# Tunable pH-Sensitive Poly(β-amino ester)s Synthesized from Primary Amines and Diacrylates for Intracellular Drug Delivery

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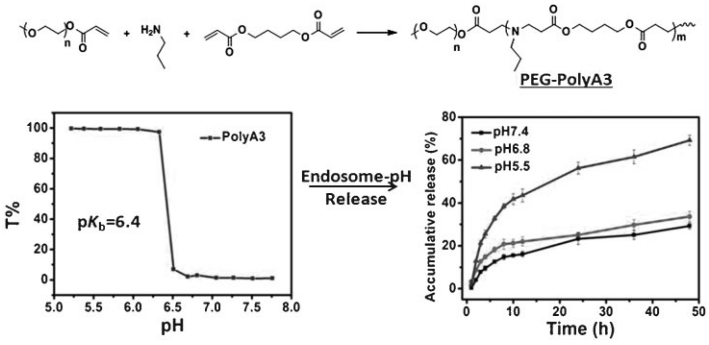
The pH sensitivity of a series of PbAEs synthesized from primary amines and diacrylates is studied. By changing alkyl groups of the amine monomers, the pKb can be tuned across a broad range (from 3.5 to 7.2). Micelles formed from a PEG-PbAE block copolymer retain the pH sensitivity of PbAE and can stably load hydrophobic molecules under neutral pH, while quickly dissociate and release their cargoes at pH ≈ 6.0. When the chemotherapy drug DOX is loaded, the micelles show efficient cell proliferation inhibition to HeLa cells and fast intracellular release. Thus, the primary-amine-based PbAEs are shown to be promising in the construction of intracellular targeting drug delivery systems.




## 1. Introduction

Nano-sized drug delivery systems based on amphiphilic copolymers have been extensively studied in recent years.[1] Stimuli-responsive nanoparticles, which are pre-programmed to alter their structure and properties during the drug delivery process, and to make them most effective at the targeting sites, have drawn more and more attention for antitumor drug delivery.[2] Among all the applied stimuli (e.g., temperature, pH, glucose, glutathione), acidic pH is particularly appealing due to the lower extracellular pH (7.2–6.5) in solid tumor than the surrounding tissues.[3] Nanovehicles applied should endure a sharp transition in response to the acidic pH and the entrapped cargoes could be quickly released, thus, lower side-effects and enhanced efficiency would be obtained.[4] Intracellular drug delivery systems (ICDDS) are regarded as a new paradigm for cancer chemotherapy. Because of the low oxygen content in the intercellular environment, tumors always present as acidic.[5] Efficient cytoplasmic drug delivery was also proved to be beneficial to overcome multidrug resistance (MDR) of cancer cells.[6]

The intracellular compartments are characterized by various different pH regions. Once being endocytosed, drug-carriers will experience a pH drop from neutral to pH around 6.0 in the early endosomes, with further

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reduction to pH = 5.0 or even lower during progression from late endosomes to lysosomes.[7] Accordingly, many drug delivery strategies based on pH-induced conformational change,[8] pH-sensitive linker[9] or combination of these have been developed.[10] However, to maximize the efficiency, precise control over drug-carriers distribution within the cell and rapid drug release in response to the different pH gradients in different cellular organelles are the two basic requirements.[11] To satisfy this, drug carriers must have a sharp transition in response to small pH changes at different intracellular locations.

Poly($\beta$-amino ester) (PbAE), originally developed by R. Langer et al. for gene delivery,[12] is a kind of pH responsive biodegradable polymers. Because of the abundant "titratable" tertiary amines, the polymer has high buffer capacity. Lee et al. studied the pH sensitivity of a series of PbAEs synthesized from secondary amines and diacrylate esters.[13] The pH sensitivity could be modulated by changing alkyl groups of the diacrylates.[14] Nanovehicles fabricated from the copolymer of poly(ethylene glycol) (PEG) and the PbAE had a sharp transition in response to pH changes and were further used for pH sensitive drug delivery and tumor imaging.[15] Stable micelles were also obtained by mixing PEG-PbAE with PEG-PLLA [PLLA = poly(L-lactic acid)], and active targeting systems could be made by conjugating ligands at the end of the PEG.[16] Shen et al. synthesized a kind of pH-sensitive polymer by condensation polymerization of diacrylate and piperazine in the presence of PEG-diacrylate macromonomer. The obtained micelles were dissociated inside tumor cells.[17] More recently, Chen et al. reported pH and reduction dual-sensitive copolymer micelles for doxorubicin (DOX) delivery. This new kind of pH-sensitive copolymer was synthesized from 4,4′-trimethylenedipiperidine, 2,2′-dithiodiethanol diacrylate, and methoxy-PEG-$NH_2$, and high efficient intracellular drug delivery was realized.[18]

Although secondary amine based PbAE has been reported for pH-sensitive drug delivery, the use of PbAEs made from primary amines were still less studied by now.[12b,19] The research into primary amines based PbAEs may provide plenty of choice for making new kinds of pH sensitive materials for drug delivery vehicles. In addition, functional groups could be easily introduced into the side chains.[20] In this work, we synthesized a series of PbAEs based on primary amines and diacrylates, and the effect of structure diversity on the pH sensitivities of these primary amine based PbAEs were investigated in detail. Furthermore, the block copolymer of PEG and PbAE from propylamine and 1,4-butanediol diacrylate (PEG-PolyA3) was prepared to evaluate the potential of this kind of polymers for intracellular targeting antitumor drug delivery.

