



Synthesis and characterization of triple-responsive PNiPAAm-S-S-P(α N₃CL-g-alkyne) copolymers bearing cholesterol and fluorescence monitor

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ABSTRACT

Real-time monitoring and controlled release of drugs are key factors in drug delivery. In this study, versatile fluorescence triple-responsive block-graft copolymers PNiPAAm-S-S-P(α N₃CL-g-alkyne)_n with a disulfide (S–S) bond as a junction point between the hydrophilic and hydrophobic chains bearing cholesteryl- and pyrenyl- side groups were synthesized. The polymer was temperature-sensitive with a lower critical solution temperature in the range of 36 °C to 44 °C. When the polymer solution was treated with reducing agents and exposed to ultrasound, significant hydrolysis of the amide bonds was noted. The partial destruction of micelles in the presence of a reducing agent and ultrasound was observed through transmission electron microscopy. The copolymers containing cholesteryl and pyrenyl possessed highly drug loading content and entrapment efficiency. Above the lower critical solution temperature, the release rate could be greatly increased and adjusted remotely under high-intensity focused ultrasound in the presence of dithiothreitol. The nanoparticles exhibited slight toxicity against HeLa cells in the concentration range of 10–300 μ g mL^{−1}. Doxorubicin (DOX)-loaded micelles facilitated the uptake of DOX by HeLa cells and were predominantly retained in the cytoplasm.

1. Introduction

During the past decade, stimuli responsive polymers have received considerable attention because of their sensitivity to change in their environment [1, 2]. They have been applied in drug delivery, tissue engineering, bio-sensing, and separation processes. Stimuli responsive polymers are sensitive to various chemical or physical stimuli (e.g., pH, oxidation, reduction, enzymes, temperature, light, and magnetic fields). Through these stimuli, the chemical or physical structures of copolymers can be altered. This can in turn induce micelles destabilization or destruction and the release of encapsulated drugs. Compared with single or dual responsiveness, multi-responsiveness may greatly enhance the versatility of these materials to meet various requirements in practical applications. Various stimuli-responsive systems that can respond to multiple environmental changes have attracted particular attention for two reasons: (i) The multi-responsive feature can be elicited concurrently or sequentially using multiple stimuli, providing more effective spatiotemporal control. (ii) Because of behavioral changes in the biological systems often result from several environmental changes, rather than only one factor, multi-responsive materials provide an ideal artificial platform for mimicking natural biological processes [3–16].

Poly(N-isopropylacrylamide) (PNiPAAm) is water-soluble at room temperature but undergoes a transition from a hydrated coil to a dehydrated globule at its lower critical solution temperature (LCST) at approximately 32 °C, close to body temperature. PNiPAAm-containing materials are biocompatible and have been approved by the United States Food and Drug Administration for clinical use. Its copolymers have been widely used in drug delivery systems and temperature-targeted therapy materials [17–19]. Redox is a type of internal stimulus for copolymer micelles. The redox-responsive copolymer micelle containing a central S–S bond has attracted increasing attention because the S–S bond can be cleaved specifically in the reducing environment inside the cell by using redox reagents such as glutathione (GSH). Cancer cells often exhibit elevated GSH levels, this potentially enhances therapeutic efficacy and reduces drug resistance in cancer cells [20–22]. Ultrasound is a widely used medical imaging and therapeutic modality. In particular, high-intensity focused ultrasound (HIFU) can enhance the cleavage of weak chemical bonds (e.g., S–S bond, and acid-labile hydrazine bond) and thus promote drug release from the micelles, leading to enhanced therapeutic efficacy [23, 24]. In our previous report, the PNiPAAm-S-S-PXCL (X=H, CH₃, Ph) possessed the multiple stimuli-responsive properties [25].

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To realize efficient drug delivery, the fluorescence technique has been considered a convenient and fundamental method for monitoring drug release in complex intracellular environments. For fluorescence detection, fluorescence probes, such as small fluorophores and fluorescent proteins, are typically introduced into polymeric drug delivery systems [26, 27].

