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pH-Dependent, Thermosensitive Polymeric Nanocarriers for Drug Delivery to Solid Tumors

Ching-Yi Chen^{1,†}, Tae Hee Kim^{2,‡}, Wen-Chung Wu^{1,3}, Chi-Ming Huang³, Hua Wei², Christopher W. Mount², Yanqing Tian^{1,§}, Sei-Hum Jang¹, Suzie H. Pun^{2,*}, and Alex K-Y Jen^{1,*}

¹Department of Materials Science and Engineering, Box 352120, University of Washington, Seattle, WA 98195-2120, USA

²Department of Bioengineering, Box 355061, University of Washington, Seattle, WA 98195, USA

³Department of Chemical Engineering, National Cheng Kung University, Tainan, Taiwan 70101

Abstract

Polymeric micelles are promising carriers for anticancer agents due to their small size, ease of assembly, and versatility for functionalization. A current challenge in the use of polymeric micelles is the sensitive balance that must be achieved between stability during prolonged blood circulation and release of active drug at the tumor site. Stimuli-responsive materials provide a mechanism for triggered drug release in the acidic tumor and intracellular microenvironments. In this work, we synthesized a series of dual pH- and temperature-responsive block copolymers containing a poly(ϵ -caprolactone) (PCL) hydrophobic block with a poly(triethylene glycol) block that were copolymerized with an amino acid-functionalized monomer. The block copolymers formed micellar structures in aqueous solutions. An optimized polymer that was functionalized with 6-aminocaproic acid (ACA) possessed pH-sensitive phase transitions at mildly acidic pH and body temperature. Doxorubicin-loaded micelles formed from these polymers were stable at blood pH (~7.4) and showed increased drug release at acidic pH. In addition, these micelles displayed more potent anti-cancer activity than free doxorubicin when tested in a tumor xenograft model in mice.

1. Introduction

Chemotherapy, the use of drugs to treat cancer, is a widely used and effective clinical approach to cancer therapy. However, current chemotherapy still faces several challenges including severe side effects and drug-resistance. Traditional chemotherapeutics generally have low circulation half-life and high volumes of distribution resulting in significant off-site toxicity. It would therefore be desirable to develop a drug delivery system that can provide more selective accumulation of drugs at tumor sites by the enhanced permeability and retention (EPR) effect,[1] thereby reducing adverse side effects on healthy normal

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*Corresponding authors. ajen@u.washington.edu (Alex K.-Y. Jen); spun@u.washington.edu (Suzie H. Pun).

†Current Address: Department of Chemical Engineering, National Chung Cheng University, Chiayi, Taiwan 62102

‡Current Address: Korea Institute of Industrial Technology.

§Current Address: The Center for Biosignatures Discovery Automation, Biodesign Institute, Arizona State University, Tempe, AZ 85287-5001, USA

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tissues. In the recent decades, intelligent materials that respond to specific stimuli, such as temperature, pH, or enzymatic activity have been developed.[2, 3] When used in anticancer drug delivery applications, the responsive nature of these materials can provide more preferential delivery of drugs to tumor sites. For example, pH-sensitive polymers have been designed for drug release in acidic tumor microenvironments,[4–6] and temperature-sensitive polymers have been synthesized as drug carriers and used in conjugation with hyperthermia treatment for localized drug delivery at tumor sites.[7, 8]

Amphiphilic block copolymers that self-assemble as core-shell polymeric micelles in aqueous solutions are one class of promising delivery systems. The hydrophobic inner core of the micelle acts as a reservoir to accommodate drugs that may have poor solubility, low stability, and/or undesirable systemic toxicity by themselves. The small particle size (typically <100 nm) and the hydrophilic surface of polymeric micelles can increase the circulation time of encapsulated drugs while facilitating passive accumulation at tumor sites via the EPR effect.[1] Due to their advantages for drug delivery including high payload, ease of administration, reduced systemic toxicity, and improved therapeutic efficacy, polymeric micelles are currently in all three stages of clinical trials.[9–11]

Ideally, polymeric micelles used for anticancer therapy should be stable during circulation but release drug cargo after extravasation to tumor sites. Premature micelle disassembly or drug release would result in fast drug clearance kinetics and delivery to normal cells while highly stable formulations may sequester drugs and display reduced antitumor efficacy.[12, 13] To address this apparent dichotomy, stimuli-responsive materials have been designed that undergo a transition in the slightly acidic tumor tissue or after internalization into acidic intracellular vesicles. One such approach has been to incorporate pH-sensitive moieties in the polymeric micelle core that become hydrophilic at acidic pH, resulting in micelle disassembly and release of drugs.[4, 14, 15] Another approach incorporates pH-sensitive monomers to modulate the lower critical solution temperature (LCST) of thermosensitive polymers. In this way, the temperature-responsive phase change of polymers can be triggered by a change in local pH. This effect has been extensively studied using the temperature-sensitive material poly(N-isopropylacrylamide), or PNIPAAm. For example, copolymerization of NIPAAm with a pH-sensitive monomer can result in a material with LCST above body temperature at neutral pH but that lowers LCST below body temperature at lower pH. These materials can therefore release encapsulated drugs more effectively in acidic environments due to the collapse of PNIPAAm.[16–20]

Polymers synthesized using oligoethyleneglycol (OEG) monomers are gaining interest as a temperature-sensitive material system that are attractive alternatives to PNIPAAm with several advantages; the polymers can be synthesized by controlled radical polymerization, have reversible phase transitions, and form effective shields against protein adsorption.[21, 22] In this work, we report a series of new pH-dependent thermosensitive copolymers comprised of a poly(ϵ -caprolactone) (PCL) hydrophobic block with a hydrophilic block synthesized by copolymerization of an OEG methacrylate (OEGMA) monomer with an N-hydroxysuccinimide methacrylate (NHSMA) monomer that is used to introduce pH-sensitive side chains. The chemical structure of the pH-sensitive side chain was selected to modulate the LCST of the OEG block such that the transition occurs in slightly acidic pH. The block copolymers form micelles that can be efficiently loaded with anticancer drugs and the release of loaded drugs is triggered by the phase transition at acidic pH.

