

## Synthesis of Self-assemble pH-responsive Cyclodextrin Block Copolymer for Sustained Anticancer Drug Delivery<sup>\*</sup>

Bei-bei Lu<sup>a</sup>, Lu-lu Wei<sup>a</sup>, Gui-hua Meng<sup>a</sup>, Jun Hou<sup>b</sup>, Zhi-yong Liu<sup>a\*\*</sup> and Xu-hong Guo<sup>a,c</sup>

<sup>a</sup> School of Chemistry & Chemical Engineering, Shihezi University/Key Laboratory of Materials-Oriented Chemical Engineering of Xinjiang Uygur Autonomous Region/Engineering Research Center of Materials-Oriented Chemical Engineering of Xinjiang Bingtuan, Shihezi 832003, China

<sup>b</sup> Department of Immunology, Shihezi University School of Medicine/Department of Pathology and Key Laboratories for Xinjiang Endemic and Ethnic Diseases, Shihezi University School of Medicine, Shihezi 832003, China

<sup>c</sup> State Key Laboratory of Chemical Engineering, East China University of Science and Technology, Shanghai 200237, China

**Abstract** Well-defined pH-responsive poly( $\epsilon$ -caprolactone)-graft- $\beta$ -cyclodextrin-graft-poly(2-(dimethylamino)ethyl-methacrylate)-*co*-poly(ethylene glycol) methacrylate amphiphilic copolymers (PCL-*g*- $\beta$ -CD-*g*-P(DMAEMA-*co*-PEGMA)) were synthesized using a combination of atom transfer radical polymerization (ATRP), ring opening polymerization (ROP) and “click” chemistry. Successful synthesis of polymers was confirmed by Fourier transform infrared spectroscopy (FTIR), proton nuclear magnetic resonance (<sup>1</sup>H-NMR), and gel permeation chromatography (GPC). Then, the polymers could self-assemble into micelles in aqueous solution, which was demonstrated by dynamic light scattering (DLS) and transmission electron microscopy (TEM). The pH-responsive self-assembly behavior of these copolymers in water was investigated at different pH values of 7.4 and 5.0 for controlled doxorubicin (DOX) release, and these results revealed that the release rate of DOX could be effectively controlled by altering the pH, and the release of drug loading efficiency (DLE) was up to 88% (*W/W*). CCK-8 assays showed that the copolymers had low toxicity and possessed good biodegradability and biocompatibility, whereas the DOX-loaded micelles remained with high cytotoxicity for HeLa cells. Moreover, confocal laser scanning microscopy (CLSM) images revealed that polymeric micelles could actively target the tumor site and the efficient intracellular DOX release from polymeric micelles toward the tumor cells further confirmed the anti-tumor effect. The DOX-loaded micelles could easily enter the cells and produce the desired pharmacological action and minimize the side effect of free DOX. These results successfully indicated that pH-responsive polymeric micelles could be potential hydrophobic drug delivery carriers for cancer targeting therapy with sustained release.

**Keywords** pH-responsive;  $\beta$ -Cyclodextrin; Self-assembly; DOX; Controlled release; Click chemistry

### INTRODUCTION

Stimuli-responsive polymers are capable of changing their physico-chemical properties to adapt to the external stimuli<sup>[1, 2]</sup>. Such materials was potentially important for their applications in biomedical fields, such as drug delivery and biosensor<sup>[3–5]</sup>. There are many different stimuli to modulate the response of polymer systems, and they can be classified as physical and chemical stimuli. These stimuli, such as pH<sup>[6, 7]</sup>, temperature<sup>[8, 9]</sup>, light<sup>[10, 11]</sup>, and CO<sub>2</sub><sup>[12, 13]</sup>, will affect the level of various energy sources and alter molecular interactions at critical onset points. Double hydrophilic block copolymers (DHBCs) are an important class of stimuli-responsive polymers. They are made from two different hydrophilic segments, one of which was completely soluble in

<sup>\*</sup> This work was financially supported by the National Natural Science Foundation of China (No. 21367022) and Bingtuan Innovation Team in Key Areas (No. 2015BD003).

<sup>\*\*</sup> Corresponding author: Zhi-yong Liu (刘志勇), E-mail: lzyongclin@sina.com

Received January 14, 2017; Revised February 28, 2017; Accepted March 1, 2017

doi: 10.1007/s10118-017-1947-0

water, while the other can alter its physical and chemical properties to become hydrophobic when subjected to external stimuli<sup>[14, 15]</sup>. Recently, much more attention has been paid to the DHBCs with pH responsive property due to their greatly improved drug bioavailability *via* either passive targeting by the enhanced permeability and retention (EPR) effect<sup>[7, 8]</sup> and potential as environmentally friendly and biocompatible drug carriers and gene vectors<sup>[16, 17]</sup>.

Star polymers comprise several polymer arms emanating from a central core<sup>[18]</sup>. The preparation of star polymers can be mainly categorized into two strategies: “core-first” and “arm-first” methods<sup>[19, 20]</sup>. The “core-first” method allows growing arms from a multifunctional core that is capable of initiating the polymerization of monomers at multiple sites. In the “arm-first” method, the linear polymer arms are first synthesized, followed by binding them together to form star polymers. Up to now, various controlled/living polymerizations including ring opening polymerization (ROP)<sup>[21, 22]</sup>, living anionic polymerization<sup>[23, 24]</sup>, atom transfer radical polymerization (ATRP)<sup>[25, 26]</sup>, nitroxide-mediated radical polymerization (NMP)<sup>[21]</sup> and reversible addition-fragmentation chain transfer (RAFT) polymerization<sup>[27, 28]</sup> have been employed to generate the desired multiarmed macromolecular architectures. In particular, in “click” chemistry<sup>[29, 30]</sup>, this technique has become a powerful and versatile synthetic strategy because of its unique advantages, such as mild and simple reaction conditions, high efficiency and selectivity, as well as exceptional tolerance towards a wide range of functional groups and reaction conditions. “click” chemistry has also been utilized to combine with various polymerization techniques to prepare many kinds of star polymers with controlled compositions<sup>[31]</sup>.

The targeted drug-delivery systems formed through various types of intermolecular forces have attracted great interest because of their therapeutic potential in drug development and cancer treatment. Many elegant drug-delivery systems based on such non-bonding host-guest interactions have been developed. It is well-known that cyclodextrins (CDs) are a range of cyclic oligosaccharides that comprise several glucopyranose linked by  $\alpha$ -(1,4) glycosidic bonds. Because of steric effect,  $\alpha$ -,  $\beta$ - and  $\gamma$ -CD containing 6, 7 or 8 D-glucose units can be produced using genetic engineering techniques<sup>[32, 33]</sup>. And they are among the best nonimmunogenic vector candidates for self-assembly of targeted drug delivery systems<sup>[34–36]</sup> due to their unique hydrophobic cavity structure, excellent biocompatibility, chemical modifiability of hydroxyl groups in the primary face, and strong ability to entrap drug molecules through molecular recognition<sup>[32, 37]</sup>.

