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Supramolecular Copolymer Micelles Based on the Complementary Multiple Hydrogen Bonds of Nucleobases for Drug Delivery

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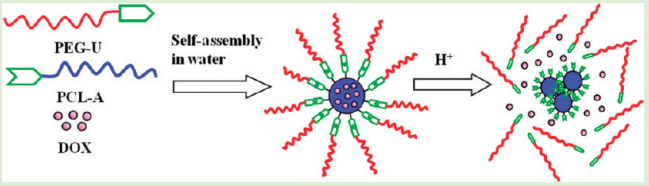
Supporting Information

ABSTRACT: Novel supramolecular copolymer micelles with stimuli-responsive abilities were successfully prepared through the complementary multiple hydrogen bonds of nucleobases and then applied for rapid intracellular release of drugs. First, both adenine-terminated poly(ε-caprolactone) (PCL-A) and uracil-terminated poly(ethylene glycol) (PEG-U) were synthesized. The supramolecular amphiphilic block copolymers (PCL-A:U-PEG) were formed based on multiple hydrogen bonding interactions between PCL-A and PEG-U. The micelles self-assembled from PCL-A:U-PEG were sufficiently stable in water but prone to fast aggregation in acidic condition due to the dynamic and sensitive nature of noncovalent interactions. The low cytotoxicity of supramolecular copolymer micelles was confirmed by MTT assay against NIH/3T3 normal cells. As a hydrophobic anticancer model drug, doxorubicin (DOX) was encapsulated into these supramolecular copolymer micelles. In vitro release studies demonstrated that the release of DOX from micelles was significantly faster at mildly acid pH of 5.0 compared to physiological pH. MTT assay against HeLa cancer cells showed DOX-loaded micelles had high anticancer efficacy. Hence, these supramolecular copolymer micelles based on the complementary multiple hydrogen bonds of nucleobases are very promising candidates for rapid controlled release of drugs.



INTRODUCTION

During the past decades, core/shell polymeric micelles self-assembled from amphiphilic copolymers in water have emerged as one of the most promising drug delivery systems.[1−6] The inner hydrophobic core of the micelles serves as a microenvironment for incorporating hydrophobic drugs by physical entrapment, while the outer hydrophilic shell maintains a hydration barrier to provide a stable interface between the hydrophobic core and the external medium.[7,8] Block copolymer micelles for drug delivery application are usually associated with several advantages. First, they improve the solubility and bioavailability of hydrophobic drugs in water.[9,10] Furthermore, the nano size and narrow size distribution of micelles can effectively avoid renal clearance and nonspecific reticuloendothelial uptake.[11] Therefore, these micelles significantly protect the incorporated drugs from fast degradation and elimination in the body and prolong drug circulation time after intravenous administration.[12,13]

Recently, stimuli-responsive micelles have gained widespread attention for programmable delivery systems in which the release of drugs can be readily controlled by exerting an appropriate stimulus (e.g., pH, temperature, glutathione, etc).[14−19] Among all these stimuli, acidic pH as an internal stimulus is particularly fascinating because tumor sites and inflammatory tissues as well as the intracellular compartments such as endosomes and lysosomes of cells have a more acidic environment.[20,21] The acidic pH at tumor sites has been considered as an ideal stimulus for the selective release of anticancer drugs in tumors to achieve tumor-targeted drug delivery.[22] Thus, micelles based on acid-labile covalent bonds such as acetal, hydrazone, *cis*-acotinyl, and *ortho*-ester are designed and applied in drug delivery field.[23−26] For example, Zhong et al.[27] prepared acid-sensitive micelles based on the block copolymer of poly(ethylene glycol) (PEG) and polycarbonate with acetal moieties, in which the hydrolysis of acetals under acidic condition led to the release of loaded anticancer drugs. Kataoka et al.[28] reported pH-sensitive polymeric micelles by attaching DOX to a PEGylated polyaspartate block copolymer via an acid-labile hydrazone bond. In addition, Heller and co-workers[29] have exploited *ortho*-ester as acid-labile linkages for design of pH-responsive micelles for drug delivery. These pH-responsive micelles based on acid-sensitive covalent bonds exhibit sustained release of drugs over tens of hours via a diffusion-controlled mechanism under acidic conditions.[30,31] However, for cancer therapy, it is often more desirable to accomplish rapid drug release after micelles arriving at the pathological sites, which may enhance the therapeutic efficacy as well as reduce probability of drug resistance in cells.[32]

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Therefore, it is necessary to design and exploit novel polymeric micelles with rapid response to acidic environment.

Compared to conventional covalent-linked polymers, supramolecular polymers based on noncovalent interactions have been found to be more sensitive to external stimuli, which offers a new route for design of drug delivery systems with rapid response abilities.[33,34] Among various noncovalent interactions, hydrogen bonding is very sensitive to pH variation. Therefore, it can be imaged that if the hydrophobic and hydrophilic blocks are linked through strong multiple hydrogen bonding interactions, a novel supramolecular copolymer with highly pH-sensitive abilities can be obtained. Ascribed to the perfect combination of amphiphilic and pH-responsive properties, self-assembled micelles with rapid controlled release property may become realizable.

In biological systems, multiple hydrogen bonding interactions occur in the adenine−uracil (A-U), adenine−thymine (A-T), and guanine−cytosine (G-C) base pairs in DNA and RNA.[35] In the present work, the complementary adenine−uracil (A:U) base pair between hydrophobic PCL-A and hydrophilic PEG-U was employed to construct supramolecular amphiphilic block copolymers (PCL-A:U-PEG). Benefiting from their amphiphilicity and noncovalent connection, PCL-A:U-PEG could self-assemble into micelles with pH-responsive abilities in aqueous solution. These novel stimuli-responsive micelles not only exhibited similar properties to conventional micelles from covalent-linked copolymers, but also responded rapidly to acidic stimulus. Moreover, the cytotoxicity and cellular uptake of the drug-loaded micelles were also evaluated using MTT assay, flow cytometry, and confocal laser scanning microscopy (CLSM).

EXPERIMENTAL SECTION

Materials. *N*,*N*-Dimethylformamide (DMF), dimethylsulfoxide (DMSO), and methylene dichloride (CH_2Cl_2) were dried over calcium hydride for 48 h and then distilled before use. Toluene was dried by refluxing with the fresh sodium-benzophenone complex under nitrogen gas and distilled just before use. ε-Caprolactone (ε-CL, 99%, J&K) was dried over calcium hydride for 24 h and then purified by distillation under reduced pressure prior to use. Uracil (U, 99%, Sigma), adenine (A, 99%, J&K), triethylaluminum ($AlEt_3$, 0.6 M in heptane, J&K), and ethylene carbonate (99%, Fluka) were all used as received. Monomethoxy poly(ethylene glycol) (PEG) with M_n = 5000 g/mol was purchased from Fluka and dried by azeotropic distillation in the presence of dry toluene. Doxorubicin hydrochloride (DOX·HCl) was purchased from Beijing Huafeng United Technology Corporation and used as received. Clear polystyrene tissue culture treated 12-well and 96-well plates were obtained from Corning Costar. All other reagents and solvents were of analytical grade and used as received unless mentioned.