2. Materials and Methods

2.1. Synthesis of block copolymers

2.1.1. Materials— β -alanine or 6-aminocaproic acid, *N,N*-diisopropylethylamine (DIPEA), *N*-hydroxysuccinimide (NHS), *N,N,N',N',N'*-pentamethyl-diethylenetriamine (PMDETA), stannous octoate ($\text{Sn}(\text{Oct})_2$), anhydrous anisole were purchased from Aldrich, and were used as received. Doxorubicin hydrochloride (DOX-HCl) was purchased from Aldrich. ϵ -Caprolactone was distilled over CaH_2 under reduced pressure. Copper bromide (CuBr) was washed with acetic acid, ether and then dried under vacuum. Ether, toluene, and triethylamine were distilled under N_2 . Millipore purified water (18.2 M Ω cm) was used in all experiments. The (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl- tetrazolium bromide) (MTT) assay, Dulbecco's Modified Eagle's Medium (DMEM) and Hoechst 33342 are commercially available from Invitrogen. *N*-hydroxysuccinimide methacrylate (NHSMA), methoxytri(ethylene glycol) methacrylate (TEGMA), and hydroxyethyl 2-bromoisobutyrate were prepared according to the published protocols.[23–25]

2.1.2. Synthesis of poly(ϵ -caprolactone) (PCL-Br)— ϵ -caprolactone (1.53 g, 13.33 mmol) and hydroxyethyl 2-bromoisobutyrate (80 mg, 0.38 mmol) were weighed into a dry flask. The reaction mixture was stirred for 5 min at 110 °C in a preheated oil bath before a drop of stannous octoate was added as a catalyst and the polymerization was allowed to proceed for 3 hours. The resulting viscous solution was rapidly cooled, upon which it solidified. The crude polymer PCL-Br was dissolved in dichloromethane (DCM) and purified by precipitating into cold methanol three times. M_n (GPC) = 4500 g/mol, M_w/M_n = 1.24. ^1H NMR (CDCl_3): 4.35 (m, 4H, $\text{OCOCH}_2\text{CH}_2\text{OCO}$), 4.06 (t, 2H, $\text{OCH}_2(\text{CH}_2)_5\text{CO}$), 2.31 (t, 2H, O $(\text{CH}_2)_5\text{CH}_2\text{CO}$), 1.94 (s, 6H, $-\text{C}(\text{CH}_3)_2-$), 1.68 (m, 4H, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CO}$), 1.39 (m, 2H, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CO}$). $\text{DP}_{\epsilon\text{-CL}}$ = 43; M_n (NMR) = 4920 g/mol.

2.1.3. Synthesis of poly[methoxytri(ethylene glycol) methacrylate-co-*N*-hydroxysuccinimide methacrylate]-b-poly(ϵ -caprolactone) [P(TEGMA-co-NHSMA)-b-PCL, P1 and P2]—P(TEGMA-co-NHSMA)-b-PCL was prepared by Atom Transfer Radical Polymerization (ATRP).[26] PCL-Br macroinitiator (100 mg, 0.02 mmol), CuBr (6 mg, 0.04 mmol), and NHSMA (37.2 mg, 0.2 mmol) were added into a Schlenk tube and kept under vacuum for 10 minutes to degas. Under nitrogen atmosphere, a solution of TEGMA (0.94 g, 4 mmol) and PMDETA (8.5 μL , 4 mmol) in anisole (0.5 mL) was added into the Schlenk tube. The solution was degassed three times by using a freeze–pump–thaw process. After stirring at ambient temperature for 30 minutes, the solution was heated at 85 °C under nitrogen for 16 hours. After cooling to room temperature, the mixture was passed through an Al_2O_3 column to remove the copper and purified by precipitating into diethyl ether three times to produce pure block copolymer (**P1**). Poly[oligo(ethylene glycol) methacrylate]-b-poly(ϵ -caprolactone) (P(TEGMA-co-EGMA)-b-PCL), the pH-insensitive control polymer (**P0**), was also synthesized using the same procedure except replacing the NHSMA monomer with an ethyleneglycol monomer that contains an average of 4.5 ethyleneoxide units per monomer, in order to increase the LCST of the polymer and ensure stability of micelles in physiologic conditions. The monomer feed ratio for the synthesis was TEGMA:EGMA:CL = 120:10:1. **P0**: M_n (GPC) = 31,410 g/mol, DP_{CL} = 43; DP_{TEGMA} = 105; DP_{EGMA} = 7; M_n (NMR) = 35500 g/mol. **P1**: M_n (GPC) = 15040 g/mol, M_w/M_n = 1.16; ^1H NMR (CDCl_3): 4.08 (t, 2H, $\text{OCH}_2(\text{CH}_2)_5\text{CO}$, and br, 2H, COOCH_2), 3.67 (br, 8H, $\text{COOCH}_2\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_3$), 3.57 (br, 2H, CH_2OCH_3), 3.40 (br, 3H, CH_2OCH_3), 2.83 (br, 4H, NHS H), 2.32 (t, 2H, O $(\text{CH}_2)_5\text{CH}_2\text{CO}$), 1.8–2.1 (br, 2H, main chain CH_2), 1.67 (m, 4H, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CO}$), 1.39 (m, 2H, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CO}$), 0.8–1.21 (br, 3H, $\alpha\text{-CH}_3$). DP_{TEGMA} = 150; DP_{NHSMA} = 7;

M_n (NMR) = 41000 g/mol. **P2**: M_n (GPC) = 12540 g/mol, M_w/M_n = 1.19, DP_{TEGMA} = 120; DP_{NHSMA} = 15; M_n (NMR) = 35500 g/mol.

2.1.4. Synthesis of poly[methoxytri(ethylene glycol) methacrylate-co-N-methacryloyl amino acid]-b-poly(ϵ -caprolactone) [P(TEGMA-co-NMAA)-b-PCL] (P1- β A, P1-ACA, and P2-ACA)—Five times excess amount of β -alanine or 6-aminocaproic acid relative to NHSMA P(TEGMA-co-NHSMA)-b-PCL was weighed into a flask with DCM/methanol (1/4 v/v), DIPEA was added under N_2 , and allowed to react for 10 hours. After removing solvents under reduced pressure, the crude products were dissolved in DCM and filtered to remove unreacted β -alanine or 6-aminocaproic acid. The solutions were concentrated and the reaction mixtures were purified by precipitating into diethyl ether twice (P1- β A, P1-ACA, and P2-ACA).

2.2. Determination of lower critical solution temperature (LCST) of polymers

Optical transmittances of aqueous polymer solutions at various pH values and temperatures were measured at 550 nm with a UV-vis spectrometer. All samples were equilibrated for 10 minutes at each temperature step before the measurements. LCST values of polymer solutions were determined at temperature showing 50% optical transmittance of micelle solutions. The concentration of polymer solutions used for the LCST study was 0.8 mg/mL.