Over the past few years, biodegradable and biocompatible saccharide-based branched amphiphiles have attracted much attention for their applications as biomaterials, and “click” chemistry is a good choice to synthesize well-defined functional polymers. Yuan *et al.*<sup>[38]</sup> prepared amphiphilic centipede-like brush copolymers with poly( $\epsilon$ -caprolactone) (PCL) and poly(ethyl ethylene phosphate) side segments *via* combination of ROP and “click” chemistry by one-pot synthesis strategy. The polymer brush could self-assemble into stable aggregates. Chen and Liu<sup>[39]</sup> synthesized a well-defined amphiphilic poly(3-caprolactone)-block-poly(glycidyl methacrylate)-graft-7-propinyloxy coumarin-block-poly(poly(ethyleneglycol)methyl ether methacrylate) (PCL-*b*-[PGMA-*g*-PC]-*b*-P(PEGMA)) with fluorescence units through ROP, RAFT and “click” chemistry, and micelles obtained from the self-assembly of amphiphilic copolymers have been used as a nano-reservoir for controlled release of doxorubicin (DOX) for bladder cancer therapy. He *et al.*<sup>[40]</sup> synthesized  $\beta$ -CD-PLA-mPEG copolymer by “click” reaction of  $\beta$ -CD-N<sub>3</sub> and alkyne-terminated  $\beta$ -PLA-mPEG. The  $\beta$ -CD-PLA-mPEG copolymer could self-assemble in aqueous solution to form micelles loaded with the hydrophobic drug (IND). Cytotoxicity results demonstrated that  $\beta$ -CD-PLA-mPEG copolymer had low toxicity to cells. Yin *et al.*<sup>[41]</sup> searched a new targeting therapy for membrane estrogen receptor-positive breast cancer. An estrogen-anchored cyclodextrin encapsulating a doxorubicin derivative Ada-DOX (CDE1-Ada-DOX) had been synthesized and evaluated in human breast cancer MCF-7 cells. It was demonstrated that a targeted therapeutic delivery of CDE1-Ada-DOX to breast cancer cells took place in a controlled manner and that the drug vector CDE1 could be potentially employed as a molecular tool to differentiate nongenomic from genomic mechanism. Poly(2-(*N,N*-dimethylamino)ethyl methacrylate) (PDMAEMA) is a weak base with  $pK_a$  at about 7.0. Under the  $pK_a$ , PDMAEMA is hydrophilic as its amine groups are protonated, while above  $pK_a$ , it is hydrophobic as its amine groups are deprotonated. As a result, PDMAEMA-based copolymers have been developed to achieve reduced

toxicity and enhanced transfection activity. Liu *et al.*<sup>[42]</sup> synthesized novel reducible and degradable brushed PDMAEMA derivatives with a relatively high molecular weight by ATRP and reduction-sensitive disulfide bonds and found them effective as gene vectors with an excellent cytocompatibility.

In recent years, cancer therapy has become increasingly significant. With the development of science and technology, several effective antitumor drugs have been developed. As a widely used chemotherapeutic drug of anthracycline, doxorubicin (DOX) has attracted numerous attentions. However, its application in clinical treatment was seriously limited due to the high toxicity to heart and liver tissues<sup>[43]</sup>. In addition, the poor water-solubility of DOX also led to a low bioavailability. In order to overcome these shortcomings, many efforts have been made to improve the therapeutic efficacy of DOX, among which the polymer-drug conjugate would be one of the most promising species. The polymeric micelles were self-assembled from amphiphilic block copolymers, which had hydrophobic core to encapsulate some poorly water-soluble drugs and hydrophilic shell to stabilize micelles, and hold enormous potential to increase the drug efficacy by means of reducing toxicity, improving water solubility, prolonging circulation time in blood and enhancing uptake by tumors<sup>[44]</sup>.

In this work, we have synthesized pH-responsive PCL-*g*- $\beta$ -CD-*g*-P(DMAEMA-*co*-PEGMA) amphiphilic copolymer by ROP, ATRP, and “click” chemistry with different PCL segments, DMAEMA and PEGMA as monomers. The star-shaped polymers could self-assemble into spherical nano-micelles in water. Meanwhile, the particle size, polydispersity index (PDI), zeta potential of the polymer micelles were evaluated by DLS and TEM and the size of the spherical micelles was about 60 nm. The reversible transformation of transparency and turbidity was proved for their stability and prolonged cycle length by DLS and UV-Vis. Moreover, the cytotoxicity tests revealed that these polymeric micelles possessed good biodegradability and biocompatibility for the HeLa cells and the DOX loaded micelles were taken up by the nuclei of cells *via* endocytosis processed by CLSM. In addition, stimuli-responsive polymeric micelles could be a potential hydrophobic drug delivery carrier for cancer targeting therapy with sustained release.

## EXPERIMENTAL

### Materials

$\beta$ -Cyclodextrin ( $\beta$ -CD, > 98%, purified by recrystallization from water twice prior to use) was purchased from Aladdin. NaOH was purchased from Fuyu Chemical Reagent Co. and used as received. Potassium iodide (KI) was supplied by Tianjin sheng'ao the chemical reagent Co., Ltd. 3-Butyn-1-ol, *p*-toluenesulfonyl chloride (TsCl) and sodium azide (NaN<sub>3</sub>) were purchased from Aladdin. DMAEMA and PEGMA ( $M_n = 500 \text{ g}\cdot\text{mol}^{-1}$ ) (Aldrich) were passed through a column of activated basic alumina to remove the inhibitors.  $\varepsilon$ -CL (Sigma-Aldrich) was distilled under reduced pressure after being treated with CaH<sub>2</sub>. Tin 2-ethylhexanoate (Sn(Oct)<sub>2</sub>, Aldrich) was distilled under reduced pressure. 2-Bromoisobutyl bromide (BIBB, Aldrich) and *N,N,N',N',N''*-pentamethyldiethylenetriamine (PMDETA) were purchased from Sigma-Aldrich. Copper(I) bromide (99.999%, Alfa Aesar) was used without further purification. Dimethylformamide (DMF), dichloromethane (CH<sub>2</sub>Cl<sub>2</sub>), toluene and triethylamine (TEA) were dried by refluxing over CaH<sub>2</sub> and distilled just before use. HeLa cells (Institute of Cells, CAS, Shanghai) were used as received. Cell Counting Kit-8 (CCK-8, Shanghai, Beyotime Biotechnology), Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), pancreatic enzymes were obtained from biological industries. 4',6-Diamidino-2-phenylindole (DAPI), 4% paraformaldehyde and TritonX-100 were purchased from Solarbio. Doxorubicin hydrochloride (DOX·HCl) was purchased from Beijing HuaFeng United Technology Co., Ltd.

