PEG-PAU-PEG copolymer solution in DMF (10 mg/mL) under stirring at room temperature followed by extensive dialysis against PB (10 mM, pH 7.4) with a molecular weight cutoff (MWCO) of 3500.

The critical micelle concentration (CMC) was determined by using pyrene as a fluorescence probe. The concentration of copolymer was varied from 1.0  $\times$  10<sup>–5</sup> to 0.1 mg/mL and the concentration of pyrene was fixed at 1.0  $\mu$ M. The fluorescence spectra were recorded using a Carry Eclipse fluorescence spectrometer at an excitation wavelength of 330 nm. The emission fluorescence at 372 and 383 nm was monitored. CMC was estimated as the cross-point when extrapolating the intensity ratio  $I_{372}/I_{383}$  at low and high concentration regions.

**pH-Dependent Hydrolysis of the Acetals in PEG-PAU-PEG Micelles.** The hydrolysis of the acetals in PEG-PAU-PEG micelles was detected by UV/vis spectroscopy by measuring the absorbance at 263 nm. The micelle dispersions (1.0 mg/mL) prepared as mentioned above were divided into three aliquots and adjusted to pH 4.0, 5.0, and 7.4 by adding 40  $\mu$ L of 4.0 M pH 4.0 and 5.0 acetate buffer or pH 7.4 PB, respectively. The solutions were incubated at 37 °C. At the desired time intervals, 80  $\mu$ L aliquot was taken out and diluted with 3.5 mL of

PB (0.1 M, pH 7.4). The absorbance at 263 nm was monitored. At the end, all samples were completely hydrolyzed by adding concentrated HCl and the absorbance at 263 nm was used to calculate extent of acetal hydrolysis.

**Size Change of PEG-PAU-PEG Micelles in Response to Acidic pH.** The change of size and size distribution in response to acidic pHs were followed by DLS measurements. Briefly, to 1.0 mL of micelle dispersions (0.5 mg/mL) prepared as above at 37 °C was added 40  $\mu$ L of acetate buffer (4 M, pH 5.0), which resulted in a final pH of 5.0. The size changes of micelles were monitored in time by DLS.

**Loading and Acid-Triggered Release of DOX.** DOX-loaded PEG-PAU-PEG micelles were prepared by dropwise adding 1.0 mL of PB (10 mM, pH 7.4) to a mixture of PEG-PAU-PEG copolymer solution (100  $\mu$ L, 10 mg/mL) in DMF and DOX solution (25  $\mu$ L, 5 mg/mL) in DMSO under stirring at room temperature followed by dialysis against PB (10 mM, pH 7.4) with a MWCO of 3500 for 12 h. The dialysis media were changed at least five times. The whole procedure was performed in the dark.

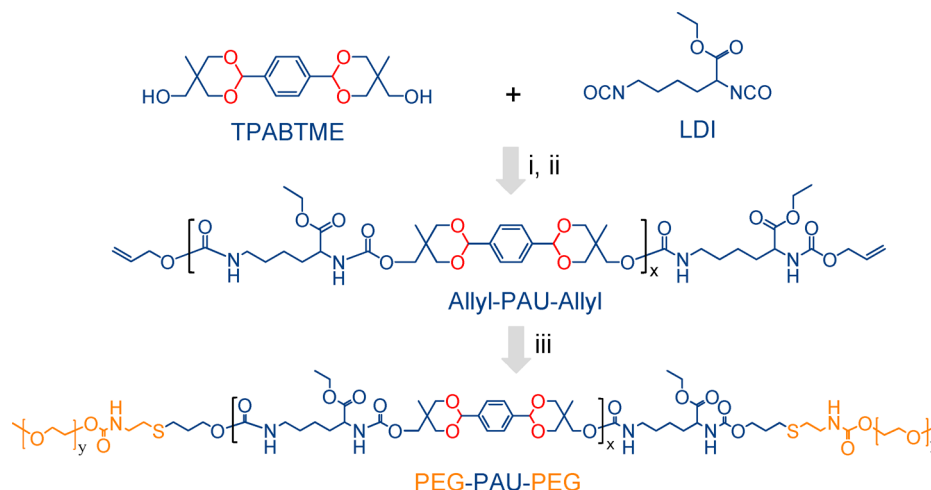
The amount of DOX was determined by a fluorescence spectrophotometer (Cary Eclipse) with the excitation at 480 nm and emission at 560 nm. To determine the drug loading content (DLC), lyophilized DOX-loaded micelles were dissolved in DMSO and analyzed with fluorescence spectroscopy, wherein the calibration curve was obtained with DOX/DMSO solution with different DOX concentrations. DLC and drug loading efficiency (DLE) were calculated according to the following formula:

$$\text{DLC (wt \%)} = \frac{\text{weight of loaded drug}}{\text{total weight of loaded drug and polymer}} \times 100$$

$$\text{DLE (\%)} = \frac{\text{weight of loaded drug}}{\text{weight of drug in feed}} \times 100$$

The drug release from DOX-loaded PEG-PAU-PEG micelles was investigated at 37 °C in three different media, i.e., (a) 10 mM acetate buffer, pH 4.0, (b) 10 mM acetate buffer, pH 5.0, and (c) 10 mM PB, pH 7.4. DOX-loaded micelles (0.1 mg/mL) were divided into three aliquots (each 0.5 mL) and immediately transferred to a dialysis tube with a MWCO of 12 000–14 000. The dialysis tube was immersed into 25 mL of corresponding buffer at 37 °C. At desired time intervals, 5 mL of release media was taken out and replenished with an equal volume of fresh media. To determine the amount of DOX released, calibration curves were run with DOX in corresponding buffer solution at pH 4.0, 5.0, and 7.4, respectively. The release experiments were conducted in triplicate, and the results presented are the average data.