Synthesis of 9-(2′-Hydroxyethyl) Adenine (HEA). A mixture containing adenine (2.11 g, 15.6 mmol), ethylene carbonate (1.40 g, 15.7 mmol), and a trace of sodium hydroxide (NaOH) in DMF (100 mL) was heated at 120 °C for 2 h. After filtration, the DMF was removed under reduced pressure and the residue was recrystallized from ethanol to give out some white powder. Yield: 70%. ^{1}H NMR (400 MHz, DMSO-d_6, 298 K) δ ppm: 8.14 (s, 1H, ArH), 8.08 (s, 1H, ArH), 7.23 (s, 2H, NH_2), 5.03 (s, 1H, OH), 4.19 (t, 2H, CH_2), 3.74 (q, 2H, CH_2).

Synthesis of PCL-A. Under an argon atmosphere, HEA (0.1260 g, 7.0×10^{-4} mol), DMSO (30 mL), and a toluene solution of $AlEt_3$ (0.1 mol/L, 0.5 mL) were added to a fresh flamed and argon-purged round-bottomed flask. The solution was stirred at 27 °C for 0.5 h and then toluene was evaporated under reduced pressure. After repeating this procedure three times, the mixture was cooled to 0 °C and 5 mL of caprolactone (0.046 mol) was added. The resulting mixture was stirred at 27 °C for 24 h under an argon atmosphere. The polymerization was terminated by adding excess acetic acid. DMSO was evaporated under reduced pressure, and the residue was precipitated into cold diethyl ether twice. The product was dried under vacuum to a constant weight. Yield: 86%. ^{1}H NMR (400 MHz, $CDCl_3$, 298 K) δ ppm: 8.3 (1H,-N=C*H*N-), 7.8 (1H, =NC*H*=N-), 5.6 (2H, -NH_2), 4.4 (2H,-NCH_2CH$_2$-), 3.9−4.2 (polycaprolactone, 2H per repeating unit,-COOCH_2-), 3.7 (2H, -NCH$_2$CH_2-), 2.1−2.4 (polycaprolactone, 2H per repeating unit, -CO=CH_2CH$_2$-), 1.5−1.8 (polycaprolactone, 2H per repeating unit, -COCH$_2$CH_2-, 2H per repeating unit, -COOCH$_2$CH_2-), 1.2−1.4 (polycaprolactone, 2H per repeating unit,-COCH$_2$CH$_2$CH_2-).

Preparation of PEG-U. PEG-U was prepared by coupling uracil to PEG. In a typical procedure: NaOH (0.27 g, 6.72 mmol) and PEG (12.0 g, 2.4 mmol) in anhydrous CH_2Cl_2 (100 mL) were cooled in an ice−water bath with stirring. After stirring for 30 min, a CH_2Cl_2 solution (40 mL) of *p*-toluenesulfonyl chloride (TsCl; 0.82 g, 4.3 mmol) was slowly added to the reaction mixture and stirred for an additional 24 h at 0 °C. The resulting mixture was poured into water and extracted with CH_2Cl_2. The combined organic extracts were washed with water and saturated sodium chloride (NaCl), dried over anhydrous magnesium sulfate ($MgSO_4$), filtered, and the filtrate was evaporated. PEG-sulfanilic acid ester was obtained with a yield of 89%. After that, PEG-sulfanilic acid ester (4.0 g, 0.8 mmol), uracil (0.18 g, 1.6 mmol), potassium carbonate (K_2CO_3; 0.22 g, 1.6 mmol) were dissolved in 60 mL of DMF in a round-bottomed flask and reacted at 80 °C for 8 h. The resultant mixture was poured into water and extracted with CH_2Cl_2. After drying over $MgSO_4$, most of the solvent was removed by evaporation. The residue was precipitated into cold diethyl ether and dried under vacuum to a constant weight. Yield: 85%. ^{1}H NMR (400 MHz, $CDCl_3$, 298 K) δ ppm: 8.4 (1H, -CON*H*CO-), 7.4 (1H, -NC*H*CH-), 5.6 (1H, -CHC*H*-CO-), 3.9 (2H, -CH$_2$CH_2N-), 3.5−3.7 (PEG, 2H per repeating unit,-OCH_2CH$_2$-, 2H per repeating unit, -O−CH$_2$CH_2-), 3.4 (3H, CH_3-OCH$_2$-). ^{13}C NMR (100 MHz, $CDCl_3$, 298 K) δ: 47.04 (-CH$_2$CH$_2$N-), 58.0 (CH$_3$O-), 67.8 (-CH$_2$CH$_2$N-), 69.7 (-CH$_2$CH$_2$O-), 71.2 (CH$_3$OCH$_2$-), 100.2 (-CHCHCO-), 146.3 (-CHCHCO-), 150.9 (-NCONH-), 163.7 (-CHCONH-).

Characterization. Both ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker AVANCEIII 400 spectrometer with $CDCl_3$, DMSO-d_6, and 1,1,2,2-tetrachloroethane-d_2 as solvents. The molecular weights and polydispersity index (PDI) were determined by gel permeation chromatography/multiangle laser light scattering (GPC-MALLS). The gel permeation chromatography system consisted of a Waters degasser, a Waters 515 HPLC pump, a 717 automatic sample injector, a Wyatt Optilab DSP differential refractometer detector, and a Wyatt miniDAWN multiangle laser light scattering detector. Three chromatographic columns (styragel HR3, HR4, and HR5) were used in series. Tetrahydrofuran (THF) was used as the mobile phase at a flow rate of 1 mL/min at 30 °C. The refractive index increment d*n*/d*c* was determined with Wyatt Optilab DSP differential refractometer at 690 nm. Data analysis was performed with Astra software (Wyatt Technology). Fourier transform infrared (FTIR) spectra were recorded on a Paragon 1000 instrument by KBr sample holder method. Absorbance measurements were carried out using Perkin-Elmer Lambda 20/2.0 UV/vis spectrometer. The calibration curve of absorbance against different concentrations of DOX was made at 485 nm. Dynamic light scattering (DLS) measurements were performed with a Malvern Zetasizer Nano S apparatus equipped with a 4.0 mW laser operating at λ = 633 nm. All samples were measured at a scattering angle of 173°. The data were the mean of three tests. Transmission electron microscopy (TEM) studies were performed with a JEOL JEM-100CX-II instrument at a voltage of 200 kV. Samples were prepared by drop-casting micelle solutions onto carbon-coated copper grids and then air-drying at room temperature before measurement.

Formation of PCL-A:U-PEG Micelles and Their Critical Micelle Concentration. Typically, both PEG-U (10 mg, 0.002 mol) and PCL-A (11 mg, 0.002 mol) were dissolved in THF (2 mL) at room temperature. After that, the polymer solution was added dropwise into 10 mL of deionized water under stirring with a magnetic bar. Then the THF was removed by vacuum distillation with a rotary evaporator. The micelle solution was dialyzed against deionized water for 24 h (MWCO = 2000), during which the water was renewed every 4 h.