### Characterization

Fourier transform infrared (FTIR) spectroscopy analysis was measured by using Nicolet Avatar 360 spectrophotometer with KBr pellets. <sup>1</sup>H-NMR data were obtained by nuclear magnetic resonance spectroscopy (NMR) using a Bruker DMX-500 NMR spectrometer with DMSO-d<sub>6</sub> or CDCl<sub>3</sub> as solvent. The molecular weight and molecular weight distribution of copolymers were measured by gel permeation chromatography (GPC) using a Viscotek TDA 302 gel permeation chromatograph and THF was used as an eluent. The transmittances of copolymers aqueous solutions at various temperatures were measured at a wavelength of 500 nm on a UV-

Visible spectrophotometer (UV-Vis). The LCST value of the copolymer solution was defined as the temperature producing a 50% decrease in transmittance. Dynamic light scattering (DLS) measurements were performed by using a BECKMAN COULTER Delasa Nano C particle analyzer at a fixed angle of 165°. Before the light scattering measurements, the sample solutions were filtered three times by using Millipore Teflon (Nylon) filters with a pore size of 0.45  $\mu\text{m}$ . All measurements were repeated three times, and the average results were adopted as the final hydrodynamic diameter ( $D_h$ ). The transmission electron microscopy (TEM) images were recorded using an H-600 transmission electron microscope (Hitachi, Japan) operating at 120 kV. Confocal laser scanning microscopy (CLSM) images were recorded using Zeiss CLSM510 microscope.

### **Synthesis of PCL-g- $\beta$ -CD-g-P(DMAEMA-co-PEGMA) with Star Amphiphilic Polymers**

#### *Synthesis of $\beta$ -CD- $N_3$*

First, mono-6-deoxy-6-(*p*-tolylsulfonyl)- $\beta$ -cyclodextrin ( $\beta$ -CD-OTs)<sup>[45]</sup> was synthesized.  $\beta$ -Cyclodextrin (21.0 g, 18.5 mmol) was dissolved in 150 mL of water solution which was prepared by dissolving 1.867 g of sodium hydroxide (NaOH) in 10 mL of water. The mixture was cooled to 0 °C and stirred at the same temperature in an ice-water bath while *p*-toluenesulfonyl chloride (TsCl) (3.167 g, 16.6 mmol) was being added in small portions. The reaction mixture was stirred vigorously for 7 h at 0 °C. The reaction mixture was filtered to separate unreacted *p*-toluenesulfonyl chloride, and then filtered. The product was dried to constant weight in a vacuum oven to yield a white solid of  $\beta$ -CD-OTs. Further, the white solid was recrystallized three times by dissolving it in 30 mL of boiling water and then cooling to room temperature.

Subsequently, a mixture of  $\beta$ -CD-OTs (3.0 g, 2.33 mmol), sodium azide (1.52 g, 23.4 mmol) and KI (0.193 g, 1.16 mmol) in water/DMF (1/1, *V/V*) was stirred at 60 °C for 24 h. The resulting solution was then concentrated to half of its volume and cooled to room temperature. Excess acetone was added to precipitate. Obtained white solid was redissolved in 10 mL water and reprecipitated in excess acetone. This process was repeated three times. The white solid was dried under vacuum at 50 °C.

#### *Synthesis of PCL-g- $\beta$ -CD- $N_3$*

In order to obtain PCL-g- $\beta$ -CD- $N_3$  with varied hydrophobic segments, different feed ratios of  $\epsilon$ -CL were successfully applied in ROP with Sn(Oct)<sub>2</sub> as catalyst. An example for typical procedure was as follows:  $\beta$ -CD- $N_3$  (1.176 g, 1 mmol),  $\epsilon$ -CL (8.0 g, 70 mmol), Sn(Oct)<sub>2</sub> (0.04 g, 0.1 mmol), and anhydrous toluene (1 mL) were added into a fresh flamed and nitrogen purged round-bottomed flask and the flask was then placed in an oil bath maintained at 120 °C for 24 h. After polymerization, the mixture was cooled to room temperature. The product was dissolved in dichloromethane, and precipitated three times in methanol. Finally, the precipitate was collected and dried under vacuum to a constant weight at 35 °C.

#### *Synthesis of 3-butyn-1-Br initiator*

A solution of 3-butyn-1-ol (1.402 g, 0.02 mmol) and triethylamine (3 mL) in 30 mL of CH<sub>2</sub>Cl<sub>2</sub> was stirred for 0.5 h under nitrogen atmosphere in a 100 mL three-necked flask, and then the flask was placed in an ice/water bath. 2.0 mL of 2-bromoisobutyl bromide was added dropwise through constant pressure funnel over 2 h, and the reaction mixture was stirred for 48 h at 30 °C. The precipitate was filtered off. Then, the filtrate was washed three times sequentially with an aqueous solution of sodium bicarbonate and water. The product was further concentrated by a rotary evaporator, and precipitated three times in diethyl ether, and dried under vacuum to a constant weight at 35 °C.

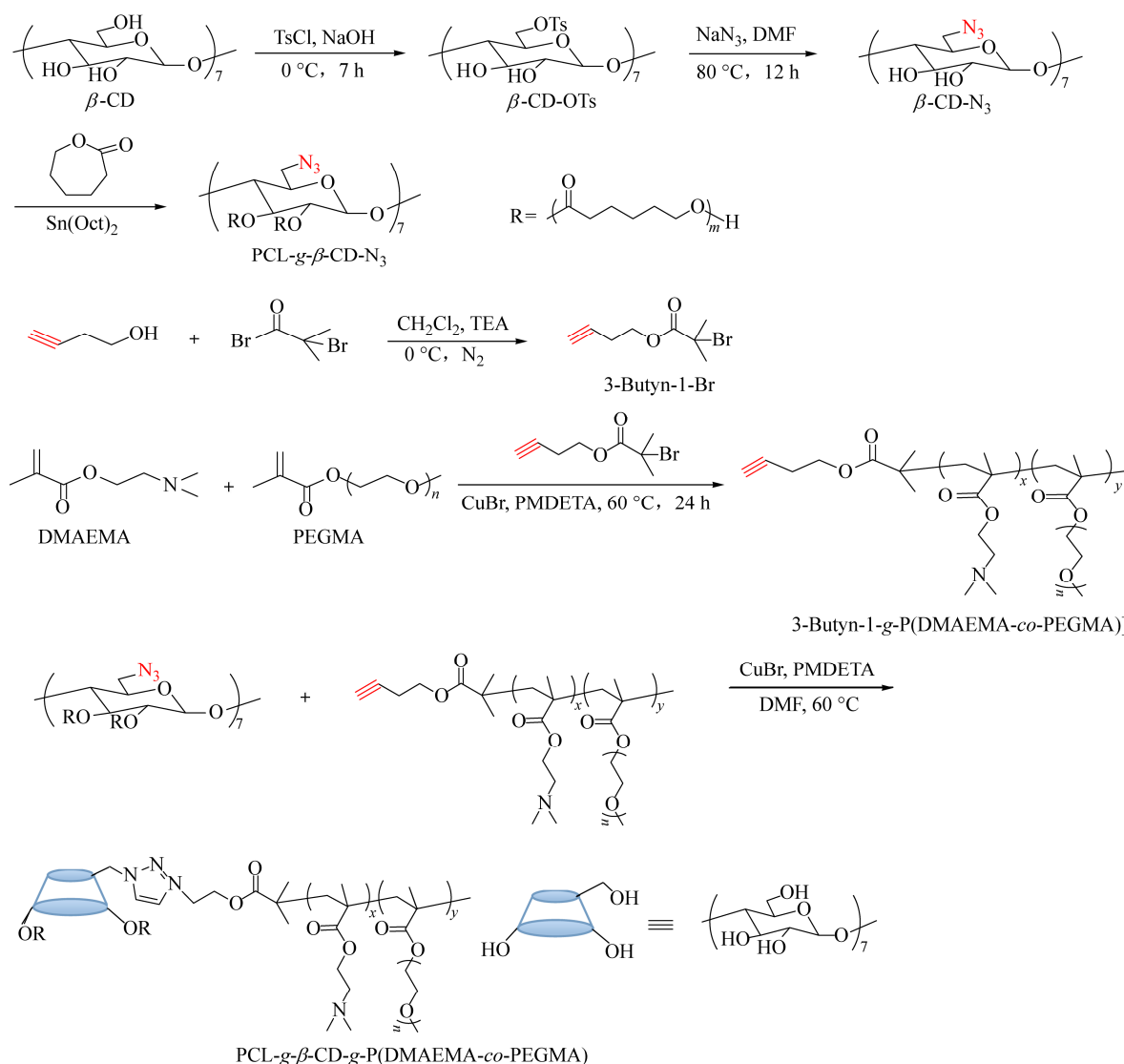
#### *Synthesis of 3-butyn-1-g-P(DMAEMA-co-PEGMA) by ATRP*