2.3. Preparation of empty or drug-loaded (doxorubicin) polymeric micelles

The empty or drug-loaded polymeric micelles were prepared by modifying reported protocols.[13, 27] In brief, a solution of the block copolymer solution (8 mg/mL in THF) mixed with or without doxorubicin (DOX) solution (1 mg/mL in DMSO) was added slowly into Britton-Robinson buffer solutions (B-R buffer solution) of different pH values. Doxorubicin hydrochloride was neutralized to DOX free base with excess amount of triethylamine before loading into the hydrophobic core of the micelles. After injecting prepared solutions into the buffer solution, the mixture was first incubated at room temperature for 6 hours and then dialyzed against buffer solution using dialysis membrane (MWCO 3500 Da) to remove solvent and unloaded DOX. The buffer solutions were replaced every 3 hours. The aqueous solutions were either diluted or condensed to desired concentrations. Finally, the solutions were filtered through a 0.45 μ m micro-filter. The amount of DOX loaded into the micelles was determined using UV-vis spectra referenced to a calibration curve of DOX in DMSO. The concentrations of drugs loaded into the micelles were calculated from the ratio of mass of drug in micelles to the total mass of the micelles.

2.4. Determination of particle size of micelles

Dynamic light scattering (DLS) measurements were used to determine particle size of the micelles. A Malvern Nano-ZS instrument equipped with a 4 mW He-Ne laser (633 nm) with an output at a scattering angle of 90° was used. Solutions of micelles were passed through a 0.45 μ m Millipore Millex-HN filter to remove dust before the DLS measurements. The samples for Transmission Electron Microscopy (TEM) were prepared by dropping 10 μ L of 0.15 mg/mL micelle suspension on the copper grid and stained with RuO_4 . The micrographs were taken by Hitachi-700 TEM operated at 80 kV.

2.5. Determination of critical micelle concentration (CMC)

The CMCs of the block copolymers in double distilled water were determined by a fluorescence probe technique using pyrene as a reference hydrophobic fluorescent probe. [28] Solutions of pyrene in acetone (1.8×10^{-4} M) were added to 20 mL vials and the acetone was removed by evaporation. Block copolymers with concentrations of 1.0×10^{-5} to 1.0 mg/mL were added to the vials containing pyrene. The final concentration of pyrene

in the block copolymer solutions were fixed at 6.0×10^{-7} M. The solutions were stirred at room temperature for 24 hours before the measurements. Excitation spectra of pyrene loaded micelles were recorded at 390 nm using a fluorescence spectrometer at room temperature. The intensity ratio of I_{337} to I_{334} was analyzed as a function of block copolymer concentration to determine the CMCs of block copolymers.

2.6. Determination of release kinetics of DOX

DOX release profiles were determined by dialysis at 37°C. Briefly, 3 mL of DOX-loaded micelles solution was placed in a molecular porous dialysis membrane (MWCO 3500 Da). Dialysis was performed against 0.3 L of B-R buffer solutions of pH 7.4 and 5.3, respectively. Samples (75 μ L) were drawn at specific time intervals from the micelles dispersion, further diluted with DMSO and then used to determine the concentration of DOX by absorbance measurements. The release percentages were calculated based on the absorbance changes.

2.7. In vitro cytotoxicity studies

A549 cells and MDA-MB-435 cells were cultured according to ATCC recommendations, and incubated at 37 °C in 5% CO₂ atmosphere. The cells were seeded in 96-well plates at a density of 10,000 cells per well, and grown for 24 (for A549) and 48 (for MDA-MB-435) hours. The cells were then incubated with a series of DOX-loaded micelles in different concentrations for 48 hours. After washing the cells with PBS buffer, fresh DMEM medium (100 μ L) and 10 μ L of MTT solution (5 mg/mL) were added to each well and the plates were incubated in 5% CO₂ at 37°C for another 3 hours. After incubation, the culture medium was taken out and 50 μ L of DMSO was added to each well and left for 10 minutes at room temperature to dissolve the internalized purple formazan crystals. The absorbance was measured at a wavelength of 540 nm using a plate reader (Tecan Infinite 500). Each experiment was conducted in four replicates per plate. Two plates were used. The results were expressed as a percentage of the absorbance of the blank control. The half maximal inhibitory concentration (IC₅₀) was determined as the concentration of DOX needed to kill 50% of cells.

2.8 Animals and tumor inoculation

Five-week-old male NCr nude mice were purchased from Taconic (Germantown, NY). All experimental procedures were performed in accordance with the protocols approved by the Institutional Animal Care and Use Committee at the University of Washington. To develop the melanoma xenografts, mice were injected under anesthesia subcutaneously in the right back with 200 μ L of single cell suspension containing 4×10^6 MDA-MB-435 cells in serum free-MEM media under anesthesia. Tumor volume was calculated by using mean diameter measured with vernier calipers and using the formula $v = 0.5 \times a \times b^2$, where a and b are the largest and the smallest diameter, respectively (short axis squared).

2.9. Xenograft tumor inhibition study

Treatment of tumors was started 2 weeks after melanoma cell inoculation when tumors reached sizes around 100–200 mm³. Following this period, 200 μ L of each of the following samples in 5% glucose solution was injected intravenously (i.v.) at a single dose of 8 mg DOX/kg of mice: free DOX, empty P1-ACA micelle, DOX-loaded P0 micelle, and DOX-loaded P1-ACA micelle. In addition, control mice were injected with 5% glucose solutions. Two days after these injections, mice received a second administration of their treatment formulation. Mouse weight and tumor size were then monitored. Data are expressed as mean $\pm$ standard error of the mean and statistical analysis was performed using unpaired one-way ANOVA analysis. Data were considered significantly different at $P < 0.05$.

3. Results and Discussion

3.1. Synthesis of temperature- and pH-sensitive block copolymers

A new amphiphilic block copolymer system based on copolymers of methoxytri(ethylene glycol) methacrylate (TEGMA), *N*-hydroxysuccinimide methacrylate (NHSMA), and a hydrophobic polycaprolactone (PCL) block (P(TEGMA-*co*-NHSMA)-*b*-PCL, P1 and P2, Scheme 1) was synthesized using an initiator containing both alkyl bromide and hydroxyl initiator groups for ATRP and living ring-opening polymerization (ROP), respectively.[29–32] The polymers, P1 and P2, were synthesized by keeping the hydrophobic PCL block constant but changing the ratio of OEGMA to NHSMA in the hydrophilic block (PCL:OEGMA:NHSMA of 43:150:7 and 43:120:15 for P1 and P2, respectively). The control polymer, P0, is a diblock copolymer of P(TEGMA-*co*-EGMA) and PCL. TEGMA and EGMA, short OEG monomers that contain 3 and 4.5 ethylene-oxide units, respectively, were selected as the thermoresponsive unit and the reactive NHSMA monomer allowed diverse synthetic flexibilities for post-functionalization. PCL was chosen as the hydrophobic block for biocompatibility and biodegradability.[33, 34] The PCL block was first synthesized by ROP of ϵ -caprolactone with $\text{Sn}(\text{Oct})_2$ as a catalyst. Then, the macroinitiator PCL block with alkyl bromide was employed for copolymerization of TEGMA and NHSMA monomers under ATRP conditions. The structures and synthetic procedures of P1 and P2 are given in Scheme 1.