The critical micelle concentration (CMC) was determined using 1,6-diphenyl-1,3,5-hexatriene (DPH) as UV probe by monitoring the absorbance at 313 nm. The concentration of block copolymer was varied from 1.0×10^{-4} to 0.2 mg/mL and the DPH concentration was fixed at 5.0×10^{-6} mol/L. The absorbance spectra of all solutions were recorded using Perkin-Elmer Lambda 20/2.0 UV/vis spectrometer.

Preparation of DOX-Loaded Micelles. Briefly, DOX·HCl and an equal molar amount of triethylamine (TEA) were dissolved in DMSO and added to a THF solution of PEG-U with an equivalent molar PCL-A at a theoretical drug loading content of 10 wt %. Then the mixture was added slowly to 5 mL of phosphate-buffered saline (PBS, 50 mM, pH 7.4). After being stirred for an additional 4 h, the solution was dialyzed against deionized water for 24 h (MWCO = 2000), during which the water was renewed every 4 h. For determination of drug loading content, the DOX-loaded micelle solution was lyophilized and then dissolved in DMF. The UV absorbance at 485 nm was measured to determine the DOX concentration.

Drug loading content (DLC) and drug loading efficiency (DLE) were calculated according to the following formula:

$$\mathrm{DLC(wt\%)} = (\text{weight of loaded drug/weight of polymer}) \times 100\%$$

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

In Vitro Release Study. A total of 6 mL of DOX-loaded micelles was transferred to a dialysis bag (MWCO = 2000). It was immersed in 75 mL of phosphate buffer (pH 7.4) or acetate buffer (pH 5.0) solutions in a shaking water bath at 37 °C to acquire sink conditions. At predetermined time intervals, 2 mL of the external buffer was withdrawn and replenished with an equal volume of fresh media. The amount of released DOX was analyzed with fluorescence (QM/TM/IM Steady-State and Time-Resolved Fluorescence Spectrofluorometer) measurement (excitation at 480 nm). The release experiments were conducted in triplicate. The results were the average data.

Cell Culture. NIH/3T3 normal cells (a mouse embryonic fibroblast cell line) and HeLa cancer cells (a human uterine cervix carcinoma cell line) were cultured in DMEM (Dulbecco's modified Eagle's medium) supplied with 10% FBS (fetal bovine serum), and antibiotics (50 units/mL penicillin and 50 units/mL streptomycin) at 37 °C in a humidified atmosphere containing 5% CO_2.

Cytotoxicity Measurements of Micelles. The relative cytotoxicity of PCL-A:U-PEG micelles against NIH/3T3 cells was estimated by MTT viability assay. In the MTT assay, NIH/3T3 cells were seeded into 96-well plates with a density of 1×10^4 cells per well in 200 μL of medium. After 24 h of incubation, the culture medium was removed and replaced with 200 μL of a medium containing serial dilutions of micelles. The cells were grown for another 48 h. Then, 20 μL of 5 mg/mL MTT assays stock solution in PBS was added to each well. After incubating the cells for 4 h, the medium containing unreacted dye was removed carefully. The obtained blue formazan crystals were dissolved in 200 μL per well DMSO and the absorbance was measured in a BioTek Elx800 at a wavelength of 490 nm.

Cellular Uptake of Micelles by HeLa Cells. The cellular uptake experiments were performed on flow cytometry and CLSM. For flow cytometry, HeLa cells were seeded in six-well plates at 6×10^5 cells per well in 1 mL complete DMEM and cultured for 24 h. Then the DOX-loaded micelles dissolved in DMEM culture medium at a final DOX concentration of 9 μg/mL were added to different wells and the cells were incubated at 37 °C for 5, 30, 60, and 180 min. Thereafter, samples were prepared for flow cytometry analysis by removing the cell growth media, rinsing with PBS, and treating with trypsin. Data for 1.0×10^4 gated events were collected and analysis was performed by means of a BD FACSCalibur flow cytometer and CELLQuest software.

For the CLSM studies, HeLa cells were seeded in six-well plates at 1×10^5 cells per well in 1 mL of complete DMEM and cultured for 24 h, followed by removing culture medium and adding DOX-loaded micelles (1 mL of DMEM medium) at a final DOX concentration of 9 μg/mL. The cells were incubated at 37 °C for predetermined intervals. Subsequently, the cells were washed with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature, and the slides were rinsed with PBS for three times. Finally, the slides were mounted and observed with a LSM510 META.

Activity Analyses. The cytotoxicity of DOX-loaded micelles and free DOX against HeLa cells was evaluated in vitro by MTT assay. HeLa cells were seeded into 96-well plates with a density of 8×10^3 cells per well in 200 μL of medium. After 24 h of incubation, the culture medium was removed and replaced with 200 μL of a medium containing serial dilutions of DOX-loaded micelles. The cells were grown for another 48 h. Then, 20 μL of 5 mg/mL MTT assays stock solution in PBS was added to each well. After incubating the cells for 4 h, the medium containing unreacted dye was removed carefully. The obtained blue formazan crystals were dissolved in 200 μL per well DMSO and the absorbance was measured in a BioTek Elx800 at a wavelength of 490 nm.