3-Butyn-1-g-P(DMAEMA-co-PEGMA) was prepared by ATRP of DMAEMA and PEGMA using 3-butyn-1-Br as an initiator and CuBr/PMDETA as catalyst. 3-Butyn-1-Br (0.076 g, 0.64 mmol), DMAEMA (3.0 g, 19.2 mmol), PEGMA (2.9 g, 9.6 mmol), CuBr (0.092 g, 0.64 mmol), PMDETA (0.337 g, 2 mmol) and DMF (20 mL) were added in a flask and the flask was degassed with three freeze-evacuate-thaw cycles. Then, the polymerization was performed at 60 °C for 24 h. After being cooled to room temperature, the reaction flask was opened to air, and diluted by DMF, and purified by dialysis against water to remove unreacted reactants using a

membrane with molecular weight cut-off of 500 Da.

*Synthesis of PCL-g- $\beta$ -CD-g-P(DMAEMA-co-PEGMA) by “click” chemistry*

The synthetic pathway is shown in Scheme 1. PCL-g- $\beta$ -CD-N<sub>3</sub> (1.0 g, 0.057 mmol), CuBr (0.151 g, 1.0 mmol), and 3-butyn-1-g-P(DMAEMA-co-PEGMA) (0.1 g, 0.40 mmol) were purged with nitrogen to remove the dissolved oxygen. PMDETA (0.168 g, 1 mmol) was added and degassed with three freeze-evacuate-thaw cycles. Then the flask was sealed and stirred at 60 °C for 24 h. The reaction mixture was exposed to air, and diluted by DMF, and purified by dialysis against water to remove unreacted reactants using a membrane with molecular weight cut-off of 500 Da. The resultant polymer PCL-g- $\beta$ -CD-g-P(DMAEMA-co-PEGMA) samples were collected by freeze-drying, which were named PCDP1, PCDP2, PCDP3 and PCDP4.



**Scheme 1** Synthesis of pH-responsive star-shaped PCL-g- $\beta$ -CD-g-P(DMAEMA-co-PEGMA) polymers

**DOX-loading and in Vitro Drug Release Assay**

For encapsulation, 100 mg of PCDP1/PCDP2 and 10 mg of DOX·HCl, were dissolved in 2 mL of DMF separately and the two solutions were mixed in a vial and stirred for 60 min. Then, a 3-fold excess of TEA was added in 2 mL of DMF overnight to obtain DOX base. Then the mixture was added dropwise using a syringe

pump to water (30 mL) under high-speed stirring. The DOX-containing suspension was then equilibrated under stirring at room temperature for 4 h, followed by thorough dialysis (molecular weight cut-off: 500 Da) against deionized water for 48 h to remove unloaded DOX, and they were named D-PCDP1 and D-PCDP2.

The DOX loading content (LC) and entrapment efficiency (EE) were determined by UV-Vis spectrophotometry at 480 nm. To determine the drug loading level, a small portion of DOX-loaded micelles was withdrawn and diluted with DMF to a volume ratio of DMF/H<sub>2</sub>O = 9/1 (*V/V*). The amount of DOX encapsulated was quantitatively determined by a UV-Vis spectrophotometer and the calibration curve used for drug loading characterization was established by the intensity of DOX with different concentrations in solutions. The DLC is defined as the weight ratio of entrapped DOX to the DOX-loaded micelles. The DLE of DOX was obtained as the weight ratio between DOX incorporated in assembled micelles and that used in fabrication.

$$\text{DLC (wt\%)} = \frac{\text{weight of loaded drug}}{\text{weight of polymer}} \times 100 \quad (1)$$

$$\text{DLE (wt\%)} = \frac{\text{weight of loaded drug}}{\text{weight of drug in feed}} \times 100 \quad (2)$$

The *in vitro* DOX release profiles from the PCDP1/PCDP2 assembled micelles were evaluated using buffers phosphate buffer saline (PBS, 10 mmol·L<sup>-1</sup>) solution with pH value 7.4. In each experiment, 4 mL of DOX-loaded nanoparticle solution was transferred into a dialysis bag (molecular weight cut-off: 500 Da), and immersed into a tube containing 60 mL of buffer solution and shaken (200 r·min<sup>-1</sup>) at 37 °C. At specified time intervals 4 mL (*V<sub>e</sub>*) of samples were taken and an equal volume of fresh buffer was added to maintain the total volume. The concentration of DOX in different samples was analyzed by UV-Vis spectrophotometry at 480 nm. The cumulative percent drug release (*E<sub>r</sub>*) was calculated using

$$E_r(\%) = \frac{V_e \sum_{i=1}^{n-1} c_i + V_0 c_n}{m_{\text{DOX}}} \times 100 \quad (3)$$

where *m<sub>DOX</sub>* represents the amount of DOX in the micelle, *V<sub>0</sub>* is the volume of the release medium (*V<sub>0</sub>* = 60 mL), *c<sub>i</sub>* represents the concentration of DOX in the *i*<sup>th</sup> sample and *c<sub>n</sub>* represents the concentration of DOX in the *n*<sup>th</sup> sample.