**MTT Assays.** The cytotoxicity of PEG-PAU-PEG micelles was determined using DOX-resistant MCF-7 human breast cancer cells (MCF-7/ADR) and mouse leukemic monocyte macrophage cells (RAW 264.7). MCF-7/ADR cells were plated in a 96-well plate (1  $\times$  10<sup>4</sup> cells/well) in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and antibiotics penicillin (100 IU/mL) and streptomycin (100  $\mu$ g/mL). After 24 h, the medium was removed and replenished by 80  $\mu$ L of fresh medium. Twenty microliters of blank PEG-PAU-PEG micelles was added leading to final micelle concentrations of 0.3, 0.9, and 1.5 mg/mL, respectively. The cells were incubated under 5% CO<sub>2</sub> atmosphere for 48 h at 37 °C. The medium was aspirated and replaced by 100  $\mu$ L of fresh medium. Ten microliters of 3-(4, 5-dimethylthiazo 1-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL) was added. The cells were incubated for another 4 h. The medium was carefully aspirated, MTT-formazan generated by live cells was dissolved in 150  $\mu$ L of DMSO, and the absorbance at a wavelength of 570 nm of each well was measured using a microplate reader (Bio-Tek, ELX808 IU). The relative cell viability (%) was determined by comparing the absorbance at 570 nm with control wells containing only cell culture medium. MTT assays in RAW 264.7 cells were performed in a similar way except that Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS, 1% L-glutamine, and antibiotics penicillin (100 IU/mL) and streptomycin (100  $\mu$ g/mL) was used.

Scheme 2. Synthesis of PEG-PAU-PEG<sup>a</sup>

<sup>a</sup>Conditions: (i) DBTDL, DMF, 65 °C, 48 h; (ii) allyl alcohol, DBTDL, DMF, 65 °C, 12 h; (iii) PEG-SH, AIBN, 1,4-dioxane, 90 °C, 48 h.

The antitumor activity of DOX-loaded PEG-PAU-PEG micelles and free DOX-HCl was also studied by MTT assays. MCF-7/ADR cells were plated in a 96-well plate ( $1 \times 10^4$  cells/well) in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and antibiotics penicillin (100 IU/mL) and streptomycin (100  $\mu\text{g}/\text{mL}$ ). After 24 h, the medium was removed and replenished by 80  $\mu\text{L}$  of fresh medium. Twenty microliters of DOX-loaded PEG-PAU-PEG micelles or free DOX-HCl were added resulting in a final DOX concentrations of 0.1, 1, 5, 10, 20, 40, and 80  $\mu\text{g}/\text{mL}$ , respectively. The cells were incubated under 5%  $\text{CO}_2$  atmosphere for 48 h at 37 °C. The medium was aspirated and replaced by 100  $\mu\text{L}$  of fresh medium and 10  $\mu\text{L}$  of MTT solution (5 mg/mL) was added. The cells were incubated for 4 h. The medium was aspirated, the MTT-formazan generated by live cells was dissolved in 150  $\mu\text{L}$  of DMSO, and the absorbance at a wavelength of 570 nm of each well was measured using a microplate reader. The cell viabilities were determined by MTT assays as described above. For MTT assays in RAW 264.7 cells, DMEM containing 10% FBS, 1% L-glutamine, and antibiotics penicillin (100 IU/mL) and streptomycin (100  $\mu\text{g}/\text{mL}$ ) was used as a culture medium and DOX concentrations ranged from 0.0001, 0.001, 0.01, 0.1, 0.5, 1, 2.5 to 5  $\mu\text{g}/\text{mL}$ .