In this study, two different amino acids, β -alanine (β A) or 6-aminocaproic acid (ACA), were used to functionalize the NHSMA monomer, introducing pH-sensitive acid groups to the copolymer and yielding the pH-dependent thermosensitive polymers, poly[(methoxytri(ethylene glycol) methacrylate-*co*-*N*-methacryloyl amino acid)]-*b*-poly(ϵ -caprolactone) [P(TEGMA-*co*-NMAA)-*b*-PCL] (P1- β A, P1-ACA, and P2-ACA, Scheme 1). The polymers were designed to differ by the relative hydrophobicity of the resulting acid side chain groups. At pH 7.4, the β A modification is the most hydrophilic, the ACA modification, which has a 6-carbon methylene linker, is intermediate in hydrophobicity, and the NHS ester, which in contrast to the β A and ACA is not ionized at pH 7.4 before hydrolysis, is the most hydrophobic. The purity and polydispersity of synthesized amphiphilic P1 and P2 were fully characterized by ^1H -NMR spectra and gel permeation chromatography (GPC). The calculated molar ratios of TEGMA, NHSMA, and CL were determined by ^1H -NMR spectra and are summarized in Table 1. The successful replacement of NHS with amino acid was evidenced by the disappearance of proton signals at 2.83 ppm from NHS moiety in the ^1H -NMR spectra.

3.2. Formulation and characterization of polymeric micelles

3.2.1. Determination of polymer LCSTs—Polymers that exhibit LCSTs are soluble in water below the LCST due to hydrogen bonding between the water and functional groups on polymers, and become increasingly insoluble above the LCST.[35] The LCST transition is entropy driven; the loss of hydrated water causes the entropy increase and leaves the polymer chains hydrophobic, inducing precipitation. In this study, the LCSTs of polymeric micelles were determined by observing the 50% optical transmittance of micelles at 550 nm using a UV-vis spectrometer as a function of temperature. The LCST of PTEGMA is around 52°C and this polymer is therefore water-soluble at body temperature.[24] To trigger drug release in the mildly acidic tumor environments, a material that undergoes a phase transition in a pH-dependent manner is desirable. Becer et al. previously showed that copolymers of methylacrylic acid (MAA) and OEGMA have pH-dependent LCSTs, but their polymers are not well-suited for this application; strongly acidic pH environments were required for LCST around 37°C for the tested MAA and OEGMA copolymers. We hypothesized that the

desired responsive polymer properties might be obtained with the inclusion of the more hydrophobic, pH-sensitive groups that result from β A and ACA functionalizations.

The LCSTs of P1 and P2 micelles show no changes in different aqueous solutions with pH ranging from 3.3 to 7.4. At neutral pH, the NHS ester is relatively stable in water with half-life on the order of several hours, so the materials do not contain significant amounts of exposed functional groups that can be ionized. In contrast, the phase transitions of micelles functionalized with β A or ACA (P1- β A, P1-ACA, and P2-ACA) are pH-dependent (Table 1 and Figure 1A). The pK_a of *N*-methacryloyl- β -alanine and *N*-methacryloyl-6-aminoheptanoic acid monomers are 4.5 and 4.7, respectively.[36] However, after incorporation into polymers, the pK_a of acids have been shown to increase due to changes in the microenvironment that reduce the acidity of the acid groups.[37, 38] Based on the pH-responsive LCST curves, the pK_a of β A and ACA are also increased to greater than 6.0 when they were incorporated in polymers (Figure 1A). Protonation of the acid groups at pH below their pK_a increases the hydrophobicity of the polymer and shifts the LCST to a lower temperature. As shown in Figure 1A, the LCSTs of these three amino acid-functionalized polymers are pH-dependent, with sharp increase at higher pH where deprotonation of the carboxylic acid groups increase the hydrophilicity of the TEGMA block. In addition, P1-ACA has lower LCSTs than P1- β A due to its long hydrophobic alkyl chain. The effect of polymer hydrophobicity on LCST behavior has been reported in temperature-sensitive polymers, such as those comprised of oligo(ethylene glycol) macromonomers[39] or PNIPAAm[40]. The effect of the density of carboxylic acid groups on the phase transition of polymeric micelles at pH 7.4 is shown in Figure 1B. At neutral pH, the carboxylic acid groups are deprotonated, increasing the hydrophilicity of the polymer. Higher acid content therefore correlates with higher LCST. The LCST of P1 is below body temperature, while P2-ACA remained soluble even at 60 °C. Thus, P1-ACA possesses the desirable stimulus-responsive behavior for tumor drug delivery. The data for the phase transition of P1-ACA as a function of temperature in aqueous solution is shown in Figure 1C. Significant phase transition at body temperature occurs in the pH range of 6–6.5. We further tested the LCST of P1-ACA as a function of polymer concentration at both pH 7.2 and pH 5.3 and showed that the LCST of P1-ACA at neutral pH remains higher than body temperature at concentrations relevant for in vivo administration (<3 mg/mL polymer) (Figure S1).

3.2.2. Determination of micelle size by dynamic light scattering—

Polymeric micelles were formed from the block copolymers by solvent evaporation. The average particle sizes of the polymer micelle solutions were determined by dynamic light scattering at room temperature (Table 1). Because the LCSTs of all materials are higher than 25 °C, the copolymers are all amphiphilic and self-assembled to polymeric micelles with PCL as a core and hydrophilic PTEGMA as shell structures. The average diameters of P1- β A, P1-ACA, and P2-ACA micelles at 25°C in pH ranging from 5.3 to 7.4 were 75 to 82 nm, 66 to 75 nm, and 62 to 70 nm, respectively. No obvious pH-dependent size variation of micelles was observed. However, the sizes of the micelles functionalized with amino acids were slightly larger than that of P1 and P2 (52 to 60 nm), possibly due to charge repulsion between carboxylic acid groups in the micelle corona that leads to slight elongation of the shell structure of micelles. The desired micelle size range for tumor delivery is bound on the lower end by the renal filtration cutoff (~ 5 nm) and at the upper end by sizes conducive to extravasation at tumor tissue and tumor penetration.[41] The pore size of tumor vasculature can be as large as 0.5 microns[42] although particles with diameters smaller than 100 nm are usually preferred for tissue penetration.[43, 44] The sizes of the P1- β A, P1-ACA, and P2-ACA polymeric micelles are within this desirable range and much smaller than most of reported pH and thermo-sensitive nanoparticles based on PNIPAAm.[17, 19, 20]

The micelle size of P1-ACA, the most promising copolymer for tumor delivery applications based on its LCST properties, was determined at different temperatures at pH 5.3 (Figure 2). At temperatures above the LCST (35.6 °C), micelle size increases significantly, with measured micelle size around 181 to 320 nm. This observation further confirms that a conformational change of the P1-ACA micelles occurs above the transition temperature. Finally, the morphology of P1-ACA micelles were confirmed by TEM imaging (Figure 3). TEM images indicate that spherical micelles with diameters of ~60–70 nm, consistent with values obtained by DLS.