### ***In Vitro Cytotoxicity***

The cytotoxic effects of polymers, free DOX and DOX-loaded polymer micelles against HeLa cells were evaluated by the standard Cell Counting Kit-8 (CCK-8). To perform cytotoxicity assay, HeLa cells were seeded at a density of 5000 cells per well on a 96-well plate and cultured for 24 h. The samples were prepared in a series of desired concentrations. Experimental wells were treated with the samples for 24 h while others were added with fresh medium as control. After 48 h incubation, 10 μL of the CCK-8 stock solution was added into each well. After incubation for another 1 h, the absorbance of each well was measured at a test wavelength of 450 nm. The cell viability of samples was calculated as follow<sup>[8]</sup>:

$$\text{Cell viability (\%)} = \frac{A_{\text{test}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \times 100 \quad (4)$$

where *A<sub>test</sub>* and *A<sub>control</sub>* represent the intensity determined for cells treated with different samples and for control cells, respectively, and *A<sub>blank</sub>* is the absorbance of wells without cells.

### ***Cellular Uptake***

The cellular uptake of drug loaded micelles and free DOX for 0.5, 4, and 24 h in HeLa cells was detected using confocal laser scanning microscopy (CLSM). Firstly, the HeLa cells were seeded in a glass base dish with a coverslip at a density of 5000 cells and cultured in DMEM supplemented with 10% FBS for 24 h. Then DOX-loaded micelles and free DOX were added, and cells were cultured in medium for 0.5, 4, and 24 h at 37 °C under 5% CO<sub>2</sub> atmosphere of incubation, and the cells were washed with PBS and stained with DAPI (10 mg·L<sup>-1</sup>) for

0.5 h. Afterwards, the culture medium was replaced with a DMEM medium containing DOX-loaded micelles. Finally, the location of intracellular fluorescence was validated using a CLSM imaging system at the excitation wavelength of 480 nm.

### Statistical Analysis

All the measurements were performed in triplicate and the data are presented as mean  $\pm$  standard deviation ( $n = 3$ ).

## RESULTS AND DISCUSSION

### Synthesis and Characterization of PCL-g- $\beta$ -CD-g-P(DMAEMA-co-PEGMA)

The whole experiment process is illustrated in Scheme 1. PCL-g- $\beta$ -CD-g-P(DMAEMA-co-PEGMA) was synthesized with different hydrophobic segments by ring-opening polymerization (ROP) and “click” chemistry. The FTIR spectra of the  $\beta$ -CD,  $\beta$ -CD-N<sub>3</sub>, PCL-g- $\beta$ -CD-N<sub>3</sub>, and PCL-g- $\beta$ -CD-g-P(DMAEMA-co-PEGMA) are shown in Fig. 1. The intensive absorption peak at 1730 cm<sup>-1</sup> of Figs. 1(c) and 1(d) are assigned to the carbonyl band of PCL in comparison with  $\beta$ -CD-N<sub>3</sub> (Fig. 1b). Moreover, the success of the “click” reaction can also be confirmed. It is clearly observed that the absorption peak at 2120 cm<sup>-1</sup> attributed to the azide group in Fig. 1(b) disappeared after the “click” reaction in Fig. 1(d). Accordingly, these results demonstrate that the “click” reaction was successfully achieved.

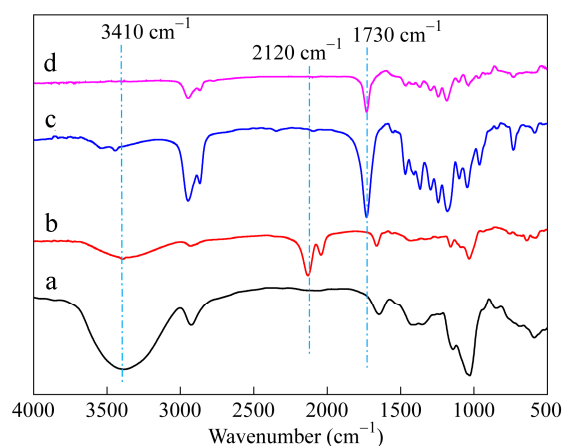


**Fig. 1** FTIR spectra of  $\beta$ -CD (a),  $\beta$ -CD-N<sub>3</sub> (b), PCL-g- $\beta$ -CD-N<sub>3</sub> (c) and PCL-g- $\beta$ -CD-g-P(DMAEMA-co-PEGMA) (d)

As shown in Fig. 2(A), the chemical shifts  $\delta = 3.19$ – $3.42$  (b and d),  $3.56$ – $3.67$  (CH<sub>2</sub>-N<sub>3</sub>, c, e),  $4.44$  (f),  $4.86$  (a),  $5.66$  (–OH, c) and  $5.70$  (–OH, b) are observed, meaning that the  $\beta$ -CD-N<sub>3</sub> was successfully synthesized. The resonance at  $\delta = 4.07$  (d),  $2.32$  (a),  $1.67$  (b), and  $1.39$  (c) are the characteristic signals of the methylene protons at different positions respectively of PCL group (Fig. 2B). The corresponding signals of all the protons are assigned in Fig. 2(C) with the typical signals listed as follows:  $\delta = 2.60$  (CH $\equiv$ C–, a) and  $2.02$  (–CH<sub>3</sub>, d). In Fig. 2(D), signals at  $\delta = 2.25$  (–N(CH<sub>3</sub>)<sub>2</sub>, i),  $2.59$  (–CH<sub>2</sub>NCH<sub>3</sub>, h) ascribed to the DMAEMA units and signals at  $\delta = 1.86$  (–CH<sub>2</sub>–, e),  $3.66$  (–CH<sub>2</sub>O–, k),  $3.38$  (–OCH<sub>3</sub>, l) assigned to the PEGMA units confirm that the block copolymer has been produced. The structural characteristics of the obtained star-shaped PCL-g- $\beta$ -CD-g-P(DMAEMA-co-PEGMA) have been determined by <sup>1</sup>H-NMR analysis in Fig. 2(E). The detailed assignments reveal all the signals are ascribed to the protons from the desired chemical structures. Specially, the appearance of a new peak at  $\delta = 7.60$  corresponds to the proton of the 1,2,3-triazole group. In addition, according to the FTIR spectrum shown in Fig. 1(d), the absorption at 2120 cm<sup>-1</sup> of the azide group completely disappeared, demonstrating the successful CuAAC “click” reaction. The chemical structure, composition and molecular weight of the copolymer were characterized by <sup>1</sup>H-NMR and GPC measurements, and these results are shown in Fig. 3 and Table 1.