## 2. Experimental Section

### 2.1. Materials

1,4-Butanediol diacrylate, 1,6-hexanediol diacrylate, 1-propylamine, 1-pentylamine, 1-octylamine and 3-propanolamine were all purchased from Alfa Aesar and used as received. Poly(ethylene glycol) monomethyl ether (mPEG, $\overline{M}_n = 5000$) and Nile red were bought from Sigma-Aldrich. DOX was obtained from Zhejiang Hisun Pharmaceutical Company. All other reagents were purchased from Sinopharm Chemical Reagent and used as received.

### 2.2. Instrumentation

$^1$H NMR spectra were recorded on a Bruker AV-400 spectrometer. Number- and weight-average molecular weights ($\overline{M}_n$, $\overline{M}_w$) and molecular weight distribution (PDI = $\overline{M}_w/\overline{M}_n$) were determined by gel permeation chromatography (GPC) using a Waters GPC system (Waters Styragel HT6E column, with OPTILAB DSP interferometric refractometer as the detector) with a mobile phase of $CH_2Cl_2$ at a flow rate of 1.0 mL min$^{-1}$ at 40 °C. Monodispersed polystyrene standards purchased from Waters with a molecular weight range of 1310–55 100 were used to generate the calibration curve. Dynamic light scattering (DLS) measurements were performed on a WyattQELS instrument with a vertically polarized He-Ne laser (DAWNEOS, Wyatt Technology). Transmission electron microscopy (TEM) images were taken from JEOL JEM-1011 transmission electron microscope with an accelerating voltage of 100 kV. UV-Vis and fluorescence spectra were measured on a UV-2401PC spectrophotometer (Shimadzu) and a fluorescence spectrometer (LS50B, Perkin-Elmer), respectively.

### 2.3. Synthesis of Poly($\beta$-amino ester)s

In a typical experiment, 1,4-butanediol diacrylate (0.010 mol, 1.982 g) and propylamine (0.010 mol, 0.591 g) were weighed into a vial. After a Teflon-coated stirring bar was added, the vial was sealed and the reaction was heated at 60 °C for 72 h. Then the reaction mixture was dissolved in $CHCl_3$ and dripped slowly into vigorously stirring petroleum ether. The precipitated polymer was collected and dried under vacuum prior to analysis. All other PbAEs were synthesized with the same procedure.

### 2.4. Synthesis of PEG-PolyA3 Block Copolymer

Poly(ethylene glycol) monomethyl ether acrylate (mPEG-Ac) was synthesized by acrylating mPEG with acryloyl chloride, following a procedure similar to that reported in literature.[14]

PEG-PolyA3 block copolymer was synthesized via one-pot Michael-type step polymerization. mPEG-Ac (0.2 mmol, 1.0 g), 1,4-butanediol diacrylate (2.0 mmol, 0.396 g), and 1-propylamine (2.2 mmol, 0.130 g) were carefully weighed into a flask, then 1 mL anhydrous $CHCl_3$ was added and the reaction was carried out at 60 °C for 72 h. The resulting viscous liquid was diluted by $CHCl_3$ and precipitated into petroleum ether. After drying in vacuum a brown viscous product was obtained which was stored at 4 °C before use.

### 2.5. Measurement of $pK_b$

The $pK_b$ values of the polymers were measured by titration method. In each case, 50 mg of polymer were dispersed in 50 mL deionized water and the pH was adjusted to 3, then the pH was tuned with the addition of 40 μL of 0.1 M NaOH solution. The $pK_b$ was determined as the inflection point of the titration curve.

### 2.6. pH-Dependent Light Transmittance

The light transmittance of the PbAE solutions at different pH was measured by UV-Vis spectrometer. In each case, polymer dissolved in deionized water was added to a series of buffer solution (0.2 M $Na_2HPO_4$/0.1 M citric acid) of pH values from 2.0–8.0, with a final concentration of 1.0 mg $mL^{-1}$. The exact pH was measured with a pH meter (pH 211, HANNA Instrument), and the turbidity change of the polymer solutions was determined from the light transmittance at 550 nm.

### 2.7. Preparation of PEG-PolyA3 Micelles

The block copolymer micelles were prepared by dialysis method. The polymer (25 mg) was dissolved in 1 mL of *N,N*-dimethylformamide (DMF), to which 2 mL phosphate buffer (PB; 0.0667 M, pH = 7.4) was slowly added under vigorous stirring. The resulting solution was stirred for another 6 h and then transferred into dialysis membrane (molecular-weight cut-off, MWCO: 3500 Da) and dialyzed against 2 L of deionized water (pH = 7.4) to remove DMF. The final solution was diluted to 25 mL and thus a 1 mg $mL^{-1}$ micelle solution was obtained.

### 2.8. Measurement of Critical Micelle Concentration (CMC)

The CMC of the block copolymer solution was estimated by fluorescence spectroscopy using pyrene as the probe. The excitation spectra were recorded from 310 to 350 nm with an emission wavelength of 392 nm. The intensity ratios of the third (338 nm) to the first (334 nm) vibronic peaks of pyrene fluorescence were plotted as a function of logarithm of polymer concentration, and CMC was corresponding to the turning point concentration.

### 2.9. pH Sensitivity Characterization of the Copolymer Micelles

The micelle sizes in aqueous solution were measured using DLS. The scattering angle was fixed at 90° and the temperature was adjusted to 25 °C. The concentration of the solution was kept at 0.1 mg $mL^{-1}$ and the micelle sizes were checked at various pH values.