3.2.3. Determination of CMC of P1-ACA—Micelles with low critical micelle concentrations (CMC), an indication of micelle stability, are necessary for intravenous applications. The CMC of the P1-ACA block copolymer solution was determined to be 5.4×10^{-3} mg/mL in aqueous solution by a pyrene fluorescence assay (Figure 4). This assay monitors a change in fluorescence characteristics of pyrene that occurs when it is encapsulated in the micellar medium. In brief, the peak excitation of pyrene shifts from 334 nm (in polar environments) to 337 nm (in nonpolar environments). The CMC of P1-ACA is much lower than those of low molecular weight surfactant, lower than most of reported Pluronic-based micelles, and similar to the PEG-poly(amino acid) block copolymers that have been used in clinical trials.[45–48]

3.3. Doxorubicin loading and release kinetics at neutral and acidic pH

As mentioned previously, P1-ACA is an attractive intelligent drug delivery carrier due to the circulation stability. The micelles will be in neutral pH and below the LCST so that micelles will be well-dispersed and stable during circulation. However, in mildly acidic conditions such as some tumor microenvironments or after cell internalization, the LCST is below body temperature resulting phase transition of polymer that facilitate drug release due to the collapse of hydrophilic shell of micelles. P1-ACA micelles were therefore loaded with doxorubicin. DOX loading slightly increased the size of P1-ACA micelles to an average diameter of ~100–110 nm but micelles remained spherical in shape (Figure S2). The LCST of DOX-loaded P1-ACA micelles did not change compared to unloaded P1-ACA micelles; LCST of unloaded vs DOX-loaded micelles was both 48.2 °C at pH 7.2 and was 33.5 °C vs 33.7 °C at pH 5.3. High drug loading (~4 wt% of DOX compared to total micelle) was achieved. The DOX release kinetics in both neutral (pH 7.4) and acidic (pH 5.3) conditions at 37°C were monitored next using a dialysis assay (Figure 5A). In neutral environment at 37°C, DOX-loaded micelles showed release of about 30 wt% of DOX from micelles after 70 hours. In acidic media, DOX release kinetics were increased, with 30 wt% release of DOX occurring within 6 hours. A turbid solution was also observed in acidic condition due to inter-particle aggregation of DOX-loaded micelles. On the other hand, the pH insensitive, DOX-loaded P0 micelles showed DOX release kinetics almost independent of pH values (Figure 5B).

3.4. Doxorubicin delivery to cultured transformed cells

The cytotoxicity of DOX-unloaded P1-ACA micelles in the concentration range of 0.01 to 0.2 mg/mL was tested against a cancer cell line, A549. No significant cytotoxicity was observed at all concentrations (Figure S3). Next, the cytotoxicity of free doxorubicin and DOX-loaded P1-ACA micelles were determined against the MDA-MB-435 cancer cell line. The half maximal inhibitory concentration (IC_{50}) of free DOX and DOX-loaded micelles to MDA-MB-435 cells was found to be 19.9 µg/mL for P0 micelles, 8.3 µg/mL for P1-ACA micelles, and 1.0 µg/mL for DOX.. The DOX-loaded micelles exhibit a less cytotoxic activity than free DOX after incubation likely due to the controlled and incomplete release of DOX from micelles over this time frame (Figure 6).

3.5. Evaluation of DOX-micelle formulations by systemic injection to tumor-bearing mice

The antitumor effect of DOX-loaded P1-ACA micelle was assessed *in vivo* using a MDA-MB-435 (melanoma) xenograft subcutaneous tumor model. To determine the effect of pH-triggered drug release, DOX-loaded P0 micelles that are not pH-sensitive were also administered as a control. Other controls included 5% glucose-injected mice (negative control), mice treated with empty micelles (negative control) and mice treated with free DOX (positive control). DOX-treated mice received 8 mg DOX/kg, around 66% of the reported LD₅₀ (median lethal dosage) of intravenously-administered DOX in mice.[49] All animals received two injections of drug spaced two days apart. The tumor size was monitored by caliper measurements for 32 days after initial injection (Figure 7A). The tumors of untreated mice and mice treated with empty P1-ACA micelles grew rapidly. Mice treated with free DOX and DOX-loaded micelles showed initial inhibition of tumor growth until 16 days after injection. After this time point, the tumor growth rate increased for both groups of mice treated with DOX and DOX-loaded P0 micelles. In contrast, the tumor size of mice treated with DOX-loaded P1-ACA micelles remained nearly constant. The ratio of tumor size of mice treated with free DOX, DOX-loaded P0, and DOX-loaded P1-ACA compared to untreated mice were 0.38, 0.72 and 0.12, respectively, at day 32. The results suggest that the pH-triggered drug release from micelles is critical for maintaining high drug potency against the cancer cells.

The average body weight of mice provides an indication of the systemic toxicity of the treatment protocols. None of the micelle-treated mice groups lost more than 5% of their body weight during the treatment, and all regained weight at similar rates after the second treatment administration (Figure 7B). In contrast, DOX-treated mice lost up to 10% of their body weight and experienced reduced weight gain for about a week after treatment. Thus, micellar DOX formulations showed low systemic toxicity in this animal model.

The utilization of nanocarriers, such as polymeric micelles and nanoparticles, has been proposed and investigated to decrease the systemic toxicity of free DOX. However, most reports of nanoparticle formulations of DOX that utilize passive, EPR-mediated delivery to tumors show reduced efficacy compared to free DOX *in vivo* when administered at the same drug dosage.[10, 13, 50–55] This effect is generally attributed to incomplete release of DOX from the nanoparticles. The formulations that are exceptions to this trend and show comparable or improved DOX efficacy have generally used pH-triggered mechanisms for drug release.[56–59] The new micelle system presented here possesses pH-dependent thermosensitive properties which can be manipulated through chemical structure and polymer composition. To our knowledge, this is the first demonstration *in vivo* of efficacious drug delivery to tumors using a dual-responsive, pH- and temperature-sensitive polymeric micelle system. The P1-ACA materials are therefore promising formulations for drug delivery to tumor sites.