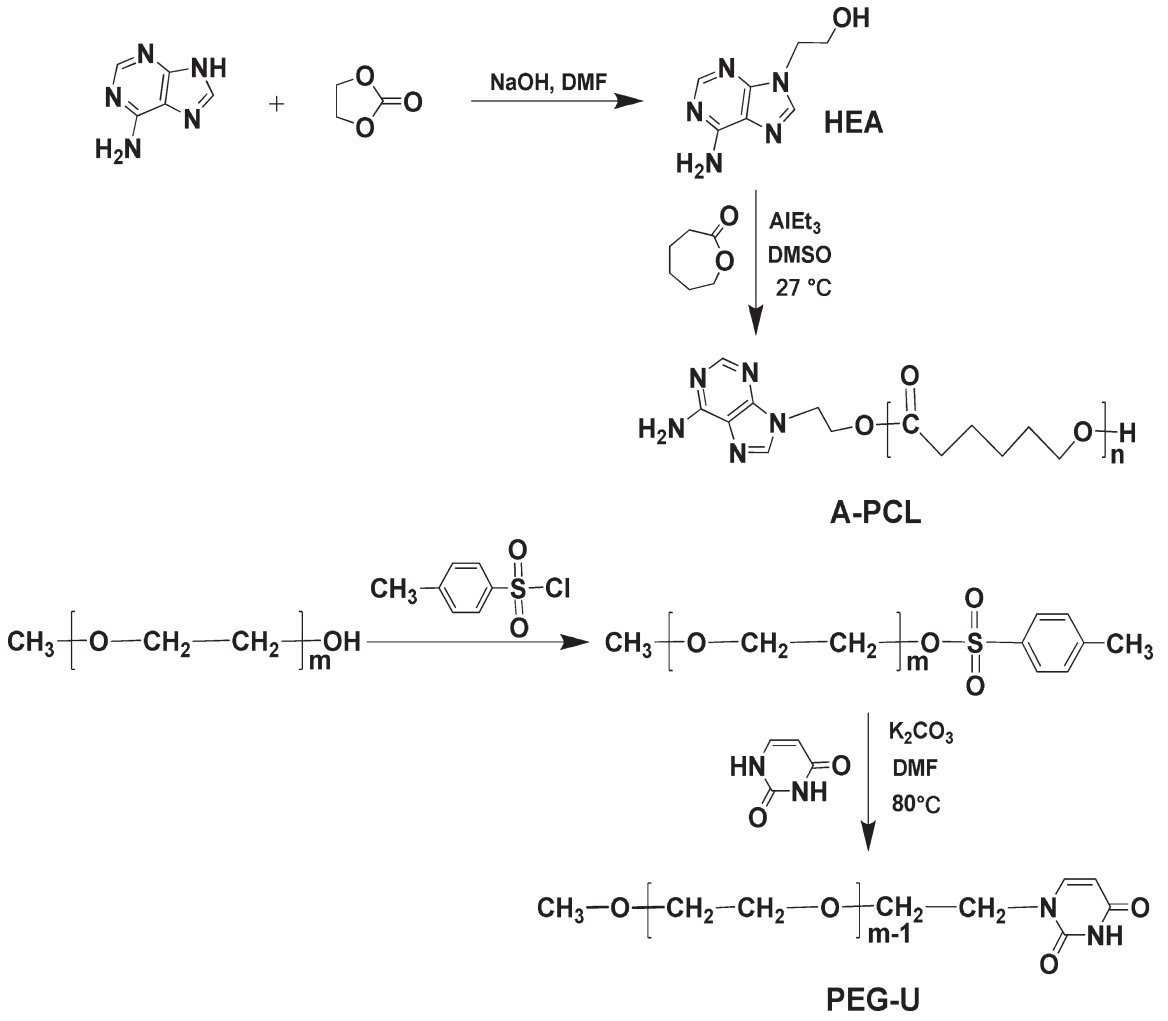
RESULTS AND DISCUSSION

Synthesis and Characterization of PCL-A and PEG-U. End-functionalized polymers can be synthesized via direct polymerization using a functional initiator or through the postmodification of polymers with reactive terminal moieties.[36−40] Scheme 1 gives the synthesis route of nucleobase-teminated PCL and PEG. First, HEA was formed through the reaction of adenine with ethylene carbonate according to previous reports,[41] which was confirmed by NMR measurements. Then adenine-terminated PCL was synthesized via the ring-opening polymerization (ROP) of ε-CL using HEA as the initiator and $AlEt_3$ as the catalyst. The obtained PCL-A was well characterized by ^{1}H NMR and GPC-MALLS. Typical ^{1}H NMR spectra of PCL-A is shown in Figure 1A. Figure 2 gives the GPC curves of PCL-A with different molecular weights and the characterization data are summarized in Table 1. In addition, number-average molecular weights are also calculated from the ^{1}H NMR integration ratios of the methylene signal on the PCL polymer backbone (-CH_2OCO-, 3.9−4.2 ppm) to the signal of methylene connected with adenine (-N-CH_2CH_2-, 4.4 ppm). These results are in agreement with those reported in previous literature.[41]

PEG-U was obtained by the reaction of PEG-sulfanilic acid ester with excess uracil in the presence of K_2CO_3. The chemical structure of PEG-U is confirmed by the ^{1}H NMR and ^{13}C NMR spectra shown in Figures 1B and S1. The peaks at 3.9 and 8.4 ppm are ascribed to the protons of CH_2 (-CH_2CH_2N-) connected with uracil and NH (-CONHCO-) on the uracil, respectively, which demonstrates the generation of PEG-U. The ^{13}C NMR results further demonstrate the successful grafting of uracil on PEG.

Hydrogen Bonding Interactions between PCL-A and PEG-U. The formation of complementary multiple hydrogen bonds between PCL-A and PEG-U was analyzed by variable-temperature ^{1}H NMR spectroscopy for the blend of two polymers with an

Scheme 1. Synthetic Route to Adenine-Terminated Poly(ε-caprolactone) (PCL-A) and Uracil-Terminated Poly(ethylene glycol) (PEG-U)



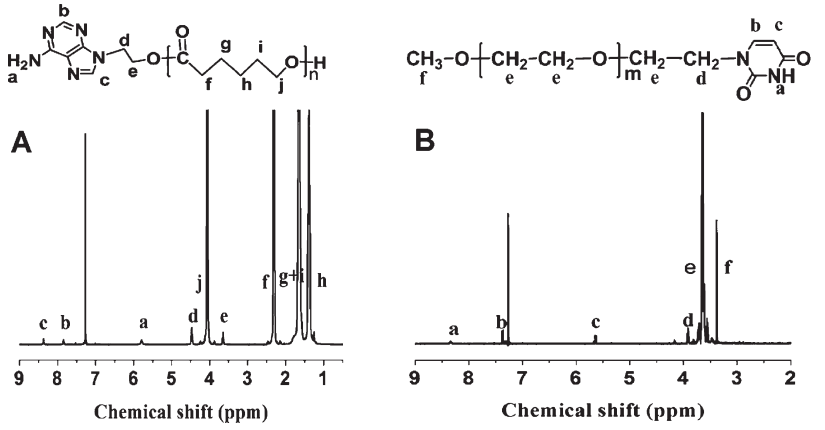


Figure 1. ^{1}H NMR spectra of adenine-terminated poly(ε-caprolactone) (PCL-A) and uracil-terminated poly(ethylene glycol) (PEG-U): (A) PCL-A, (B) PEG-U (400 MHz, $CDCl_3$).

equivalent mole in 1,1,2,2-tetrachloroethane-d_2. The temperature dependence of the NH and NH_2 proton chemical shift of the PCL-A and PEG-U complex (1:1) at 2.5 wt % in 1,1,2,2-tetrachloroethane-d_2 is shown in Figure 3. The chemical shift of the NH resonance moves upfield systematically from 8.7 to 7.9 ppm when the temperature is raised from 25 to 100 °C. The gradual decrease in the NH resonance with the increase of temperature can be attributed to the dissociation of the complementary hydrogen

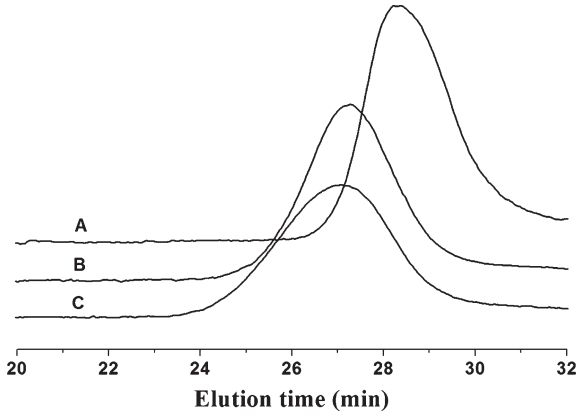


Figure 2. Gel permeation chromatography (GPC) curves of adenine-terminated poly(ε-caprolactone) (PCL-A): (A) PCL_{25}-A, (B) PCL_{40}-A, (C) PCL_{49}-A.