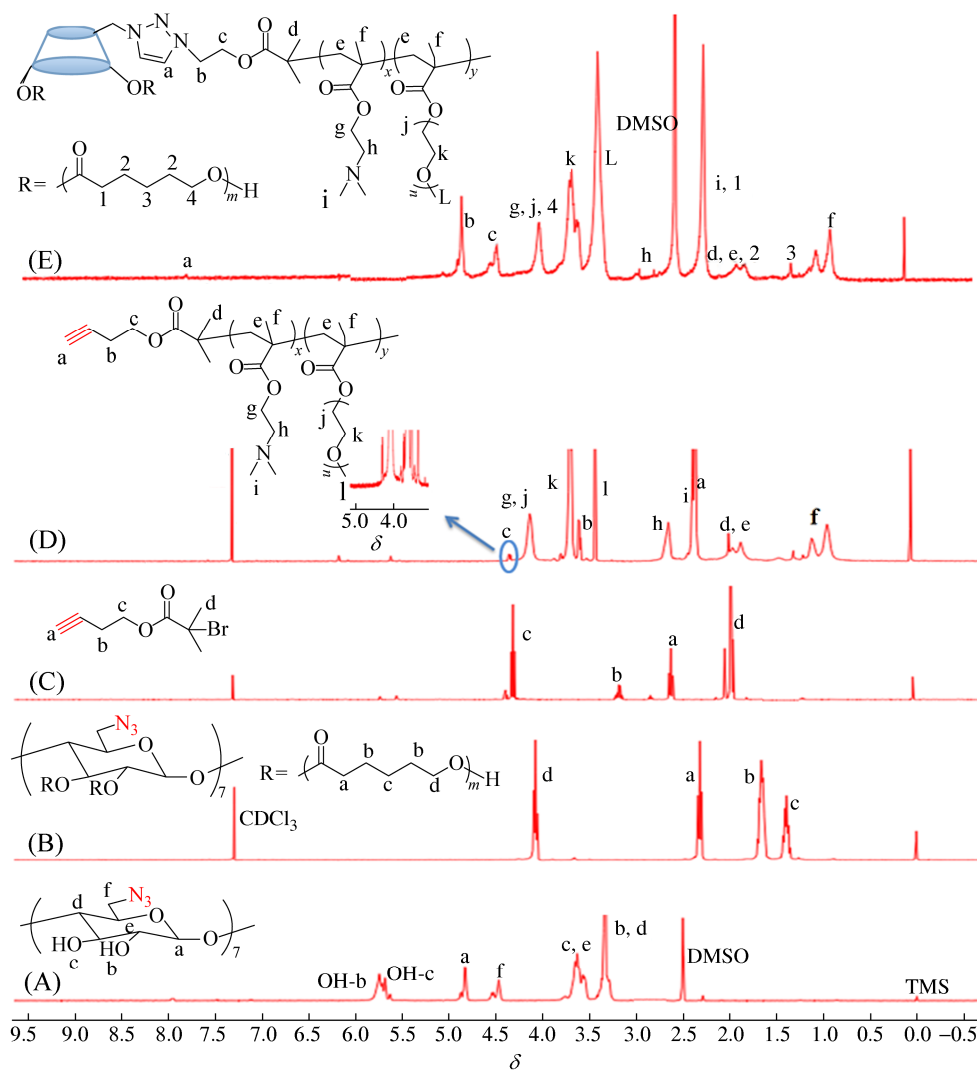


**Fig. 2**  $^1\text{H}$ -NMR spectra of  $\beta\text{-CD-N}_3$  (A), PCL-g- $\beta\text{-CD-N}_3$  (B), 3-butyne-1-Br (C), 3-butyne-1-g-P(DMAEMA-co-PEGMA) (D) and PCL-g- $\beta\text{-CD-g-P(DMAEMA-co-PEGMA)$  (E)

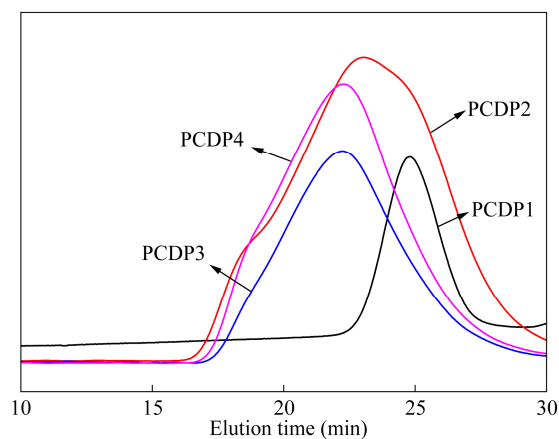


**Fig. 3** Evolution of GPC chromatograms of PCL-g- $\beta\text{-CD-g-P(DMAEMA-co-PEGMA)$  star-shaped copolymers with different molecular weights

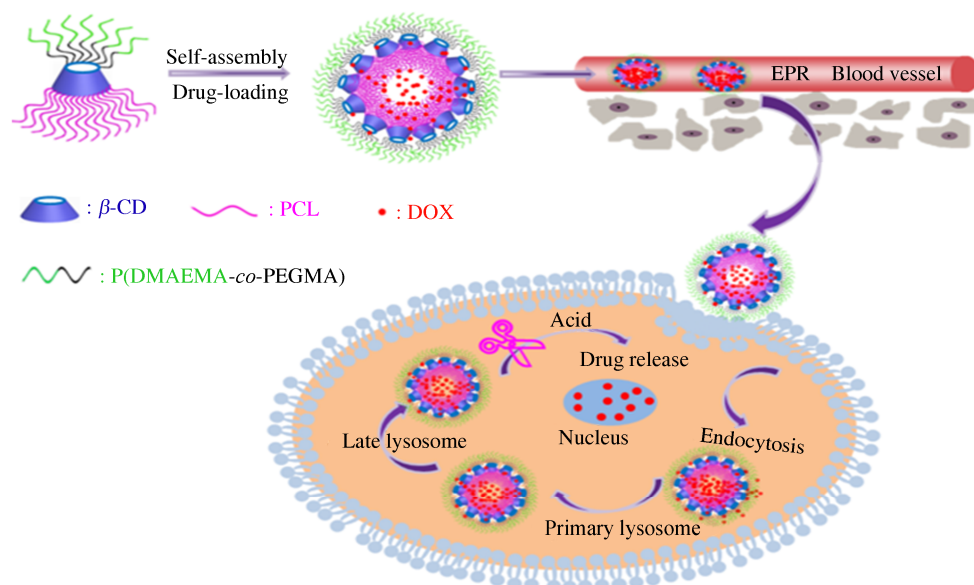
**Table 1** Characterization of PCL-*g*- $\beta$ -CD-*g*-P(DMAEMA-*co*-PEGMA) copolymers

| Samples <sup>a</sup> | [ $\beta$ -CD-N <sub>3</sub> ]/[PCL] (mol%/mol%) | $M_{n, GPC} \times 10^{-4}$ <sup>b</sup> (g·mol <sup>-1</sup> ) | $M_{n, NMR} \times 10^{-4}$ <sup>c</sup> (g·mol <sup>-1</sup> ) | PDI <sup>b</sup> |
|----------------------|--------------------------------------------------|-----------------------------------------------------------------|-----------------------------------------------------------------|------------------|
| PCDP1                | 1/5                                              | 5.83                                                            | 6.05                                                            | 1.28             |
| PCDP2                | 1/10                                             | 7.32                                                            | 7.15                                                            | 1.51             |
| PCDP3                | 1/15                                             | 8.67                                                            | 8.72                                                            | 1.42             |
| PCDP4                | 1/20                                             | 9.26                                                            | 9.50                                                            | 1.35             |

<sup>a</sup> Polymerization conditions: [monomer]<sub>0</sub>/[3-butyne-1-Br]<sub>0</sub>/[CuBr]<sub>0</sub>/[PMDETA]<sub>0</sub> = 100/1/1/2 (molar ratio); <sup>b</sup> Measured by GPC calibrated with PS standards with DMF used as eluent; <sup>c</sup> Calculated from <sup>1</sup>H-NMR data

### Formation and Characterization of the Blank and DOX-loaded D-PCDP1 and D-PCDP2 Micelles

As shown in Scheme 2, as an amphiphilic polymer, when the concentration was above the critical micelle concentration (CMC), PCL-*g*- $\beta$ -CD-*g*-P(DMAEMA-*co*-PEGMA) could self-assemble into micelles in selective solvent. The hydrophilic P(DMAEMA-*co*-PEGMA) arm chains were mainly in the corona of the micelles, whereas the hydrophobic  $\beta$ -CD side chains in the star-shaped polymer were mainly in the core of the micelles. The hydrophobic  $\beta$ -CD have been extensively used for drug delivery system because of the larger core space. DOX was physically incorporated into blank micelles PCDP1 and PCDP2, producing loaded ones named as D-PCDP1 and D-PCDP2. The physico-chemical properties of the blank and DOX-loaded micelles were characterized by DLS analysis.