4. Conclusion

We have developed a new drug delivery system based on a responsive amphiphilic block copolymer, P(TEGMA-*co*-NMAA)-*b*-PCL, that can self-assemble into polymeric micelles with uniform nanoscale size and exhibit pH-dependent, thermal response. The 6-aminocaproic acid-functionalized copolymer demonstrates phase transition at slightly acidic pH, as demonstrated by LCST and particle size determination. When loaded with doxorubicin, the pH-sensitive phase transition of these formulations results in increased drug release in acidic environments. The micelles show improved anti-tumor efficacy in xenograft tumor models compared to free doxorubicin and with lower associated systemic toxicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Maeda H, Wu J, Sawa T, Matsumura Y, Hori K. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. *J Control Release*. 2000; 65:271–284. [PubMed: 10699287]
2. Alarcon CDH, Pennadam S, Alexander C. Stimuli responsive polymers for biomedical applications. *Chem Soc Rev*. 2005; 34:276–285. [PubMed: 15726163]
3. Torchilin VP. Micellar nanocarriers: Pharmaceutical perspectives. *Pharm Res*. 2007; 24:1–16. [PubMed: 17109211]
4. Bae Y, Nishiyama N, Fukushima S, Koyama H, Yasuhiro M, Kataoka K. Preparation and biological characterization of polymeric micelle drug carriers with intracellular pH-triggered drug release property: Tumor permeability, controlled subcellular drug distribution, and enhanced in vivo antitumor efficacy. *Bioconjugate Chem*. 2005; 16:122–130.
5. Ulbrich K, Subr V. Polymeric anticancer drugs with pH-controlled activation. *Adv Drug Deliv Rev*. 2004; 56:1023–1050. [PubMed: 15066758]
6. Vaupel P, Kallinowski F, Okunieff P. Blood-flow, oxygen and nutrient supply and metabolic microenvironment of human tumors - a review. *Cancer Res*. 1989; 49:6449–6465. [PubMed: 2684393]
7. Chilkoti A, Dreher MR, Meyer DE. Design of thermally responsive, recombinant polypeptide carriers for targeted drug delivery. *Adv Drug Deliv Rev*. 2002; 54:1093–1111. [PubMed: 12384309]
8. Chung JE, Yokoyama M, Yamato M, Aoyagi T, Sakurai Y, Okano T. Thermo-responsive drug delivery from polymeric micelles constructed using block copolymers of poly(N-isopropylacrylamide) and poly(butylmethacrylate). *J Control Release*. 1999; 62:115–127. [PubMed: 10518643]
9. Gaucher G, Dufresne MH, Sant VP, Kang N, Maysinger D, Leroux JC. Block copolymer micelles: preparation, characterization and application in drug delivery. *J Control Release*. 2005; 109:169–188. [PubMed: 16289422]
10. Xiong XB, Falamarzian A, Garg SM, Lavasanifar A. Engineering of amphiphilic block copolymers for polymeric micellar drug and gene delivery. *J Control Release*. 2011; 155:248–261. [PubMed: 21621570]
11. Shin HC, Alani AWG, Cho H, Bae Y, Kolesar JM, Kwon GS. A 3-in-1 polymeric micelle nanocontainer for poorly water-soluble drugs. *Mol Pharm*. 2011; 8:1257–1265. [PubMed: 21630670]
12. Chen HT, Kim SW, Li L, Wang SY, Park K, Cheng JX. Release of hydrophobic molecules from polymer micelles into cell membranes revealed by Forster resonance energy transfer imaging. *Proc Natl Acad Sci U S A*. 2008; 105:6596–6601. [PubMed: 18445654]
13. Kim TH, Mount CW, Gombotz WR, Pun SH. The delivery of doxorubicin to 3-D multicellular spheroids and tumors in a murine xenograft model using tumor-penetrating triblock polymeric micelles. *Biomaterials*. 2010; 31:7386–7397. [PubMed: 20598741]
14. Gillies ER, Frechet MJ. pH-responsive copolymer assemblies for controlled release of doxorubicin. *Bioconjugate Chem*. 2005; 16:361–368.
15. Lee ES, Gao ZG, Bae YH. Recent progress in tumor pH targeting nanotechnology. *J Control Release*. 2008; 132:164–170. [PubMed: 18571265]