Table 1. Characterization of Adenine-Terminated Poly(ε-caprolactone) (PCL-A) Polymers

polymers PCL-A	M_n[a] (^{1}H NMR)	M_n[b] (GPC)	M_w[b] (GPC)	PDI[b] (GPC)
1	2800	2870	2890	1.01
2	3600	4590	4950	1.08
3	4500	5630	6470	1.15

[a] Determined by ^{1}H NMR. [b] Determined by gel permeation chromatography/multiangle laser light scattering (GPC-MALLS) using THF as the eluent.

bonds.[41,42] However, as soon as the sample is cooled from 100 to 25 °C, the NH resonance returns to its original position at 8.7 ppm. Similarly, the NH_2 resonance exhibits the same change in the range of 5.74−5.35 ppm. All of these data suggest that complementary hydrogen bonds generate between PCL-A and PEG-U, and the supramolecular diblock copolymers are thermoreversible in 1,1,2,2-tetrachloroethane-d_2.

To further evaluate the hydrogen bonding interactions between PCL-A and PEG-U, FTIR was performed at various temperatures (from 25 to 160 °C). Figure S2 shows the partial IR spectra of PCL-A and PEG-U complexes in the bulk state. The bands between 1750 and 1710 cm^{-1} come from the C═O stretching vibration. It can be found that the C═O stretching peak shifts to higher frequency from 1724 to 1734 cm^{-1} as the temperature raises from 25 to 160 °C. When the sample is cooled from 160 to 25 °C, the C═O stretching peak returns to its original position at 1724 cm^{-1}. According to previous literature, the C═O stretching bands shift to lower frequencies upon hydrogen bond formation.[43] It is concluded that the complementary hydrogen bonds dissociate with increasing temperature. The results of the FTIR spectra suggest that polymer chains are linked through complementary hydrogen bonds to form a supramolecular diblock copolymer with thermoreversibility.

Formation of Micelles and pH-Triggered Destabilization. PCL-A and PEG-U could form supramolecular diblock copolymer through complementary hydrogen bonding interactions. Owing to the presence of the hydrophobic PCL-A and hydrophilic PEG-U domains, the supramolecular amphiphilic block copolymer self-assembled into micelles in aqueous solution. Micelles of PCL-A:U-PEG block copolymers were prepared by dialysis method and the resulting micelles had a yield of 92% (weight of resulting micelles/weight of copolymers in feed). The CMC of these supramolecular copolymers were investigated by UV/vis spectra using DPH as a hydrophobic probe. The relationship of the content of PCL with the CMC is listed in Table 2. As the content of PCL in copolymers increases, the CMC gradually decreases.

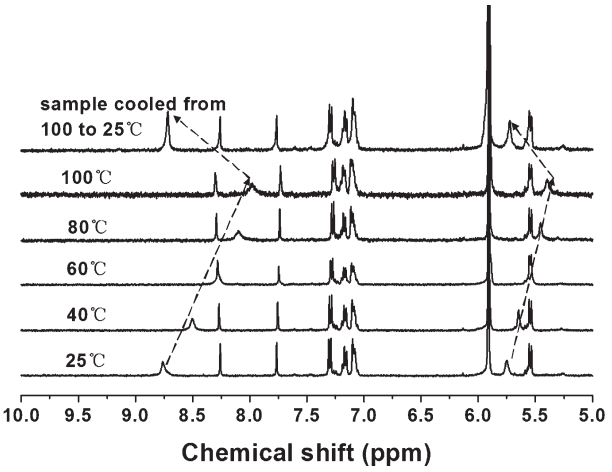


Figure 3. ^{1}H NMR NH and NH_2 chemical shifts of the adenine-terminated poly(ε-caprolactone) (PCL-A) and uracil-terminated poly(ethylene glycol) (PEG-U) complex (1:1) as a function of temperature. Sample was allowed to equilibrate for 10 min at each temperature (400 MHz, 1,1,2,2-tetrachloroethane-d_2).

Table 2. Properties of the PCL-A:U-PEG Micelles

sample	CMC[a] (mg/mL)	diameter[b] (nm)	PDI[b]
PCL_{25}-A:U-PEG_{114}	6.5×10^{-2}	142	0.097
PCL_{40}-A:U-PEG_{114}	3.9×10^{-2}	156	0.093
PCL_{49}-A:U-PEG_{114}	1.8×10^{-2}	172	0.139

[a] Critical micelle concentration (CMC) was determined by UV/vis spectrometer. [b] Diameter and polydispersity index (PDI) of PCL-A:U-PEG micelles were determined by dynamic light scattering (DLS).

To further study the properties of supramolecular copolymer micelles, both DLS and TEM measurements were performed. DLS results show that all of the PCL-A:U-PEG micelles exhibit unimodal size distribution with the mean diameter from 142 to 172 nm and the detailed data are summarized in Table 2. Apparently, the average diameter of the micelles increases as the PCL increases from M_n = 2.8 k to M_n = 5.6 k with the same PEG block. This indicates that a long PCL block can enhance its assembly and lead to a large core. The morphologies of supramolecular copolymer micelles for PCL_{49}-A:U-PEG_{114} visualized by TEM are shown in Figure 4B1. The copolymer aggregates into approximate spherical micelles in aqueous solution and the micelle sizes determined by TEM are in accordance with the data from DLS in Figure 4A1.

The hydrogen bonding interactions between hydrophobic PCL-A core and hydrophilic PEG-U shell made copolymer micelles unstable at acidic pH. To evaluate the responsive ability, the copolymer micelles were treated with pH 5.0 acetate buffer (50 mM) and the particle sizes were followed by DLS measurements at different time intervals. Figure 5A shows that PCL_{49}-A:

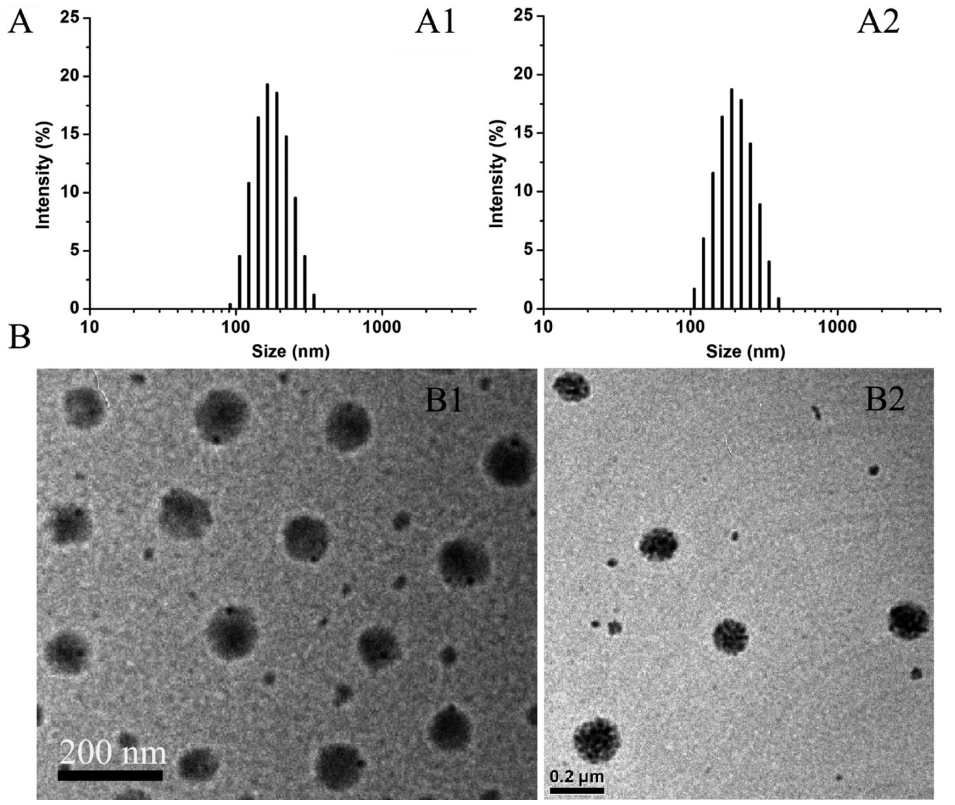


Figure 4. Characterization of PCL_{49}-A:U-PEG_{114} and doxorubicin (DOX)-loaded micelles. (A) The size distribution of PCL_{49}-A:U-PEG_{114} micelles determined by DLS: (A1) PCL_{49}-A:U-PEG_{114} micelles and (A2) DOX-loaded copolymer micelles. (B) TEM images of self-assembled micelles: (B1) PCL_{49}-A:U-PEG_{114} micelles and (B2) DOX-loaded copolymer micelles.

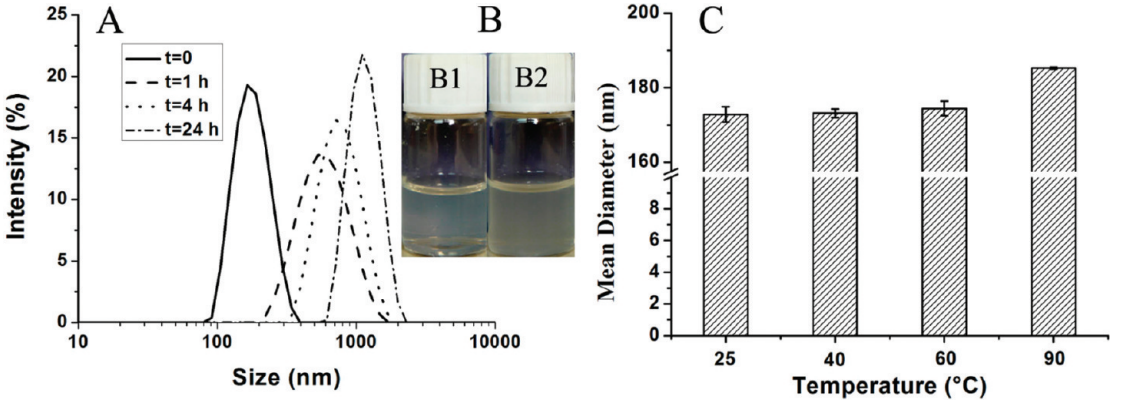


Figure 5. Characterization of PCL_{49}-A:U-PEG_{114} micelles: (A) Size change of pH-sensitive micelles over time at pH 5.0 at 25 °C monitored by DLS; (B) Photographs of copolymer micelles over 48 h (B1) pH = 7.4, (B2) pH = 5.0; (C) The size of PCL_{49}-A:U-PEG_{114} micelles at different temperatures determined by DLS. Error bars represent the standard deviation (n = 3).

U-PEG_{114} micelles aggregate rapidly at pH 5.0. The size of copolymer micelles increases from 172 nm to about 722 nm in 4 h, and reaches over 1000 nm after 24 h. The photographs in Figure 5B show that the micelle solution becomes turbid at low pH value after 48 h, while still keeps clear under physiological pH condition (pH 7.4). The driving force for the change of micelle size at low pH value is attributed to the protonation of the nucleobase nitrogen atoms, which leads to the shedding of hydrophilic PEG shell from the micelles and the aggregation of hydrophobic PCL core.[44] In contrast, no change in micelle size is observed after 48 h at pH = 7.4.

Thermal Stability of PCL-A:U-PEG Micelles. The stability of supramolecular block copolymers was studied at different temperatures. As shown in Figure 5C, the effect of temperature on PCL-A:U-PEG micelles is very slight at the temperature below 60 °C and the average diameter is around 172 nm. When self-assembled micelles are heated from 60 to 90 °C, the micelle size reaches 185 nm. It is related to the dissociation of complementary hydrogen bonds at high temperature. Therefore, the stability of prepared micelles at physiological temperature offers the possibility for the introduction to the body with the potential of therapeutics delivery.

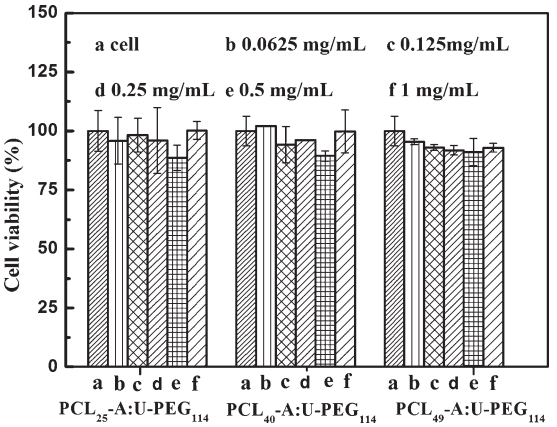


Figure 6. Cell viability of NIH/3T3 cells against nanomicelles after cultured for 48 h with different micelle concentrations.

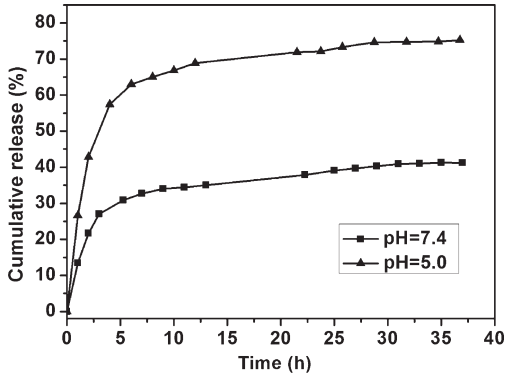


Figure 7. In vitro release profiles of doxorubicin (DOX) from PCL_{49}-A:U-PEG_{114} micelles at different pH values (7.4 and 5.0) at 37 °C.