### 2.10. Polymer Degradation Studies

Hydrolysis of the polymer was tested in pH = 7.4 and 5.5 phosphate-buffered saline (PBS) at 37 °C. PEG-PolyA3 (0.06 g) was dissolved in 10 mL buffers separately. At timed intervals (24 h and 48 h), the buffer solution was dialyzed against deionized water for 2 h (MWCO 3500 Da), then frozen immediately in liquid nitrogen and lyophilized. Then $^1H$ NMR was measured and the remaining amount of PolyA3 block was calculated according to the integral ratio of the signal of methylene protons in PolyA3 and PEG.

### 2.11. Nile Red Encapsulation and pH-Triggered Release

Hydrophobic molecule Nile red was applied as a probe to test the encapsulate stability and pH-triggered release behavior of the micelles, following a method described by Ryu.[21] PEG-PolyA3 (22.5 mg) and Nile red (2.5 mg) were dissolved in 1 mL of DMF. After stirring for 30 min, 2 mL of PBS (pH = 7.4) was added and the mixture was stirred for another 6 h. Then the solution was dialyzed against 2 L deionized water to remove the organic solvent. Excess insoluble Nile red was removed by filtration using a 0.45 μm filter and the final solution was lyophilized. Cargo-encapsulated micelle solution in ampoule (1 mg $mL^{-1}$ in PBS) was placed at 37 °C with a shaking rate of 100 rpm, and the fluorescence intensity was measured at different time intervals. The fluorescence detector was set at an excitation wavelength of 543 nm and the emission intensities from 570 to 720 nm were measured. The fluorescence intensities of the micelles at pH = 6.8 and 5.5 were measured immediately after the pH adjustment.

### 2.12. DOX Encapsulation and Release

DOX-loaded micelles were prepared by a method similar to Nile red. Briefly, DOX (5 mg) dissolved in 1 mL DMF was stirred with 2 equivalents of triethylamine, and 45 mg PEG-PolyA3 in 1 mL DMF was added and stirred for 30 min. Then 4 mL PBS (pH = 7.4) was added dropwise under vigorous stirring and the mixture was stirred for another 6 h. After dialysis against deionized water and lyophilization, a red powder was obtained. The drug loading content (DLC%) and drug loading efficiency (DLE%) were calculated according to

$$\text{DLC}\,\% = \frac{\text{weight of drug in micelle}}{\text{weight of drug-loaded micelle}} \times 100\%$$

$$\text{DLE}\,\% = \frac{\text{weight of drug in micelle}}{\text{total weight of feedingdrug}} \times 100\%$$

The pH-dependent DOX release was conducted as below: 3 mg DOX-loaded polymeric micelle powder was added to a dialysis membrane tube (MWCO 3500 Da), which was then incubated in 30 mL PBS at different pH (7.4, 6.8 and 5.5) at 37 °C with a shaking rate of 100 rpm. At a predetermined time, 3 mL of incubated solution was taken out and replaced with fresh PBS. The DOX content of the sample was determined by measuring the emission fluorescence intensity at 590 nm with an excitation wavelength of 490 nm.

### 2.13 Cellular Uptake and Cytotoxicity Assay

The observation of drug endocytosis was performed with confocal laser scanning microscopy (CLSM). Henrietta Lacks (HeLa) cells were seeded in 6-well plates at $2 \times 10^5$ cells per well in Dulbecco's modified Eagle medium (DMEM) and cultured for 24 h, and then treated with free DOX and DOX-loaded micelles at a final concentration of 5 μg $mL^{-1}$ for 1 and 4 h. Cells were washed three

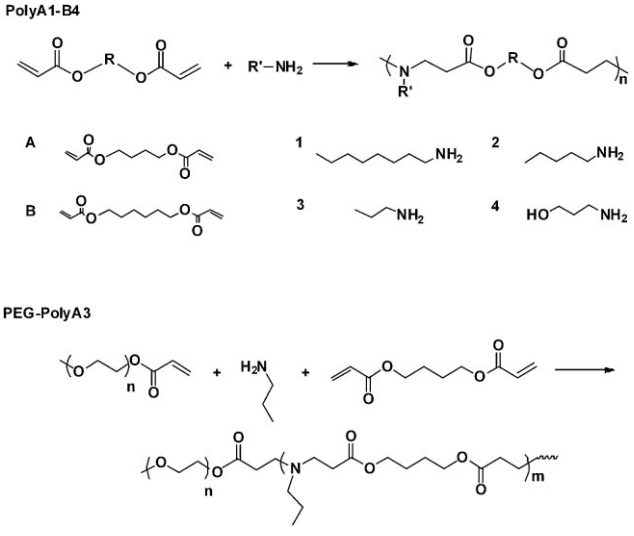
times with PBS, and fixed with fresh 4% paraformaldehyde for 30 min at room temperature. The cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) for cell nucleus following the manufacturer's instructions. The coverslips were mounted and visualized under a CLSM (Carl Zeiss LSM 700).

The cytotoxicities of blank micelles, DOX-loaded polymeric micelles and free DOX were assessed with methyl thiazolyl tetrazolium (MTT) viability assay against HeLa cells. The cells were seeded in 96-well plates at ca. 10 000 cells per well in 100 μL complete DMEM containing 10% fetal bovine serum, supplemented with 50 U mL$^{-1}$ penicillin and 50 U mL$^{-1}$ streptomycin, and incubated at 37 °C in 5% $CO_2$ atmosphere for 24 h, followed by removing culture medium and adding micelles or DOX at different concentrations. 48 h later, 20 μL of a 5 mg mL$^{-1}$ MTT assay stock solution in PBS were added to each well. After incubating for another 4 h, the medium containing unreacted MTT was removed carefully. The obtained blue formazan crystals were dissolved in 150 μL dimethyl sulfoxide (DMSO) per well and the absorbance was measured in a Bio-Rad 680 microplate reader at a wavelength of 492 nm. Cell viability (%) was calculated by $A_{sample}/A_{control} \times 100$, where $A_{sample}$ and $A_{control}$ are denoted as absorbance of the sample and control wells, respectively.