**Confocal Laser Scanning Microscopy (CLSM) Measurements.** The cellular uptake and intracellular release behaviors of DOX-loaded PEG-PAU-PEG micelles were followed with CLSM using RAW 264.7 and MCF-7/ADR cells. MCF-7/ADR cells were plated in a 24-well plate ( $5 \times 10^4$  cells/well) in RPMI 1640 media supplemented with 10% FBS, 1% L-glutamine, and antibiotics penicillin (100 IU/mL) and streptomycin (100  $\mu\text{g}/\text{mL}$ ) for 24 h. The media were aspirated and replaced by 400  $\mu\text{L}$  of fresh media. One hundred microliters of DOX-loaded micelles or free DOX-HCl (drug dosage: 15.0  $\mu\text{g}/\text{mL}$ ) was added. The cells were incubated with DOX-loaded micelles or free DOX-HCl for 4, 6, or 8 h at 37 °C in a humidified 5%  $\text{CO}_2$  containing atmosphere. The culture medium was removed, and the cells were rinsed three times with phosphate buffered saline (PBS, pH 7.4, 10 mM). The cells were then fixed with 4% paraformaldehyde for 20 min and washed with PBS for three times. The cell nuclei were stained with 4, 6-diamidino-2-phenylindole (DAPI, blue) for 20 min and washed with PBS three times. Fluorescence images of cells were obtained with a confocal laser scanning microscope (TCS SP2). For RAW 264.7 cells, DMEM containing 10% FBS, 1% L-glutamine, and antibiotics penicillin (100 IU/mL) and streptomycin (100  $\mu\text{g}/\text{mL}$ ) was used as a culture medium and DOX dosage was fixed at 5  $\mu\text{g}/\text{mL}$ .

**Flow Cytometry Analysis.** The cellular uptake and intracellular drug release behaviors of DOX-loaded PEG-PAU-PEG micelles were studied in MCF-7/ADR cells using flow cytometry. In Brief, MCF-7/ADR cells were seeded in a 6-well plate ( $1 \times 10^6$  cells/well) using

RPMI-1640 media supplemented with 10% FBS, 1% L-glutamine, antibiotics penicillin (100 IU/mL), and streptomycin (100  $\mu\text{g}/\text{mL}$ ) for 24 h. DOX-loaded PEG-PAU-PEG micelle dispersion or free DOX-HCl solution in 400  $\mu\text{L}$  of PBS was added to each well (DOX dosage: 10.0  $\mu\text{g}/\text{mL}$ ). After 4 h incubation at 37 °C, the cells were digested by 0.25 w/v% trypsin and 0.03 w/v% EDTA. The suspensions were centrifuged at 1500 rpm for 3 min at 4 °C, washed twice with PBS, and resuspended in 500  $\mu\text{L}$  of PBS. Fluorescence histograms were recorded with a BD FACS Calibur flow cytometer (Beckton Dickinson, U.S.A.) and analyzed using Cell Quest software. We analyzed 20 000 gated events to generate each histogram. The gate was arbitrarily set for the detection of red fluorescence.

## RESULTS AND DISCUSSION

**Synthesis of pH-Sensitive Degradable PEG-PAU-PEG Triblock Copolymer.** PEG-PAU-PEG triblock copolymers were readily obtained from polycondensation reaction between LDI and a diacetal-containing diol, TPABTME, followed by thiol-ene click conjugation with thiolated PEG at both ends (Scheme 2). TPABTME was synthesized at a high yield (94%) by reacting TPA with TME in the presence of catalytic amount of PTSA in toluene at 120 °C for 8 h followed by washing with 10 mM pH 7.4 PB (Scheme S1).  $^1\text{H}$  NMR showed besides signals attributable to the aromatic protons of TPA moiety ( $\delta$  7.40) and peaks due to TME moiety ( $\delta$  4.66, 3.15–3.89, 1.14, and 0.68) also acetal protons at  $\delta$  5.40 (Figure S1). The integral ratios of signals at  $\delta$  7.40, 5.40, and 0.68/1.14 (methyl protons of TME moiety) were close to 2:1:3, supporting successful synthesis of TPABTME. The structure of TPABTME was further confirmed by elemental analysis and  $^{13}\text{C}$  NMR spectrum (Figure S2).

The polycondensation reaction between LDI and TPABTME was carried out at varying LDI/TPABTME molar ratios from 1/0.97, 1/0.96 to 1/0.93 in DMF using DBTDL as a catalyst and terminated with allyl alcohol. The results of polymerization are summarized in Table 1. FTIR of Allyl-PAU-Allyl revealed absorption band characteristic of urethane stretch at  $3350\text{ cm}^{-1}$  (Figure S3).  $^1\text{H}$  NMR showed clearly signals assignable to LDI moieties ( $\delta$  1.16, 1.32, 1.38, 1.60, 1.65, 2.96, 3.64, 4.07), TPABTME moieties ( $\delta$  0.73, 1.16, 3.65, 3.74, 3.82, 3.92, 4.20, 5.40, 7.40), terminal vinyl protons ( $\delta$  5.90, 5.16, 5.27) and urethane protons ( $\delta$  7.12, 7.18, 7.56, 7.63) (Figure 1). The  $M_n$  of resulting Allyl-PAU-Allyl copolymers could be

**Table 1.** Synthesis of pH-Sensitive Degradable Poly(acetal urethane)s

entry	polymers	LDI/TPABTME feed ratio	$M_n$ (kg/mol)		
			$^1\text{H NMR}^a$	GPC <sup>b</sup>	PDI <sup>b</sup>
1	Allyl-PAU <sub>42</sub> -Allyl	1:0.97	12.3	18.3	1.8
2	Allyl-PAU <sub>26</sub> -Allyl	1:0.96	7.7	10.2	1.5
3	Allyl-PAU <sub>14</sub> -Allyl	1:0.93	4.3	7.7	1.3