In Vitro Cytotoxicity of PCL-A:U-PEG Micelles. The cytotoxicity of PCL-A:U-PEG micelles was evaluated by MTT assay using NIH/3T3 normal cells. The MTT assay is based on the ability of a mitochondrial dehydrogenation enzyme in viable cells to cleave the tetrazolium rings of the pale-yellow MTT and form formazan crystals with dark-blue color. Figure 6 shows the cell viability after 48 h incubation with the micelles of PCL_{25}-A:U-PEG_{114}, PCL_{40}-A:U-PEG_{114}, and PCL_{49}-A:U-PEG_{114}, respectively, at different concentrations (the total amount of PCL-A and PEG-U in aqueous solution). The results demonstrate that no obvious cytotoxicity against NIH/3T3 cells is observed even the concentration of copolymer micelles is up to 1 mg/mL. Therefore, these PCL-A:U-PEG micelles exhibit low cytotoxicity to NIH/3T3 cells.

Drug Loading and pH-Triggered Drug Release. To assess the potential of supramolecular copolymer micelles based on molecular recognition of uracil and adenine nucleobases, DOX was used to evaluate the drug loading and release properties. DOX is one of the most potent anticancer drugs in the treatment of different types of solid malignant tumors and known to interact with DNA by interaction and inhibition of macromolecular biosynthesis.[45,46] It is well-known that physical entrapment of hydrophobic drugs in polymeric micelles is mainly driven by the hydrophobic interactions between the drug and the hydrophobic segments of polymers.[30] Therefore, supramolecular block copolymer PCL_{49}-A:U-PEG_{114} with the high PCL content was selected as a drug carrier. Hydrophobic DOX was well-encapsulated into the hydrophobic inner cores of the polymeric micelles (PCL_{49}-A:U-PEG_{114}) with a loading efficiency of about 15%. The theoretical DLC was set 10% and the final DLC of PCL_{49}-A:U-PEG_{114} was 1.6%. It should be noted that DOX-loaded micelles show a lightly increased average size of about 198 nm, measured by DLS and TEM (Figure 4A2 and B2), wherein the polydispersity of polymeric micelles is fairly low, indicating narrow size distribution.

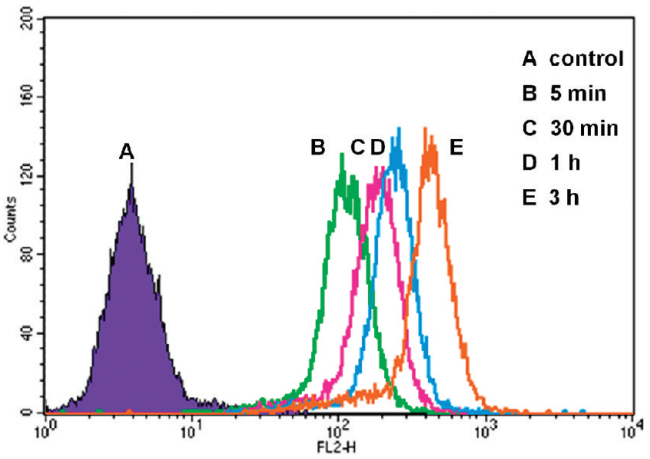


Figure 8. Flow cytometry histogram profiles of HeLa cells that were incubated with self-assembled PCL_{49}-A:U-PEG_{114} micelles containing core-encapsulated doxorubicin (DOX).

The in vitro release behavior of DOX-loaded micelles was investigated by dialysis in two different buffered solutions (pH 7.4 and 5.0) at 37 °C to assess the feasibility of using PCL_{49}-A:U-PEG_{114} micelles as an anticancer drug delivery carrier. As illustrated in Figure 7, the release profiles show that DOX is released quickly from the micelles in the first stage and then the drug release could be sustained over a prolonged time. It is obvious that the DOX release from micelles at pH 5.0 is much faster than the release at pH 7.4, indicating a pH-dependent drug release profile. At pH 7.4, the release rate of DOX is low with less than 40% of the DOX released in 40 h, while a noticeably increased release rate of DOX is observed at pH 5.0 with more than 65% of the DOX released within 5 h. This is in accordance with the previous observation that PCL_{49}-A:U-PEG_{114} micelles were destabilized and formed aggregates in response to pH 5.0 acetate buffer. Gao et al.[30] reported that less than 20% DOX was released from PCL-PEG micelles at pH 5.0 in one month. Therefore, the fast release of DOX in acidic environment could be attributed to the protonation of the amino group of nucleobase and shedding of micelle shell at acidic condition. This pH-dependent releasing behavior is of particular interest in achieving the tumor-targeted DOX delivery with micelles. Therefore, micelles from supramolecular block copolymers may represent a highly promising approach to achieve fast controlled drug release.

Cell Internalization. The cellular uptake of DOX-loaded PCL_{49}-A:U-PEG_{114} micelles was evaluated by flow cytometry using HeLa cancer cells. After DOX-loaded micelles with DOX concentration of 9 μg/mL were added to culture medium, the HeLa cells were cultured at 37 °C for the predetermined time intervals. Histograms of cell-associated DOX fluorescence for HeLa cells are displayed in Figure 8. Remarkably, strong DOX