## 3. Results and Discussion

### 3.1. Synthesis and Characterization of the PbAEs

Linear PbAE, containing both tertiary amines and degradable esters in their backbones, was first developed by Langer et al. by the reaction of bifunctional amines with diacrylates.[12a] For this addition reaction, usually a long reaction time is needed, and higher temperature will accelerate the reaction rate.[22] In this work, bulk polymerization with no solvent was applied, and the polymerization was carried out for 72 h at 60 °C. The synthesis strategy is shown in Scheme 1 (PolyA1-B4). The $\overline{M}_n$ and PDI are listed in Table 1. The GPC curves of the obtained polymers were all monomodal with $\%\overline{M}_n$ ranging from $3.2 \times 10^3$ to $8.4 \times 10^3$ and PDIs being below 2.0.




*Scheme 1.* Synthetic strategy for the preparation of PbAEs and PEG-PolyA3.

*Table 1.* GPC and pH sensitivity results of PbAEs and PEG-PolyA3 studied in this work.

| Sample | $\overline{M}_n$ [a] [kDa] | $\overline{M}_w$ [a] [kDa] | PDI | p$K_b$ [b] | pH-UV trans [c] |
|---|---|---|---|---|---|
| PolyA1 | 3.6 | 4.7 | 1.3 | 3.5 | 4.0 |
| PolyB1 [d] | 8.4 | 13.1 | 1.5 | – | – |
| PolyA2 | 5.9 | 10.4 | 1.8 | 5.4 | 5.4 |
| PolyB2 | 5.4 | 9.1 | 1.7 | 4.4 | 4.3 |
| PolyA3 | 3.2 | 4.2 | 1.3 | 6.4 | 6.4 |
| PolyB3 | 4.6 | 7.5 | 1.6 | 5.6 | 5.5 |
| PolyA4 | 4.7 | 8.8 | 1.9 | 7.2 | 7.2 |
| PolyB4 | 6.0 | 11.3 | 1.9 | 6.7 | 6.9 |
| PEG-PolyA3 [e] | 7.8 | 9.6 | 1.2 | 6.4 | – |

[a] For GPC measurement, $CH_2Cl_2$ was used as the eluent, and the molecular weight and PDI are reported relative to polystyrene standards; [b] The p$K_b$ values were measured by titration; [c] The pH-UV *trans*-point represented the pH when light transmissivity of the polymer solution became 50%; [d] The p$K_b$ value and pH-UV trans of PolyB1 were not measured because it was still insoluble when pH was adjusted to 3; [e] The pH sensitivity of PEG-PolyA3 was not studied by UV-vis spectrometry.

### 3.2. pH Sensitivity Study of the PbAEs

The PbAEs reported above showed outstanding pH sensitive properties in a wide pH region. The acid-base titration profiles are shown in Figure 1A, B and C. All the polymers from PolyA1 to PolyB4 exhibited pH buffering capacities, while the buffering regions were quite different. The different monomer structure had a distinct impact on the p$K_b$ values. For example, as the monomers changed from 1-propylamine to 1-octylamine (PolyA3 to PolyA1), the p$K_b$ values decreased from 6.4 to 3.5 (Figure 1A). Increasing the alkyl chain length in main chains resulted in a decrease of the p$K_b$ value (Figure 1B). For the PbAEs prepared from 1-propylamine and 1-pentylamine, the p$K_b$ values decreased from 6.4 and 5.4 to 5.6 and 4.4, respectively, as the bisacrylate esters were changed from butane-1,4-diol diacrylate to hexane-1,6-diol diacrylate. When hydrophilic groups were introduced, the p$K_b$ values shifted to higher regions. As can be seen in Figure 1C, the p$K_b$ values of polymers synthesized from 3-propanolamine (7.2 for PolyA4, 6.7 for PolyB4) were higher than those from 1-propylamine (6.4 for PolyA3, 5.6 for PolyB3).

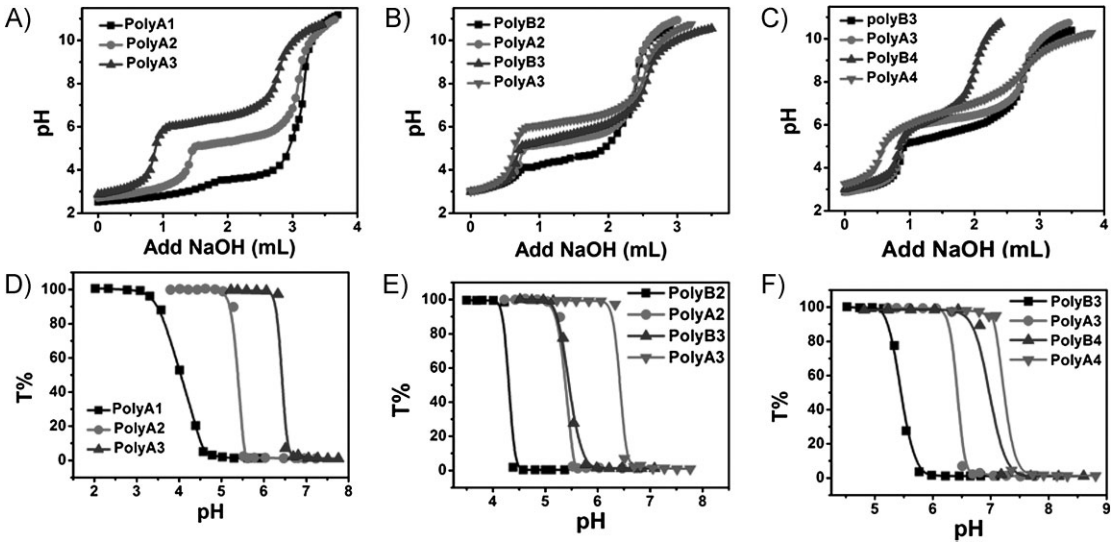



*Figure 1.* Titration (A, B, C) and pH-UV *trans*-curves (D, E, F) of PolyA1-B4.