<sup>a</sup>Determined by  $^1\text{H NMR}$ . <sup>b</sup>Determined by GPC (eluent: DMF, flow rate: 0.8 mL/min, standards: PMMA, 40 °C).

determined by comparing the integrals at  $\delta$  5.90 and  $\delta$  2.96, which were attributed to the terminal vinyl protons and methylene protons of LDI neighboring to urethane bond, respectively. The results showed that Allyl-PAU-Allyl had  $M_n$  values varying from 4.3 to 7.7 to 12.3 kg/mol, which increased with increasing LDI/TPABTME molar ratios. The number of acetal groups in Allyl-PAU-Allyl chain was determined to be 42, 26, and 14 by comparing the integrals at  $\delta$  5.90 (terminal vinyl protons) and  $\delta$  5.40 (acetal protons). The corresponding PAUs were denoted as PAU<sub>42</sub>, PAU<sub>26</sub>, and PAU<sub>14</sub>, respectively (Table 1). GPC measurements showed that these Allyl-PAU-Allyls had moderate polydispersity indices of 1.3–1.8 and  $M_n$  ranging from 7.7, 10.2 to 18.3 kg/mol, which increased in parallel with those determined by  $^1\text{H NMR}$  end-group analyses. It should be noted that PMMA standards were used for the calibration of GPC columns, which likely accounts for the deviation in  $M_n$  values.

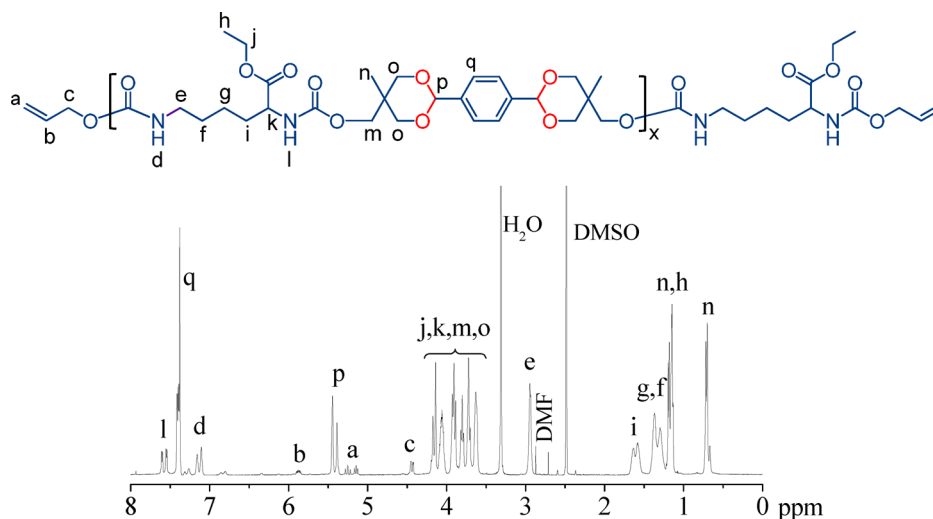
PEG-PAU-PEG triblock copolymers were finally obtained by thiol-ene click reaction between Allyl-PAU-Allyl and PEG-SH in 1,4-dioxane at 90 °C for 48 h.  $^1\text{H NMR}$  detected besides signals assignable to PAU block also resonances owing to the methyl and methylene protons of PEG at  $\delta$  3.24 and 3.51 (Figure 2). Notably, peaks due to vinyl protons ( $\delta$  5.12, 5.25, 5.90) completely disappeared and a new peak attributable to the methylene protons neighboring to the thiol ether appeared at  $\delta$  2.74, confirming successful conjugation of PEG to Allyl-PAU-Allyl. The  $M_n$  values of PEG-PAU-PEG were determined to be 15.2, 18.9, and 23.2 kg/mol by comparing the integrals of signals at  $\delta$  7.40 (aromatic protons of PAU block) and  $\delta$  3.51 (methylene protons of PEG block) (Table 2). Importantly,

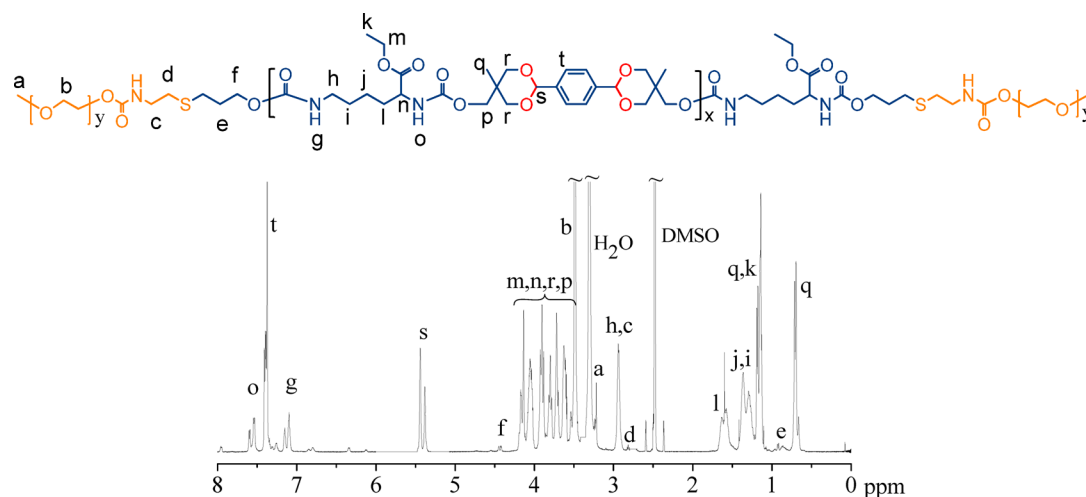
GPC curves revealed that all three PEG-PAU-PEG copolymers had a unimodal distribution with polydispersity indices of 1.2–1.7, indicating the absence of free PEG and PAU homopolymers (Figure S4). The  $M_n$  values determined by GPC were 21.5, 24.2, and 28.3 kg/mol, respectively, which were in accordance with those determined by  $^1\text{H NMR}$ . It is evident that we have successfully synthesized PEG-PAU-PEG triblock copolymers.