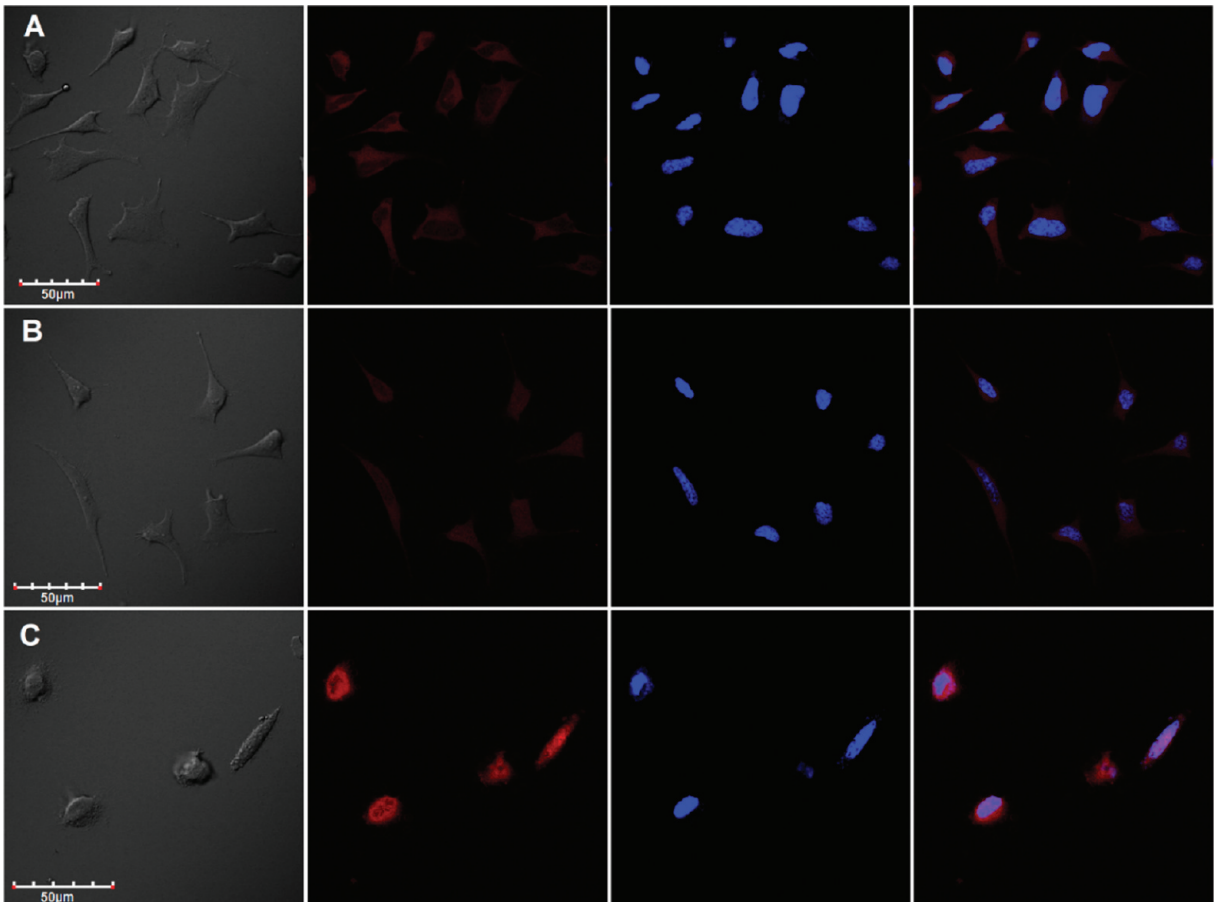


Figure 9. Confocal laser scanning microscopy (CLSM) images of HeLa cells that incubated with doxorubicin(DOX)-loaded PCL_{49}-A:U-PEG_{114} micelles for (A) 30 min, (B) 6 h, and (C) 24 h. Cell nucleuses were stained with Hoechest 33342.

fluorescence intensity is observed in the cells after 5 min incubation with DOX-loaded PCL_{49}-A:U-PEG_{114} micelles, which is most likely due to significantly faster intracellular release of DOX from PCL_{49}-A:U-PEG_{114} micelles. After a longer incubation time of 3 h, the relative geometrical mean fluorescence intensities of DOX-loaded micelle pretreated cells are about 50 fold of nonpretreated cells. The curves show that the gradual increase intensity of fluorescence with increasing incubation time can be attributed to the cellular uptake of DOX-loaded micelles.[47,48]

The cellular uptake and intracellular release behaviors of DOX-loaded micelles by HeLa cells were further investigated by CLSM. HeLa cells were cultured at 37 °C for 30 min, 6 h, and 24 h, respectively, after DOX-loaded micelles were added to culture medium with a DOX concentration of 9 μg/mL. Then the nucleus was stained with Hochest33342 and the pretreated cells were observed under CLSM. As shown in Figure 9, the DOX fluorescence is observed mainly in the cytoplasm of the cells when the cells are cultured in the DOX-loaded PCL_{49}-A:U-PEG_{114} micelles for 30 min and 6 h. However, the fluorescence is partly localized in the cell nucleus when cells are incubated with the DOX-loaded micelles for 24 h. These results indicate that DOX-loaded PCL_{49}-A:U-PEG_{114} micelles may be taken up by the cells through a nonspecific edocytosis mechanism and the DOX molecules are released in endocytic compartments.[47,49] Therefore, the novel PCL-A:U-PEG micelles are not only transported efficiently but also resided in cytoplasm. These properties of PCL-A:U-PEG micelles are very important for its use as an ideal drug carrier.

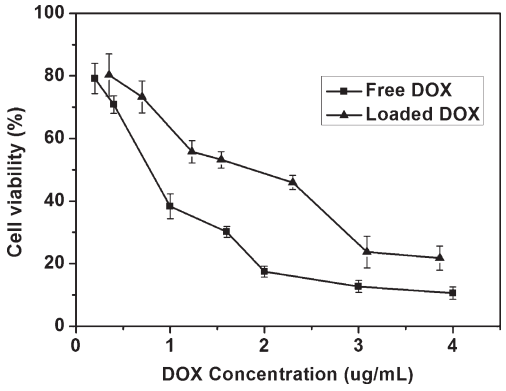


Figure 10. Cell viability of HeLa cells against doxorubicin (DOX)-loaded PCL_{49}-A:U-PEG_{114} micelles after cultured for 48 h with different DOX doses.

In Vitro Cytotoxicity of Drug-loaded Micelles. The in vitro cytotoxicity of DOX-loaded PCL_{49}-A:U-PEG_{114} copolymer micelles compared with that of free DOX was evaluated by MTT assay against HeLa cancer cells. The HeLa cells were treated with the DOX-loaded PCL_{49}-A:U-PEG_{114} micelles and free DOX at different DOX dose from 0.2 to 4 μg/mL. The HeLa cell proliferation results are shown in Figure 10. It is found that the

loaded DOX dose required for 50% cellular growth inhibition (IC_{50}) is 1.6 μg/mL. This result demonstrates that DOX-loaded PCL_{49}-A:U-PEG_{114} micelles are able to enter the cells and produce the desired pharmacological action. On the other hand, the IC_{50} of free DOX is 0.75 μg/mL, which exhibits higher inhibition compared with DOX-loaded micelles. It is well reported by previous literatures that the free drug is more potent than the loaded drug.[30,49] The lower cytotoxicity of DOX-loaded micelles can be attributed to the time-consuming DOX release from micelles and delayed nuclear uptake in HeLa cells, which have been proved by the in vitro DOX release and internalization studies by CLSM.

CONCLUSIONS

We have successfully developed novel stimuli-responsive micelles self-assembled from supramolecular amphiphilic block copolymer PCL-A:U-PEG. This study demonstrates that PCL-A:U-PEG micelles have strong response to mildly acid pH and are capable of rapidly releasing DOX inside the cells to yield significantly enhanced drug efficacy. The resulting micelles are nontoxic. Supramolecular copolymer micelles based on multiple hydrogen bonding interactions are very appealing drug carriers, because they can potentially combine the advantages of traditional covalent-linked copolymer micelles and dynamic supramolecular properties. Hence, such stimuli-responsive micelles are very promising candidates for improvements in drug delivery systems.

ASSOCIATED CONTENT

Supporting Information. ^{13}C NMR spectrum of uracil-terminated poly(ethylene glycol) (PEG-U) in $CDCl_3$ and variable temperature Fourier transform infrared (FTIR) spectra in the 1800−1650 cm^{-1} region of adenine-terminated poly(ε-caprolactone) (PCL-A) and uracil-terminated poly(ethylene glycol) (PEG-U) complexes. This material is available free of charge via the Internet at http://pubs.acs.org.

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