The above results indicated that the p$K_b$ values could be modulated from slightly acidic to highly acidic by changing the alkyl groups of the monomers. For longer alkyl chains, a higher proton concentration was needed to protonate the tertiary amine chains, thus p$K_b$ values were shifted to lower regions. However, when hydrophilic groups were introduced, the p$K_b$ values shifted to the opposite direction. The influence of structure on pH sensitivity was also reported in other publications. For example, when L-phenylalanine was introduced into the poly(L-histidine) block, the p$K_b$ value was obviously decreased and intracellular pH responsive micelle drug formulation was obtained by Bae's group.[6d] Thus these primary amine based PbAEs provided a pool of candidates for the construction of pH-responsive drug delivery carriers.

### 3.3. Synthesis and Characterization of PEG-PolyA3

To prepare pH-responsive carriers, amphiphilic copolymers with one hydrophilic block and one pH sensitive hydrophobic block are needed. Thus, in the following work, the block copolymer of PEG and PbAE from primary amine was prepared to evaluate the feasibility of this kind of polymers for pH sensitive drug delivery.

PolyA3, with a p$K_b$ value of 6.4 (Table 1), was chosen as the pH sensitive block for the amphiphilic copolymer. The pH transition point of PolyA3 was just among the early endosome region, thus it provided high potential for intracellular drug delivery. The PEG-PolyA3 copolymer (Scheme 1) was synthesized by Michael-addition step polymerization of mPEG-Ac, 1,4-butanediol diacrylate and 1-propylamine with a feed ratio of 1:10:11. The $^1$H NMR spectrum of the block copolymer is shown in Figure 2. The resonances at $\delta = 2.44$ (f) and 2.77 (g) were assigned to the protons of the newly formed methylene between the tertiary amine and the esters. The disappearance of the resonances signals around $\delta = 6.0$ indicated the complete conversion of the diacrylate monomers. GPC measurements of the copolymer gave $\overline{M}_n = 7.8 \times 10^3$ Da with a PDI of 1.2 (Table 1). These results indicate that the polymers were formed through the conjugate addition of the amines to the acrylate moieties of the diacrylates and that the reaction was quite complete.

### 3.4. Degradation Study

PbAE is a kind of biodegradable material, but the degradation behavior is quite complicated according to the literature. Lynn et al. found that the PbAE hydrochloride salt degraded more slowly at pH = 5.1 than at pH = 7.4,[12a]

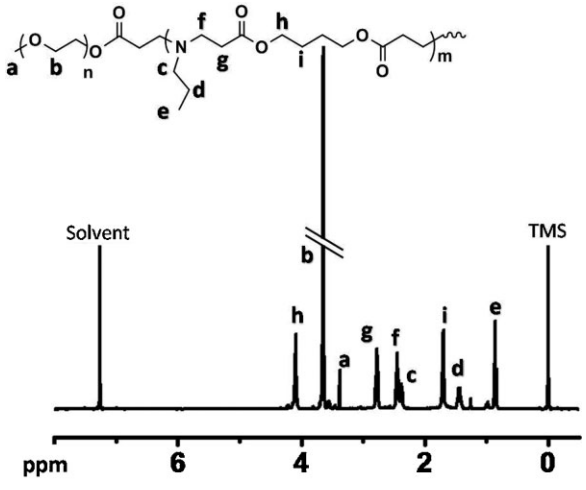



*Figure 2.* $^1$H NMR spectrum of PEG-PolyA3 (400 MHz, $CDCl_3$).

while Shen et al. reported that the PbAE-PEG copolymer degraded more quickly at pH = 5.5 than at pH = 7.4.[17] We studied the hydrolysis of PEG-PolyA3 by $^1$H NMR. After being incubated in PBS for timed intervals, the PEG-PolyA3 copolymer was placed in a dialysis tube (MWCO 3500 Da), and the remaining amount of PolyA3 block was measured according to the integral ratio of the methylene in PolyA3 and PEG (which neither degrade nor pass through the 3500 Da dialysis membrane). As shown in Figure 3, peaks at $\delta = 4.2$ and 3.6 correspond to protons of the methylene in PolyA3 and PEG chains, respectively. After incubation in pH = 7.4 PBS for 48 h, the intensity at $\delta = 4.2$ showed little change, while it decreased quickly in pH = 5.5 buffer (64% of the ester linkages remained after 24 h and 37% remained after 48 h). The results reflected that the synthesized PolyA3 was relatively stable in neutral environment while gradually degraded under acidic conditions. We concluded that water accessibility played a crucial role in the rate of degradation. In neutral environment, the copolymer aggregated into micelles, and the PolyA3 core was hydrophobic, thus the degradation was slow. In contrast, under acidic conditions, PolyA3 became hydrophilic, and the approach of the ester main chains to water was much easier, thus resulting in fast degradation. The effect of hydrophilicity on the degradation rate was also testified on PEG-PolyA4 ($pK_b = 7.2$), and fast hydrolysis in pH = 7.4 PBS was seen (peaks at $\delta = 4.2$ quickly disappeared after 24 h, data not shown).