**Formation and pH-Responsivity of PEG-PAU-PEG Micelles.** PEG-PAU-PEG micelles were prepared via the solvent exchange method. DLS showed that all micelles had a narrow size distribution (PDI: 0.09–0.12) (Figure 3A). The average sizes of micelles varied from 92 to 122 nm, which increased with increasing  $M_n$  of PAU block. TEM micrograph revealed that PEG-PAU-PEG micelles had a homogeneous distribution with particle sizes close to those determined by DLS (Figure 3B). The CMC measurements using pyrene as a fluorescence probe showed that PEG-PAU-PEG had particularly low CMC values of 0.56–1.22 mg/L (Table 3), indicating that PEG-PAU-PEG triblock copolymer forms micelles with high stability.

The hydrolysis of acetals in PEG-PAU<sub>26</sub>-PEG micelles was investigated at 37 °C under three different pH conditions (i.e., pH 4.0, 5.0 and 7.4). The extent of acetal hydrolysis could be determined using UV/vis spectroscopy by monitoring the absorbance at 263 nm, which is the characteristic absorbance of TPA (hydrolysis product). The results showed that hydrolysis rate of acetals in PEG-PAU<sub>26</sub>-PEG micelles was highly pH dependent, in which 99.6%, 58.7%, and 14.8% acetals were degraded in 12 h at pH 4.0, 5.0 and 7.4, respectively (Figure 3C). The half-lives of acetals were determined to be 3.2 h at pH 4.0 and 9.6 h at pH 5.0. In comparison, less than 20% acetals were hydrolyzed even after 24 h at pH 7.4.

The size change of PEG-PAU<sub>26</sub>-PEG micelles in response to acidic pH was followed by DLS measurements. The results showed that micelles underwent rapid and remarkable swelling at pH 5.0, in which average size increased from ca. 100 to 160 nm in 2 h and reached over 800 nm in 12 h (Figure 3D). Interestingly, only unimers were detected after 24 h incubation at pH 5.0, indicating that acetal hydrolysis has led to complete dissolution of polymers. Notably, pH-sensitive micelles containing pendant acetal groups only swelled to large particles

**Figure 1.**  $^1\text{H NMR}$  spectrum (400 MHz,  $\text{DMSO-}d_6$ ) of Allyl-PAU<sub>26</sub>-Allyl (Table 1, entry 2).

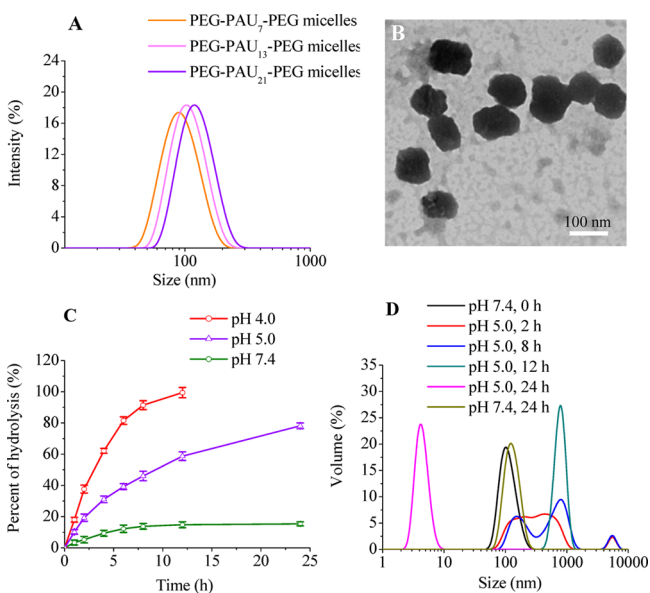


**Figure 2.**  $^1\text{H}$  NMR spectrum (600 MHz,  $\text{DMSO}-d_6$ ) of PEG-PAU<sub>26</sub>-PEG (Table 2, entry 2).

**Table 2.** Synthesis of PEG-PAU-PEG Triblock Copolymers

entry	polymers	$M_n$ (kg/mol)		
		$^1\text{H}$ NMR <sup>a</sup>	GPC <sup>b</sup>	PDI <sup>b</sup>
1	PEG-PAU <sub>42</sub> -PEG	23.2	28.3	1.7
2	PEG-PAU <sub>26</sub> -PEG	18.9	24.2	1.2
3	PEG-PAU <sub>14</sub> -PEG	15.2	21.5	1.2

<sup>a</sup>Determined by  $^1\text{H}$  NMR. <sup>b</sup>Determined by GPC (eluent: DMF, flow rate: 0.8 mL/min, standards: PMMA, 40 °C).