**Scheme 2** Illustration of pH-responsive self-assembly of the amphiphilic copolymer of PCL-*g*- $\beta$ -CD-*g*-P(DMAEMA-*co*-PEGMA) for the efficient intracellular release of anti-cancer drugs triggered by the acidic microenvironment inside the tumor tissue

The hydrodynamic diameter, polydispersity index (PDI), and zeta potential of micelles were measured by DLS, TEM and are shown in Figs. 4 and 5 and Table 2. It can be seen that blank micelles and D-PCDP micelles presented a nanoparticle size with a narrow particle size distribution. When DOX was loaded into micelles in the core and adsorbed on the surface, the zeta potentials of the drug-loaded micelles were lower than those of blank micelles, resulting from decreased charge density due to larger particle sizes (shown in the Table 2). TEM images shown in Fig. 5 depict the morphologies of the micelles produced from PCDP1 and PCDP2 at 30 °C and confirm their correspondence to spherical particles in aqueous solution. When the temperature surpassed their corresponding LCST, the micelles started to associate and form spherical nanoparticles due to the dehydration of P(DMAEMA-*co*-PEGMA) corona, which should be the hydrophobic  $\beta$ -CD cores stabilized by pH-responsive P(DMAEMA-*co*-PEGMA) coronas. At 30 °C, TEM images showed the relative micelle diameters of about 40

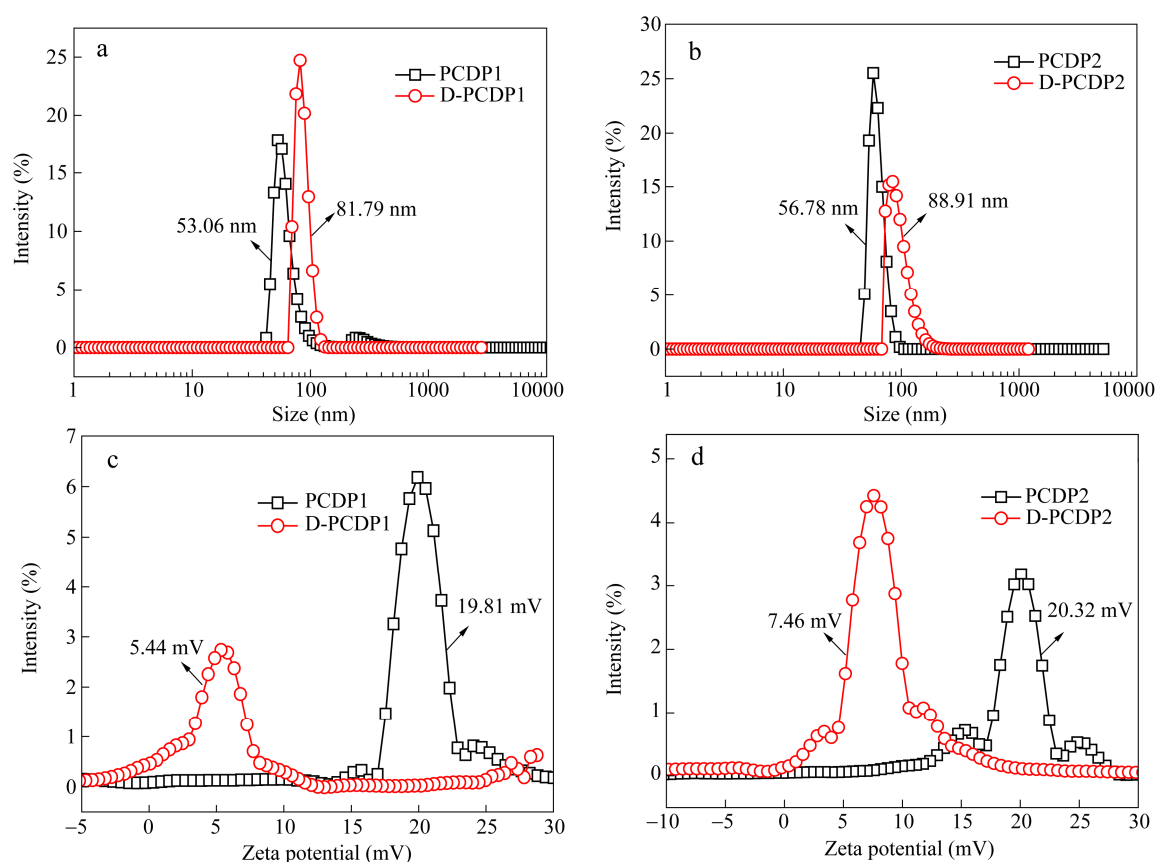


**Fig. 4** The particle size distribution curves of PCDP1 and D-PCDP1(a) and PCDP2 and D-PCDP2 (b); Zeta potentials of PCDP1 and D-PCDP1 (c) and PCDP2 and D-PCDP2 (d)

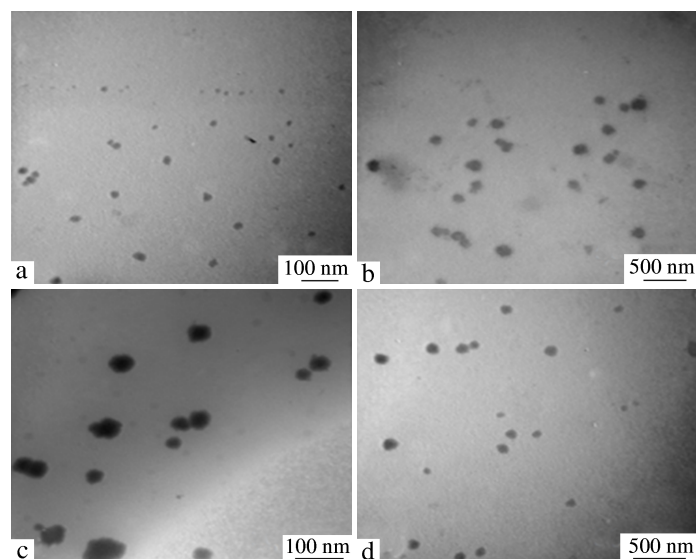


**Fig. 5** TEM images of polymer micelles PCDP1 (a, b) and PCDP2 (c, d)

and 50 nm in Figs. 5(a) and 5(c) (bar = 100 nm), and DLS showed those values of approximately 53 and 56 nm in Fig. 4. The change tendency of diameter values was consistent with DLS result while the diameter of the spheres measured by DLS was larger than that in TEM images. This was because the DLS results directly

reflected the dimension of micelles in solution, whereas TEM images corresponded to micelles under dry state.