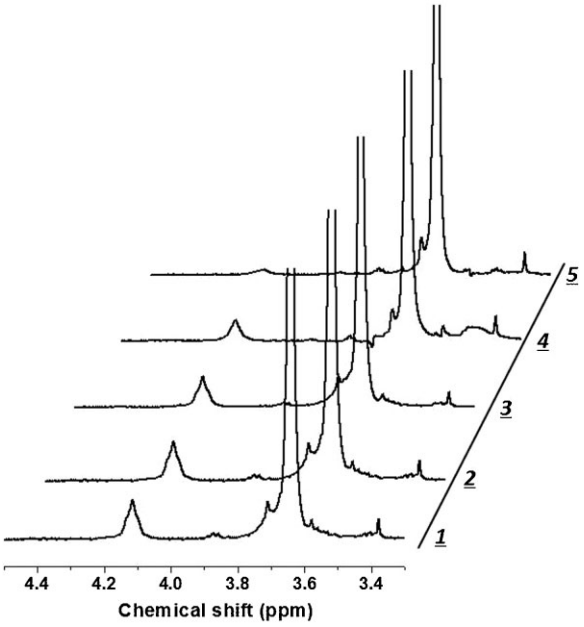



*Figure 3.* Degradation study of PEG-PolyA3: *1* is the $^1$H NMR spectrum of the polymer before incubation in PBS. *2* and *3* are the $^1$H NMR spectra of the resultant polymer after incubation in pH = 7.4 PBS for 24 h and 48 h, respectively. *4* and *5* are the corresponding results in pH = 5.5 PBS for 24 h and 48 h, respectively. All the spectra were measured after lyophilization and were obtained in $CDCl_3$.

### 3.5. Micelle Formation and Solution Behavior

Amphiphilic copolymers are capable of providing various self-assembled structures in the selective solvents. In this work, pH sensitive micelles were prepared and the pH related solution behavior was studied. Acid-base titration showed that the PEG-PolyA3 copolymer preserved the good pH sensitivity of PolyA3, and the $pK_b$ value was also 6.4 (Figure 4A). The PEG-PolyA3 polymeric micelles were fabricated using the solvent displacement method and deionized water (pH = 7.4) was used for dialysis. The CMC was determined by the reported fluorescent method using pyrene as a fluorescent probe. As the concentration of the block copolymer increased gradually, the pyrene transferred to the less polar micelle core, and the maximum excitation peak shifted from 338 to 334 nm. The measured CMC was $7.6 \times 10^{-3}$ mg mL$^{-1}$ (Figure 4B). TEM showed that the copolymer aggregated to uniformly spherical micelles at neutral environment with a mean hydrodynamic diameter $R_h$ of about 90 nm (Figure 4C). DLS gave a similar result ($R_h$, $74.3 \pm 17.9$ nm) and narrow size distribution was seen (Figure 4C, inserted). These results indicated that these polymeric micelles were well dispersed in aqueous media and homogeneous nano-sized micellar structures were formed.

The pH-dependent demicellization behavior of PEG-PolyA3 block copolymer was assessed by the DLS. The average sizes of the polymer micelles under different pH conditions were measured. Results are shown in Figure 4D. As expected, at pH above 7.0, the micelles had constant sizes around 80 nm. However, between pH = 5.8 and 6.8, the micelles became quite unstable and would expand or disassemble due to the ionization extent of the tertiary amine moieties of the PolyA3 block. When the pH decreased below 5.8, no signal was detected. This indicated that the pH-sensitive block copolymers were completely dissolved and did not form any micellar structures. These results showed that the PEG-PolyA3 block copolymer micelles formed from 1-propylamine had a distinct pH-dependent demicellization behavior. In addition, the demicellization pH was just among the endosome pH region. Thus it could be used for intracellular pH targeting drug delivery systems.

### 3.6. Encapsulation Stability and Tunable in vitro Guest Release

An effective drug delivery system should be able to stably encapsulate the loaded drugs and release them in response to a biologically relevant trigger. Herein, Nile red was used as a probe to test the encapsulation stability and the pH