16. Cheng C, Wei H, Shi BX, Cheng H, Li C, Gu ZW, et al. Biotinylated thermoresponsive micelle self-assembled from double-hydrophilic block copolymer for drug delivery and tumor target. *Biomaterials*. 2008; 29:497–505. [PubMed: 17959241]
17. Lo CL, Lin KM, Hsiue GH. Preparation and characterization of intelligent core-shell nanoparticles based on poly(D,L-lactide)-g-poly(N-isopropyl-acrylamide-co-methacrylic acid). *J Control Release*. 2005; 104:477–488. [PubMed: 15911047]
18. Loh XJ, del Barrio J, Toh PPC, Lee TC, Jiao DZ, Rauwald U, et al. Triply triggered doxorubicin release from supramolecular nanocontainers. *Biomacromolecules*. 2012; 13:84–91. [PubMed: 22148638]
19. Soppimath KS, Tan DCW, Yang YY. pH-triggered thermally responsive polymer core-shell nanoparticles for drug delivery. *Adv Mater*. 2005; 17:318–+.
20. Zhang LY, Guo R, Yang M, Jiang XQ, Liu BR. Thermo and pH dual-responsive nanoparticles for anti-cancer drug delivery. *Adv Mater*. 2007; 19:2988–+.
21. Lutz JF. Polymerization of oligo(ethylene glycol) (meth)acrylates: Toward new generations of smart biocompatible materials. *J Polym Sci Pol Chem*. 2008; 46:3459–3470.
22. Ma HW, Hyun JH, Stiller P, Chilkoti A. "Non-fouling" oligo(ethylene glycol)-functionalized polymer brushes synthesized by surface-initiated atom transfer radical polymerization. *Adv Mater*. 2004; 16:338–+.
23. Batz HG, Franzman G, Ringsdorf H. Model reactions for synthesis of pharmacologically active polymers by way of monomeric and polymeric reactive esters. *Angew Chem-Int Edit Engl*. 1972; 11:1103–1104.
24. Han S, Hagiwara M, Ishizone T. Synthesis of thermally sensitive water-soluble polymethacrylates by living anionic polymerizations of oligo(ethylene glycol) methyl ether methacrylates. *Macromolecules*. 2003; 36:8312–8319.
25. Sarbu T, Lin KY, Spanswick J, Gil RR, Siegwart DJ, Matyjaszewski K. Synthesis of hydroxy-telechelic poly(methyl acrylate) and polystyrene by atom transfer radical coupling. *Macromolecules*. 2004; 37:9694–9700.
26. Matyjaszewski K, Xia JH. Atom transfer radical polymerization. *Chem Rev*. 2001; 101:2921–2990. [PubMed: 11749397]
27. Ma YH, Cao T, Webber SE. Polymer micelles from poly(acrylic acid)-graft-polystyrene. *Macromolecules*. 1998; 31:1773–1778.
28. Inoue T, Chen GH, Nakamae K, Hoffman AS. An AB block copolymer of oligo(methyl methacrylate) and poly(acrylic acid) for micellar delivery of hydrophobic drugs. *J Control Release*. 1998; 51:221–229. [PubMed: 9685920]
29. He XH, Liang LY, Xie MR, Zhang YQ, Lin SL, Yan DY. Synthesis of novel linear PEO-b-PS-b-PCL triblock copolymers by the combination of ATRP, ROP, and a click reaction. *Macromol Chem Phys*. 2007; 208:1797–1802.
30. Meier MAR, Aerts SNH, Staal BBP, Rasa M, Schubert US. PEO-b-PCL block copolymers: Synthesis, detailed characterization, and selected micellar drug encapsulation behavior. *Macromol Rapid Commun*. 2005; 26:1918–1924.
31. Ohno K, Tsujii Y, Fukuda T. Synthesis of a well-defined glycopolymers by atom transfer radical polymerization. *J Polym Sci Pol Chem*. 1998; 36:2473–2481.
32. Wang JS, Matyjaszewski K. Controlled living radical polymerization - atom-transfer radical polymerization in the presence of transition-metal complexes. *J Am Chem Soc*. 1995; 117:5614–5615.
33. Hu Y, Jiang XQ, Ding Y, Zhang LY, Yang CZ, Zhang JF, et al. Preparation and drug release behaviors of nimodipine-loaded poly(caprolactone)-poly(ethylene oxide)-polylactide amphiphilic copolymer nanoparticles. *Biomaterials*. 2003; 24:2395–2404. [PubMed: 12699677]
34. Lee JW, Hua F, Lee DS. Thermoreversible gelation of biodegradable poly(epsilon-caprolactone) and poly(ethylene glycol) multiblock copolymers in aqueous solutions. *J Control Release*. 2001; 73:315–327. [PubMed: 11516508]
35. Jeong B, Kim SW, Bae YH. Thermosensitive sol-gel reversible hydrogels. *Adv Drug Deliv Rev*. 2002; 54:37–51. [PubMed: 11755705]

36. Suzuki K, Nishiyama N, Nemoto K, Asakura T, Nakai H. Effects of N-methacryloyl-omega-amino acid primer pretreatment on the bond strength of the resin to acid-etched dentin. *J Biomed Mater Res.* 1997; 37:261–266. [PubMed: 9358320]
37. Dong HT, Du HB, Wickramasinghe SR, Qian XH. The effects of chemical substitution and polymerization on the pK(a) values of sulfonic acids. *J Phys Chem B.* 2009; 113:14094–14101. [PubMed: 19780534]
38. Schuwer N, Klok HA. Tuning the pH Sensitivity of poly(methacrylic acid) brushes. *Langmuir.* 2011; 27:4789–4796. [PubMed: 21425827]
39. Jones JA, Novo N, Flagler K, Pagnucco CD, Carew S, Cheong C, et al. Thermoresponsive copolymers of methacrylic acid and poly(ethylene glycol) methyl ether methacrylate. *J Polym Sci Pol Chem.* 2005; 43:6095–6104.
40. Kuckling D, Adler HJP, Arndt KF, Ling L, Habicher WD. Temperature and pH dependent solubility of novel poly(N-isopropylacrylamide) copolymers. *Macromol Chem Phys.* 2000; 201:273–280.
41. Fox ME, Szoka FC, Frechet JMJ. Soluble polymer carriers for the treatment of cancer: the importance of molecular architecture. *Accounts Chem Res.* 2009; 42:1141–1151.
42. Yuan F, Dellian M, Fukumura D, Leunig M, Berk DA, Torchilin VP, et al. Vascular-permeability in a human tumor xenograft - molecular-size dependence and cutoff size. *Cancer Res.* 1995; 55:3752–3756. [PubMed: 7641188]
43. Goodman TT, Olive PL, Pun SH. Increased nanoparticle penetration in collagenase-treated multicellular spheroids. *Int J Nanomed.* 2007; 2:265–274.
44. Wong C, Stylianopoulos T, Cui JA, Martin J, Chauhan VP, Jiang W, et al. Multistage nanoparticle delivery system for deep penetration into tumor tissue. *Proc Natl Acad Sci U S A.* 2011; 108:2426–2431. [PubMed: 21245339]
45. Alakhov V, Klinski E, Li SM, Pietrzynski G, Venne A, Batrakova E, et al. Block copolymer-based formulation of doxorubicin. From cell screen to clinical trials. *Colloid Surf B-Biointerfaces.* 1999; 16:113–134.
46. Oerlemans C, Bult W, Bos M, Storm G, Nijssen JFW, Hennink WE. Polymeric micelles in anticancer therapy: targeting, imaging and triggered release. *Pharm Res.* 2010; 27:2569–2589. [PubMed: 20725771]
47. Yamamoto T, Yokoyama M, Opanasopit P, Hayama A, Kawano K, Maitani Y. What are determining factors for micelle carriers? Consideration stable drug incorporation into polymeric on physical and chemical characters of the micelle inner core. *J Control Release.* 2007; 123:11–18. [PubMed: 17716772]
48. Kim TH, Mount CW, Dulken BW, Ramos J, Fu CJ, Khant HA, et al. Filamentous, mixed micelles of triblock copolymers enhance tumor localization of indocyanine green in a murine xenograft model. *Mol Pharm.* 2012; 9:135–143. [PubMed: 22118658]
49. Bertazzoli C, Rovero C, Ballerini L, Lux B, Balconi F, Antongiovanni V, et al. Experimental systemic toxicology of 4'-epidoxorubicin, a new, less cardiotoxic anthracycline antitumor agent. *Toxicol Appl Pharmacol.* 1985; 79:412–422. [PubMed: 3862266]
50. Du YZ, Weng Q, Yuan H, Hu FQ. Synthesis and antitumor activity of stearate-g-dextran micelles for intracellular doxorubicin delivery. *ACS Nano.* 2010; 4:6894–6902. [PubMed: 20939508]
51. Hu FQ, Liu LN, Du YZ, Yuan H. Synthesis and antitumor activity of doxorubicin conjugated stearic acid-g-chitosan oligosaccharide polymeric micelles. *Biomaterials.* 2009; 30:6955–6963. [PubMed: 19782395]
52. Park JH, Kwon S, Lee M, Chung H, Kim JH, Kim YS, et al. Self-assembled nanoparticles based on glycol chitosan bearing hydrophobic moieties as carriers for doxorubicin: In vivo biodistribution and anti-tumor activity. *Biomaterials.* 2006; 27:119–126. [PubMed: 16023198]
53. Son YJ, Jang JS, Cho YW, Chung H, Park RW, Kwon IC, et al. Biodistribution and anti-tumor efficacy of doxorubicin loaded glycol-chitosan nanoaggregates by EPR effect. *J Control Release.* 2003; 91:135–145. [PubMed: 12932645]
54. Tsai HC, Chang WH, Lo CL, Tsai CH, Chang CH, Ou TW, et al. Graft and diblock copolymer multifunctional micelles for cancer chemotherapy and imaging. *Biomaterials.* 2010; 31:2293–2301. [PubMed: 20042234]