**Figure 3.** (A) Size distribution of PEG-PAU-PEG triblock copolymer micelles determined by DLS. (B) TEM image of PEG-PAU<sub>26</sub>-PEG micelles. (C) pH-dependent hydrolysis of acetals in PEG-PAU<sub>26</sub>-PEG micelles at 37 °C. Data are presented as mean  $\pm$  standard deviation ( $n = 3$ ); (D) Size changes of PEG-PAU<sub>26</sub>-PEG micelles in acetate buffer (pH 5.0, 10 mM) or PB (pH 7.4, 10 mM) at 37 °C in time.

instead of dissolution at acidic pH.<sup>41</sup> By contrast, little change of PEG-PAU-PEG micelles size was observed over 24 h at pH 7.4 under otherwise the same conditions.

**Loading and *in Vitro* Release of DOX.** In this study, DOX was used as a model lipophilic drug to investigate the drug encapsulation and release behaviors of PEG-PAU-PEG

micelles. The theoretical DLC was set at 20 wt %. The results showed that PEG-PAU-PEG micelles had DLCs of 8.0–9.5 wt %, which corresponded to DLEs of 35–42% (Table 3). DLC increased with increasing length of hydrophobic PAU block, likely due to a stronger interaction with DOX. The loading of DOX resulted in about 40 nm increase in the micelle size while maintaining a low PDI. The *in vitro* release studies performed at 37 °C and different pHs demonstrated a much faster release of DOX at acidic pHs than at physiological pH (Figure 4). For example, approximately 96% and 73% of DOX was released within 48 h from PEG-PAU<sub>42</sub>-PEG micelles at pH 4.0 and 5.0, respectively. In contrast, less than 30% of drug was released at pH 7.4 under otherwise the same conditions. At the same pH, drug release rate increased, though not significantly, with increasing PAU molecular weights.

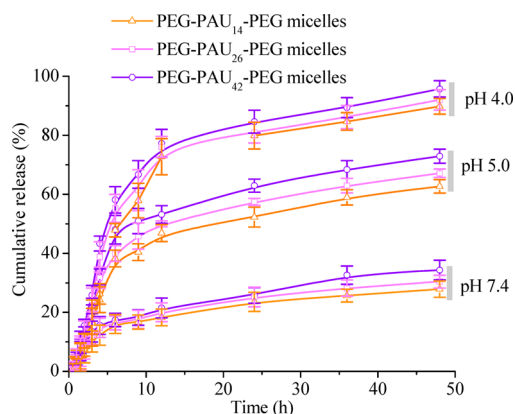
#### Antitumor Activity, Cellular Uptake and Intracellular Release Behaviors of DOX-Loaded PEG-PAU-PEG Micelles.

The antitumor activity of PEG-PAU-PEG micelles was investigated via MTT assays in RAW 264.7 cells and drug-resistant MCF-7/ADR cells. The results revealed that DOX-loaded PEG-PAU-PEG micelles displayed significant antitumor activity toward RAW 264.7 cells with a low  $\text{IC}_{50}$  of 0.94–1.58  $\mu\text{g}$  DOX equiv/mL, close to that observed for free DOX-HCl (Figure 5A). DOX-loaded PEG-PAU-PEG micelles showed also effective growth inhibition of MCF-7/ADR cells with an  $\text{IC}_{50}$  of 7.36–9.58  $\mu\text{g}$  DOX equiv/mL (Figure 5B). In contrast, free DOX-HCl exhibited marginal cytotoxicity toward MCF-7/ADR cells under otherwise the same conditions. This is in line with previous reports that nanosystems with triggered intracellular drug release behavior could effectively reverse drug resistance.<sup>42–44</sup> Notably, all PEG-PAU-PEG micelles displayed a low cytotoxicity toward RAW 264.7 and MCF-7/ADR cells even at a high concentration of 1.5 mg/mL (Figure 6). We have also performed MTT assays with potential degradation products from acetal hydrolysis, TPA and TME, which showed low cytotoxicities up to a tested concentration of 200  $\mu\text{g}$ /mL (Figure S5). These results indicate that PEG-PAU-PEG triblock copolymer micelles have good biocompatibility and mediate efficient intracellular drug delivery. It should be noted that PEG-PAU-PEG micelles can further be functionalized with a tumor-homing ligand to enhance anticancer efficacy and specificity.