In this study, we linked hydrophilic block of poly(*N*-isopropylacrylamide) (PNiPAAm) to the grafted alkyne hydrophobic blocks of poly(α -azo-caprolactone) (P α N₃CL-g-alkyne)_n with the S–S bonds as junction points to form temperature/reductive/ultrasonic triple-sensitivity amphiphilic PNiPAAm-S-S-P(α N₃CL-g-alkyne)_n copolymers. Cholesterol was selected as the hydrophobic moiety because of its bio-compatibility and its potential interaction with cholesterol receptors on cell surface [28, 29]. To monitor the drug release kinetics, the fluorescent pyrene was introduced into the polymeric drug delivery systems [30]. We hypothesized that the triple-responsive block-graft PNiPAAm-S-S-P(α N₃CL-g-alkyne)_n with pendant cholesterol and pyrene would form a fluorescent targetable micellar drug delivery systems. Possible morphologies and structure changes of the drug-loaded micelles under the stimuli of temperature, HIFU, and reducing agent are presented in Scheme 1.

2. Experimental

2.1. Synthesis of PNiPAAm-S-S-P(α N₃CL)_n diblock copolymers

The polymerization reaction to create PNiPAAm₁₀-S-S-P(α N₃CL)₁₀ proceeded as follows: 2-(2-Pyridyldithio)-ethanol (Py-S-S-(CH₂)₂OH, 0.12 g, 0.64 mmol), serving as an initiator, and α -ClCL (0.95 g, 6.4 mmol) were introduced into a flask, and subsequently dissolved in 50 mL of toluene under a dry nitrogen stream. Subsequently, 39 mg of SnOct₂ (1.5 wt% based on the weight of Py-S-S-(CH₂)₂OH and α -ClCL) was added to the flask. The flask was purged using nitrogen and re-fluxed for 24 h, and the solution was vacuum-concentrated under reduced pressure. The resulting product, Py-S-S-P(α ClCL)₁₀, was dissolved in CHCl₃, and subsequently precipitated into excess *n*-hexane/diethyl ether (*v/v* 5:1) two times while being stirred. The purified polymer was dried in vacuo at 50 °C for 24 h and analyzed (1.05 g; 90% yield). M_n = 1830 g mol^{−1}, PDI = 1.21; ¹H NMR (CDCl₃) δ (ppm): 8.49, 7.61–7.70, 7.11 (*CH* of pyridinyl), 4.35 (t, CH₂O of initiator), 4.29 (m, *CH*Cl of PCL), 4.21 (t, CH₂O of PCL), 3.69 (m, CH₂OH of PCL), 3.08 (t, SCH₂ of initiator), 1.91 (m, C₆H₂ of PCL), 1.89–1.45 (m, C₇H₂, C₈H₂ of PCL). The resonance peaks were assigned to the corresponding hydrogen atoms of Py-S-S-P(α ClCL)₁₀. Subsequently, 2.74 g of Py-S-S-P(α ClCL)₁₀ (1.50 mmol, 15 equiv. of α -ClCL) was dissolved in 10 mL of DMF in a glass reactor, to which NaN₃ (18 mmol) was then added. The mixture was stirred at room temperature overnight. The insoluble salt was removed through filtration and DMF elimination in vacuo. The resulting product was dissolved in CHCl₃ and precipitated into excess *n*-hexane/ethyl ether (*v/v* 4:1) twice while being stirred. The purified polymer was dried in vacuo at 50 °C for 24 h and analyzed (2.99 g; 96% yield). M_n = 2080 g mol^{−1}, PDI = 1.24. ¹H NMR (CDCl₃) δ (ppm): 8.49, 7.61–7.70, 7.11 (*CH* of pyridinyl), 4.35 (t, CH₂O of initiator), 4.21 (t, CH₂O of PCL), 3.88 (m, CHN₃ of PCL), 3.69 (m, CH₂OH of PCL), 3.08 (t, SCH₂ of initiator), 1.91 (m, C₆H₂ of PCL), 1.89–1.45 (m, C₇H₂, C₈H₂ of PCL). The resonance peaks were assigned to the corresponding hydrogen atoms of Py-S-S-P(α N₃CL)₁₀. Py-S-S-P(α N₃CL)₁₀ (0.46 g, 0.21 mmol, 1 equiv., M_n = 2080 g mol^{−1}) was subsequently dissolved in THF (10 mL) with acetic acid (0.4 mL) and stirred at room temperature for 30 min. PNiPAAm₁₀-SH (0.44 g, 0.22 mmol, M_n = 1980 g mol^{−1}) was then added, and the mixture was stirred for 24 h at room temperature under nitrogen. The solution was subsequently precipitated into excess *n*-hexane/diethyl ether (*v/v* 5:1) two times with stirring. The purified polymer, PNiPAAm₁₀-S-S-P(α N₃CL)₁₀, was dried in vacuo at 50 °C for 24 h and analyzed (0.80 g; 85% yield). M_n = 4460 g mol^{−1}, PDI = 1.56. Figs. 1A and S1C (in the

supplementary information) show the representative ¹H NMR and IR spectra of PNiPAAm₁₀-S-S-P(α N₃CL)₁₀.