55. Xiong XB, Ma ZS, Lai R, Lavasanifar A. The therapeutic response to multifunctional polymeric nano-conjugates in the targeted cellular and subcellular delivery of doxorubicin. *Biomaterials*. 2010; 31:757–768. [PubMed: 19818492]
56. Kim D, Lee ES, Park K, Kwon IC, Bae YH. Doxorubicin loaded pH-sensitive micelle: antitumoral efficacy against ovarian A2780/DOX(R) tumor. *Pharm Res*. 2008; 25:2074–2082. [PubMed: 18449626]
57. Ko J, Park K, Kim YS, Kim MS, Han JK, Kim K, et al. Tumoral acidic extracellular pH targeting of pH-responsive MPEG-poly (beta-amino ester) block copolymer micelles for cancer therapy. *J Control Release*. 2007; 123:109–115. [PubMed: 17894942]
58. Lu PL, Chen YC, Ou TW, Chen HH, Tsai HC, Wen CJ, et al. Multifunctional hollow nanoparticles based on graft-diblock copolymers for doxorubicin delivery. *Biomaterials*. 2011; 32:2213–2221. [PubMed: 21176954]
59. Talelli M, Iman M, Varkouhi AK, Rijcken CJF, Schiffelers RM, Etrych T, et al. Core-crosslinked polymeric micelles with controlled release of covalently entrapped doxorubicin. *Biomaterials*. 2010; 31:7797–7804. [PubMed: 20673684]

Figure 1. LCST of pH-dependent and thermo-sensitive polymeric micelles. (A) The LCSTs of P1-βA, P1-ACA, and P2-ACA micelle solutions as a function of pH value (B) Phase transition of P1, P2, P1-ACA, and P2-ACA micelles with 0, 3.4, and 8.4 mol% of amino acid groups at neutral condition as a function of temperature, respectively. (C) Phase transition of P1-ACA micelle solution as a function of temperature at different pH buffer solution.

Figure 2.

The particle size distribution of P1-ACA micelles at different temperature in pH 5.3 buffer solution as measured by dynamic light scattering.

Figure 3.
Transmission electron micrograph of P1-ACA micelles.

Figure 4. Critical micelle concentration determination of P1-ACA micelles. Plot of the intensity ratio I_{337}/I_{334} of pyrene as a function of P1-ACA concentration in aqueous solution.

Figure 5. The cumulative DOX release profiles from (A) P1-ACA and (B) P0 micelles in buffer solutions of pH 5.3 and pH 7.4 at 37°C.

Figure 6.

Determination of IC_{50} of free DOX, DOX-loaded P1-ACA micelles and DOX-loaded P0 micelles to MDA-MB-435 cells. Cell viability was determined by MTS assay and expressed as % viability compared to control untreated cells. By student t-test, IC_{50} of the three samples are significantly different ($p < 0.01$).

Figure 7.

In vivo tumor reduction in xenograft tumor-bearing mice dosed with empty P1-ACA micelles, DOX, and DOX loaded in P0 or P1-ACA micelles. (A) Growth inhibition of subcutaneous melanoma tumors induced by multiple intravenous injections of 5% glucose, empty P1-ACA micelles, free DOX, DOX-loaded P0 and P1-ACA micelles (8 mg DOX/kg mouse) (b) Normalized (to $t = 0$) body weights of treated mice. * indicate a statistically significant difference from P1-ACA micelles and untreated mice, or mice treated with empty micelles, DOX, or DOX-loaded P0 micelles, using the one-way ANOVA analysis, $p < 0.05$.

Scheme 1.
Synthesis of P(TEGMA-*co*-NMAA)-*b*-PCL.

Table 1

Characterization and LCSTs of the polymeric micelles at different pH values

Polymer	Mole Ratio (%) ^b			pH 7.4		pH 6.1		pH 5.3	
	CL	TEGMA	NHSMA or NMAA ^b	M _n NMR ^c	PDJ ^d	LCST ^e	D (nm)/ PDJ ^f	LCST ^e	D (nm)/ PDJ ^f
P1	12	84.9	3.1	41000	1.16	34.5	59/0.289	34.6	58/0.279
P1-βA^a	12.1	85.2	2.7	40800	1.16	61.8	80/0.432	40.2	75/0.351
P1-ACA^a	12	84.6	3.4	41100	1.16	48.2	68/0.309	36.9	66/0.289
P2	13.9	78.4	7.7	35500	1.19	34.1	52/0.289	34.2	53/0.256
P2-ACA^a	13.8	77.8	8.4	35800	1.19	— ^g	62/0.269	40.6	71/0.337

^a post-functionalization of NHS group by β-alanine (βA) or 6-aminocaproic acid (ACA).

^b Mole ratio of the polymers was analyzed from ¹H-NMR. NHSMA for **P1** and **P2**, and NMAA for **P1-βA**, **P1-ACA** and **P2-ACA**

^c Molecular weight was determined from ¹H-NMR.

^d polydispersity index of the polymers was determined by GPC.

^e LCST (°C) was determined by observing the 50% optical transmittance of micelles at 550 nm using a UV-vis spectrometer.

^f Average diameter of polymeric micelles and polydispersity index were determined by DLS at room temperature.

^g The LCST does not occur at temperature higher than 60°C in pH 7.4 buffer solution