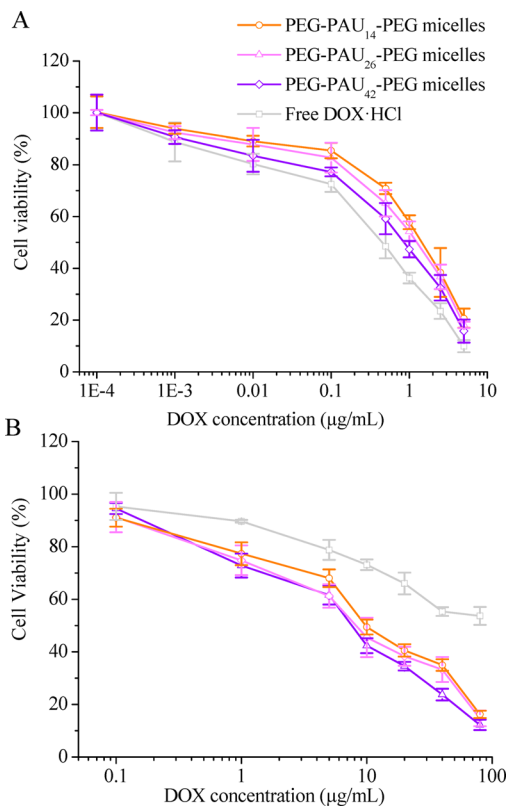
**Table 3. Characteristics of Blank and DOX-Loaded PEG-PAU-PEG Micelles<sup>a</sup>**

micelles	blank micelles		DOX-loaded micelles		DLC <sup>c</sup> (wt %)	DLE <sup>c</sup> (%)	CMC <sup>d</sup> (mg/L)
	size <sup>b</sup> (nm)	PDI <sup>b</sup>	size <sup>b</sup> (nm)	PDI <sup>b</sup>			
PEG-PAU <sub>42</sub> -PEG	122	0.12	165	0.13	9.5	42	0.56
PEG-PAU <sub>26</sub> -PEG	104	0.10	158	0.11	8.6	38	0.83
PEG-PAU <sub>14</sub> -PEG	92	0.09	135	0.10	8.0	35	1.22

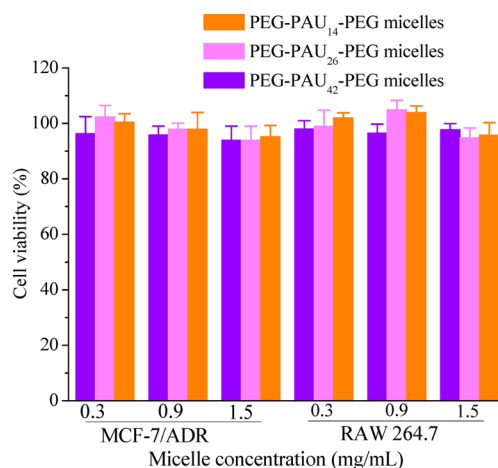
<sup>a</sup>Theoretical drug loading content = 20 wt %. <sup>b</sup>Determined by DLS. <sup>c</sup>Determined by fluorescence spectrophotometer. <sup>d</sup>Critical micelle concentration determined by fluorescence microscopy using pyrene as a probe.



**Figure 4.** Cumulative release of DOX from PEG-PAU-PEG micelles at different pHs (37 °C). Data are presented as the average  $\pm$  standard deviation ( $n = 3$ ).



**Figure 5.** Antitumor activity of DOX-loaded PEG-PAU-PEG micelles as a function of drug concentrations. (A) RAW 264.7 cells; (B) MCF-7/ADR cells. The cells were incubated with DOX-loaded PEG-PAU-PEG micelles for 48 h. Free DOX·HCl was used as a control. The cell viabilities were determined by MTT assays. Data are presented as mean  $\pm$  standard deviation ( $n = 4$ ).

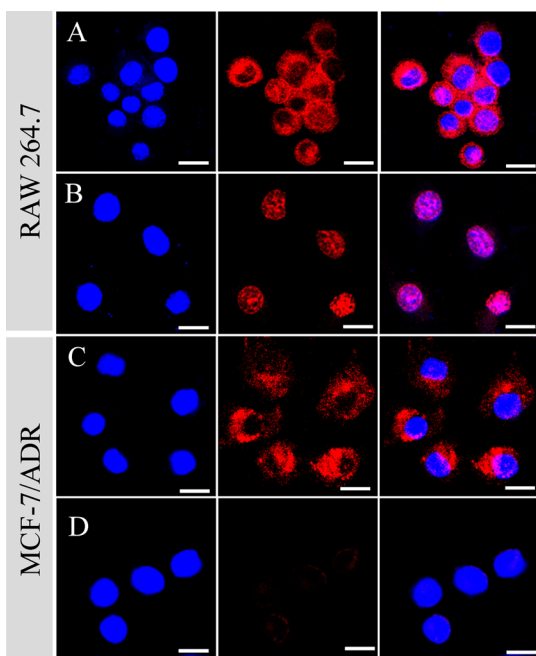


**Figure 6.** MTT assays of PEG-PAU-PEG micelles in RAW 264.7 and MCF-7/ADR cells. The cells were incubated with micelles for 48 h. Data are presented as the average  $\pm$  standard deviation ( $n = 4$ ).