2.2. Typical click reaction

PNiPAAm₁₀-S-S-P(α N₃CL)₁₀ (580 mg, 0.13 mmol, 1.3 equiv. of azide) was transferred into a glass reactor containing 10 mL THF. Two types of alkyne, pyrenylmethyl 4-pentynoate (PyrePA, 120 mg, 0.39 mmol), and cholesteryl 4-pentynoate (CholPA, 440 mg, 0.91 mmol), were added to the reactor. Subsequently, CuI (2.5 mg, 0.013 mmol) and triethyl amine (6.5 mg, 0.065 mmol) were added. The solution was stirred at 40 °C for 24 h. The cycloaddition copolymer was precipitated in ether through stirring. Subsequently, the precipitated polymer was purified through dialysis (molecular weight cutoff [MWCO], 3.5 kDa) against THF (500 mL) for 2 days. The purified polymer, PNiPAAm₁₀-S-S-P(α N₃CL₁₀-g-PyrePA₃/CholPA₇), was dried in vacuo at 50 °C for 24 h to afford a brown solid (702 mg, yield: 78%). Figs. 1D and S1D (in the supplementary information) show the representative ¹H NMR and IR spectra of PNiPAAm₁₀-S-S-P(α N₃CL₁₀-g-PyrePA₃/CholPA₇).

2.3. LCST measurements

Optical transmittance of the aqueous polymer solution (1 mg mL^{−1}) at various temperatures was measured at 500 nm using a UV–Vis spectrophotometer (Jasco V-550, Japan). The temperature of the sample cells was regulated using a temperature controller (Jasco ETC-505T, Japan). The heating rate was set at 0.1 °C min^{−1}. The LCST values for the polymer solutions were determined at the temperatures at which the optical transmittance was 50%.

2.4. Self-assembly behavior of polymers

Polymeric micelles of PNiPAAm-S-S-P(α N₃CL-g-alkyne)_n polymers were prepared through dialysis. In brief, a solution of the PNiPAAm-S-S-P(α N₃CL-g-alkyne)_n polymer (30 mg) in DMF (5 mL) was placed in a dialysis bag with a molecular weight cutoff (MWCO) of 1.0 kDa and subsequently dialyzed against deionized water at an ambient temperature for 24 h. The water was replaced at 2 h intervals. The obtained aqueous solutions were subsequently used for analyzing critical micelle concentration (CMC), and dynamic light scattering (DLS), and performing transmission electron microscopy (TEM).

The CMCs of the copolymers were determined through fluorescence spectroscopy with pyrene as the probe. Briefly, the polymer samples were equilibrated for 10 min before measurements were taken. Aliquots of pyrene in acetone solution (6.1 × 10^{−5} M, 10 μ L) were added to glass vials and air-dried to remove the acetone. Polymer solutions of varying concentrations were added to the pyrene at 1 mL each, and left to stand for 24 h. The final pyrene concentration in each vial was 6.1 × 10^{−7} M. The fluorescence spectra were recorded using a Hitachi F-4500 fluorescence spectrometer equipped with a 20 kW xenon discharge lamp. The slit width was 10 nm, and square cells (1.0 × 1.0 cm) were used. The excitation spectra were scanned at wavelengths from 300 to 360 nm, with an emission wavelength of 395 nm at 25 °C.

The average particle size, size distribution (PD), and zeta potential of the micelles in the aqueous solution were measured using DLS. Measurements were recorded after an aqueous micellar solution (C = 0.3 g L^{−1}) was filtered by using a microfilter with an average pore size of 0.2 μ m (Advantec MFS, Inc., Dublin, CA, USA).

The morphologies of the self-assembled micelles were observed through TEM (JEM 1200-EXII, Tokyo, Japan). Drops of the micelle solution (0.3 g L^{−1}, not containing a staining agent) were placed on a carbon film coated copper grid (400 mesh) and dried at room temperature. The micelles were observed at an accelerating voltage of 100 kV.

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