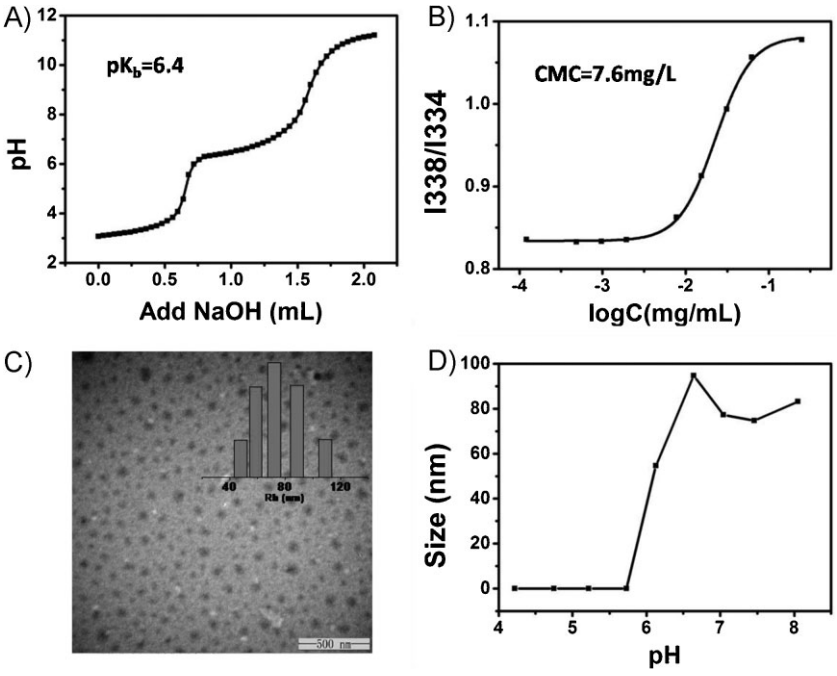



*Figure 4.* Characterization of the PEG-PolyA3 copolymer micelles: (A) Titration curve of PEG-PolyA3 copolymer obtained by adding 0.1 M NaOH at 20 °C, the p$K_b$ is 6.4. (B) The CMC of the formed micelles, derived from the plot of intensity ratio vs. copolymer concentration in PBS at pH = 7.4, which is 7.6 mg L$^{-1}$. (C) TEM micrograph and DLS results (inserted), the bar at the right foot is 500 nm. (D) pH induced demicellization behavior measured by DLS at a concentration of 0.1 mg mL$^{-1}$.

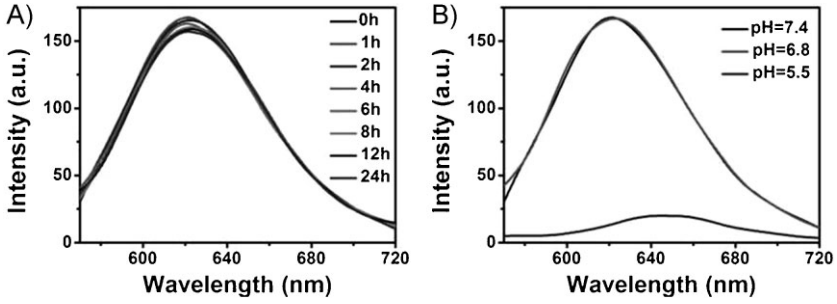



*Figure 5.* Nile red release from PEG-PolyA3 micelles in response to time and pH: (A) Fluorescent intensity of Nile red encapsulated micelles during a 24 h period in pH = 7.4 buffer; (B) Fluorescent intensity in different pH conditions (Spectrum was recorded immediately after the pH adjustment).

induced release behavior. The intensity of Nile red emission from 570 nm to 720 nm was monitored. As shown in Figure 5A, at pH = 7.4, the intensity showed negligible changes within 24 h. When the pH was changed to 6.8 (pH at extracellular environment[8a]), the result was similar to that at pH = 7.4. However, when the pH was changed to 5.5 (pH range of late endosome), the intensity was significantly reduced due to the release and precipitation of the insoluble Nile red molecules in water (Figure 5B). This is in accordance with the results in Figure 4D. The micelles were relatively stable under neutral conditions, while when the cargo-loaded micelles were dispersed in an acidic environment at pH lower than 6.0, the entrapped molecules were quickly released due to demicellization.

### 3.7. In Vitro DOX Release

Considering the results we obtained through Nile red release, the usage of the polymer micelles for chemotherapeutic drug delivery was further studied. Chemotherapy drug DOX was loaded into the PEG-PolyA3 micelles using the solvent displacement method, similar to that used before. The designed DLC% was 10%, and the resulting DLC% was 7.8%, with DLE% of approximately 76%.

The pH-dependent DOX release from the PEG-PolyA3 micelles was investigated using dialysis method. As shown in Figure 6, at pH = 7.4 and 6.8, less than 30% of DOX was released in the test duration (48 h). However, the release rate was much more accelerated when pH was decreased to 5.5. Over 40% of DOX was released within the first 10 h, and up to 70% was released after 48 h. These results were in accordance with that of Nile red. At pH = 5.5, demicellization occurred, thus the loaded DOX was released and quickly penetrated through the dialysis membrane. In addition, on the basis of the aforementioned results, PEG-PolyA3 was gradually degraded during the test period. This also contributed to the fast and sustained DOX release at pH = 5.5. These release profiles showed that the pH-sensitive drug carriers were not only beneficial for minimizing drug loss in circulation and extracellular environments, but also for fast release inside acidic tumor organelles, which could enhance the overall therapeutic efficacy.

### 3.8. Cellular Uptake and Cell Viability Assay

The cellular uptake and intracellular distribution of free DOX and loaded DOX under pH = 7.4 were investigated using CLSM (Figure 7). Free DOX entered cells rather quickly and was mostly distributed in the nuclei. The time-dependent cellular uptake of the loaded DOX was also seen since much stronger fluorescence was seen after 4 h

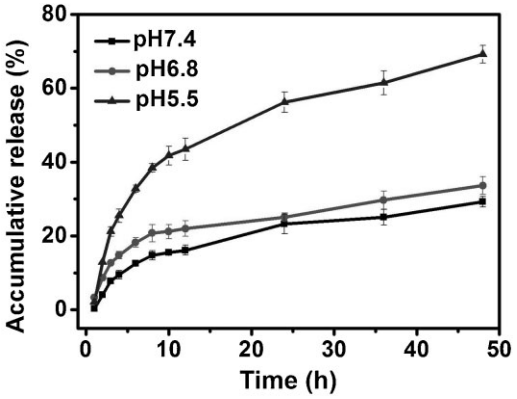



*Figure 6.* Release profiles of DOX-loaded micelles in different pH buffers. DOX concentration was measured by UV-vis absorbance at 490 nm. Three independent samples were detected for each condition.