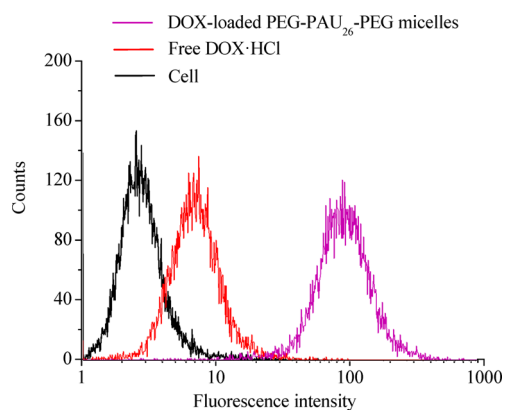
The cellular internalization and drug release performance of DOX-loaded PEG-PAU<sub>26</sub>-PEG micelles were examined in RAW 264.7 and MCF-7/ADR cells by CLSM and flow cytometry. Notably, RAW 264.7 cells following 4 h incubation with DOX-loaded PEG-PAU<sub>26</sub>-PEG micelles showed strong DOX fluorescence in the cytoplasm and perinuclei regions (Figure 7A), indicating efficient internalization of micelles and rapid drug release in the cells. DOX fluorescence became stronger in the cell nuclei and cytoplasm after a longer incubation time of 8 h (Figure S6A). In line with MTT assays, free DOX·HCl was more easily delivered into the nuclei of RAW 264.7 cells (Figure 7B). CLSM observations confirmed that PEG-PAU<sub>26</sub>-PEG micelles could also efficiently transport and release DOX into MCF-7/ADR cells in 4 h (Figure 7C), while little fluorescence was detected in the MCF-7/ADR cells following 4 h incubation with free DOX·HCl in accordance with their strong drug resistance (Figure 7D). This effective reversal of drug resistance of DOX-loaded PEG-PAU-PEG micelles is most probably due to a combination of cellular uptake via the endocytosis mechanism and pH-triggered intracellular drug release. Flow cytometry demonstrated that MCF-7/ADR cells treated for 4 h with DOX-loaded PEG-PAU<sub>26</sub>-PEG micelles had ca. 10-fold higher intracellular free DOX level than those with free DOX·HCl (Figure 8).

## CONCLUSIONS

We have shown that acid degradable PEG-PAU-PEG triblock copolymer micelles are noncytotoxic and mediate efficient pH-triggered intracellular DOX delivery, resulting in potent anticancer effects. This represents, to the best of our knowledge, a first development of poly(acetal urethane)s that are subject to main chain degradation under endo/lysosomal



**Figure 7.** CLSM images of RAW 264.7 and MCF-7/ADR cells incubated with DOX-loaded PEG-PAU<sub>26</sub>-PEG micelles and free DOX-HCl for 4 h. DOX dosages were 5 and 15  $\mu\text{g}/\text{mL}$  for RAW 264.7 and MCF-7/ADR cells, respectively. The scale bars correspond to 15  $\mu\text{m}$  in all the images. For each panel, images from left to right show cell nuclei stained by DAPI (blue), DOX fluorescence in cells (red), and overlays of two images. (A) RAW 264.7 cells incubation with DOX-loaded PEG-PAU<sub>26</sub>-PEG micelles, 4 h; (B) RAW 264.7 cells incubation with free DOX-HCl, 4 h; (C) MCF-7/ADR cells incubation with DOX-loaded PEG-PAU<sub>26</sub>-PEG micelles, 4 h; (D) MCF-7/ADR cells incubation with free DOX-HCl, 4 h.



**Figure 8.** Flow cytometry of MCF-7/ADR cells following 4 h incubation with DOX-loaded PEG-PAU<sub>26</sub>-PEG micelles and free DOX-HCl (DOX dosage: 10  $\mu\text{g}/\text{mL}$ ). Cell counts were 20 000.

pH conditions. PEG-PAU-PEG triblock copolymer micelles have several advantages: (i) they are easy to prepare and noncytotoxic; (ii) they have good stability with a low critical micelle concentration at physiological pH while undergo fast degradation under a mildly acidic environment; and (iii) drug release is triggered by endo/lysosomal pH resulting in effective reversal of drug resistance. In the following, we will develop tumor-targeted PEG-PAU-PEG triblock copolymer micelles using heterobifunctional PEG and investigate their anticancer drug delivery *in vivo*.

## ■ ASSOCIATED CONTENT

### ■ Supporting Information

<sup>1</sup>H NMR and <sup>13</sup>C NMR of TPABTME; FTIR of Allyl-PAU-Allyl; Cytotoxicity of TME and TPA in RAW 264.7 and MCF-7/ADR cells; GPC curves of Allyl-PAU<sub>42</sub>-Allyl and PEG-PAU<sub>42</sub>-PEG; CLSM images of RAW 264.7 and MCF-7/ADR cells following 8 h incubation with DOX-loaded PEG-PAU<sub>26</sub>-PEG micelles. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biomac.5b00625.

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### Notes

The authors declare no competing financial interest.

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