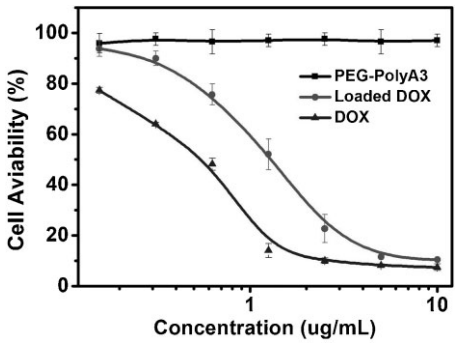



*Figure 8.* Cytotoxicity of HeLa cells treated by free DOX, DOX-loaded micelles and blank micelles for 48 h. Data are presented as the average standard deviation ($n = 3$).

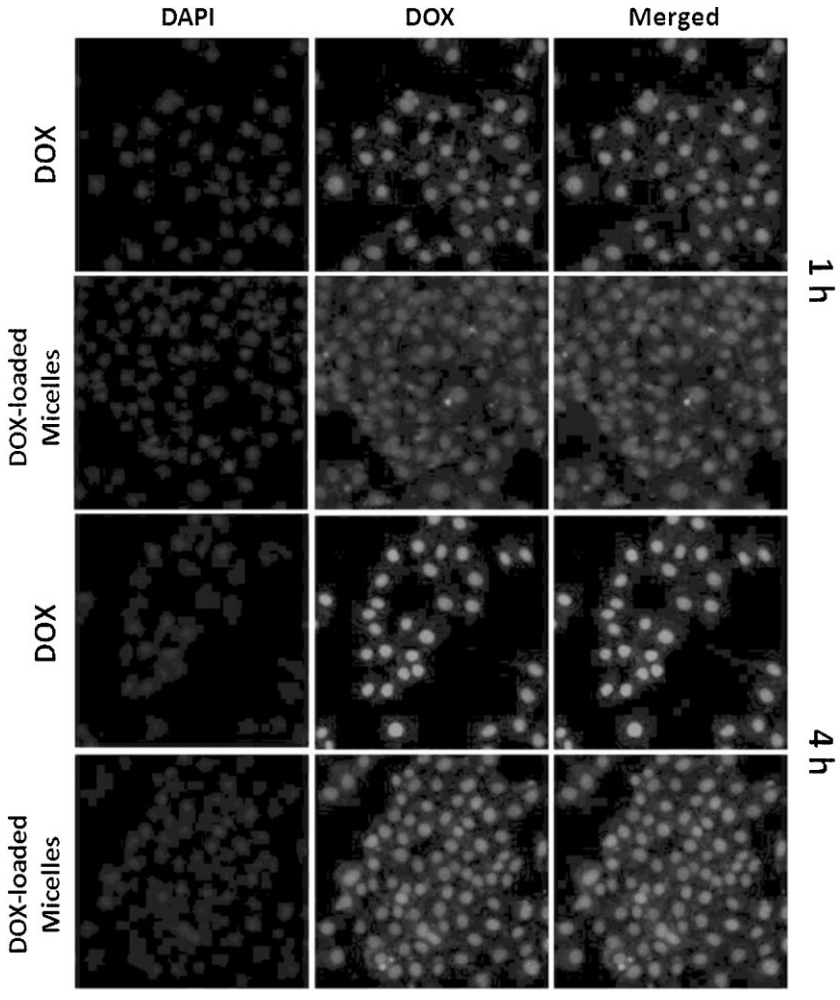



*Figure 7.* Confocal laser microscopic observation of HeLa cells after incubation with free DOX and DOX-loaded micelles for 1 h and 4 h.

than after 1 h. However, fluorescence could be detected both in the cytoplasm and nucleus for the DOX loaded micelles after both 1 h and 4 h. This is in accordance with the different cellular uptake ways. Free DOX quickly diffused through the cell membrane and concentrated in the nuclei,[23] while the DOX loaded micelles were taken up via the endocytosis pathway,[24] thus distribution in both cytoplasm and nucleus was observed.

The cytotoxicity of DOX-loaded micelles toward HeLa cells were carried out by MTT assay. The cell viabilities were investigated by treating HeLa cell lines with different concentrations of blank micelles and DOX-loaded micelles for 48 h. Free DOX was used as control. As expected (Figure 8), the blank micelles were non-toxic at the tested concentrations, while the DOX-loaded micelles showed a dose dependent cytotoxicity to HeLa celles. The $IC_{50}$ of loaded DOX (1.29 $\mu g\ mL^{-1}$) was about two times higher than that of free DOX (0.54 $\mu g\ mL^{-1}$). The toxicity of free DOX was reduced due to the endocytosis pathway and intracellualar release of the polymeric micelles, as seen in the cellular uptake images.[6d] In addition, this cytoplasmic drug delivery way can bypass the cell membrane-associated multidrug resistance and in vivo biodistribution could be changed by the enhanced permeation and retention (EPR) effect of the polymeric micelles. Thus, the intracellular pH-sensitive drug-delivery systems provide a potential drug formulation for cancer chemotherapy.

## 4. Conclusion

A series of PbAEs based on primary amines and diacrylates were prepared and the pH sensitivities were studied. Increase of the alkyl chain length resulted in a decrease in the p$K_b$ values. PEG-PbAE block copolymer from 1-propylamine and 1,4-butanediol diacrylate, with a p$K_b$ value of 6.4, was further prepared to test the pH-sensitive behavior. The copolymer aggregated into uniform micelles at neutral pH, while quickly demicellizes at pH around 5.5. When loaded with hydrophobic molecules Nile red and anti-tumor drug DOX, pH dependent release behavior was observed. In vitro cell experiments revealed that the DOX-loaded micelles could be quickly endocytosized and had obvious inhibition on the proliferation of HeLa cells. Therefore, the primary amine based PbAEs, with tunable pH sensitive regions, hold vast potential for the design of new kinds of intracellular targeting drug delivery systems.

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