**Table 2** The average particle sizes, polydispersity index (PDI), zeta potentials of the blank and DOX-loaded micelles

| Micelle | Blank      |       |           | DOX-load   |       |           |           |           |
|---------|------------|-------|-----------|------------|-------|-----------|-----------|-----------|
|         | $D_h$ (nm) | PDI   | Zeta (mV) | $D_h$ (nm) | PDI   | Zeta (mV) | DLC (wt%) | DLE (wt%) |
| PCDP1   | 53.06      | 0.179 | 19.81     | 81.79      | 0.154 | 5.44      | 5.27      | 52.7      |
| PCDP2   | 56.78      | 0.186 | 20.32     | 88.91      | 0.209 | 7.46      | 5.64      | 56.4      |

Stability assay in terms of transmittance and particle size of the PCDP1 and PCDP2 micelles was investigated by DLS and UV-Vis, and the polymer micelles presented reversible transformation of transparency and turbidity during the alternating cooling and heating cycles as shown in Fig. 6(a). It was clearly pointed out that the phase transition of micelles was reversible, which offered protection for drugs from untimely structure disintegration and premature drug release until reaching a disease site and demonstrated that the micelles were stable. There was no obvious change of the particle size for PCDP1 and PCDP2 micelles in water during 10 days (Fig. 6b), indicating that the micelles had a good long-term stability without precipitation or phase separation. These results revealed that polymers could be used as a drug delivery and release.

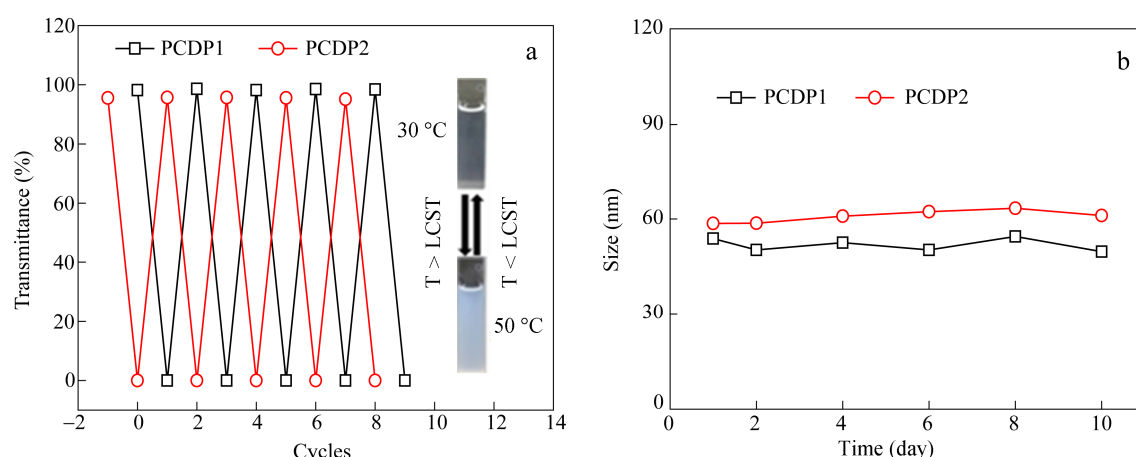


**Fig. 6** Plots of transmittance as a function of temperature for polymer micelle (a), particle size of the PCDP1 and PCDP2 micelles at room temperature (b)

### Cytotoxicity Test

To evaluate the cell viability of the DOX-loaded micelles and the blank micelles, CCK-8 assays were used. Cytotoxicity is one of the critical issues in the design of gene or drug carriers. *In vitro* cytotoxicity test for PCL- $\beta$ -CD-g-P(DMAEMA-*co*-PEGMA) (PCDP1 and PCDP2) micelles showed that the cells possessed excellent viability at high concentrations. It was demonstrated that the cell viability still remained about 95% after 48 h of incubation as shown in Fig. 7. This may be attributed to the biocompatibility imparted by brush P(DMAEMA-*co*-PEGMA) segments. Moreover, the growth inhibition test of HeLa cells was performed to evaluate the antitumor activity of polymer micelles and results showed that the polymer micelles could keep the drug activity to effectively inhibit the growth of cancer cells as well as reduce the toxicity of drug.

The drug-loaded polymer micelles were further investigated to evaluate the potential therapeutic efficacy. The *in vitro* cytotoxicity of DOX-loaded PCL- $\beta$ -CD-g-P(DMAEMA-*co*-PEGMA) (D-PCDP1 and D-PCDP2) micelles for 48 h was compared with that of free DOX determined by CCK-8 assay against HeLa cells. To analyze the activity of DOX-loaded nanoparticles, HeLa cells were cultured in the solutions of free DOX and DOX-loaded micelles at different DOX concentrations ranging from 0.125  $\mu\text{g}\cdot\text{mL}^{-1}$  to 2  $\mu\text{g}\cdot\text{mL}^{-1}$  for 48 h, respectively. As shown in Fig. 8, the viability of HeLa cells depends on DOX concentration. It could be found that DOX-loaded micelles displayed potentials for inhibition of HeLa cell proliferation similar to free DOX. The

inhibition of cancer cell growth was attributed to the intracellular DOX released from the DOX-loaded micelles and final entry into the nuclei of HeLa cells. Moreover, the DOX-loaded polymer micelles and free DOX doses required for 50% cellular growth inhibition ( $IC_{50}$ ) were 0.628, 0.871 and  $1.039 \mu\text{g}\cdot\text{mL}^{-1}$  respectively. These results showed that polymeric micelles had little toxicity.

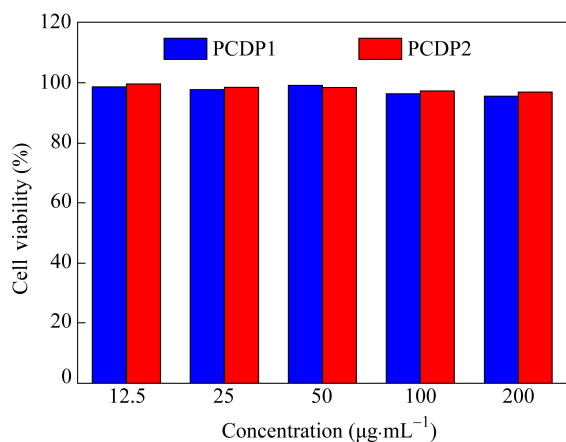


**Fig. 7** *In vitro* cell viability of HeLa cells treated with PCDP1 or PCDP2 at different concentrations after incubation of 48 h

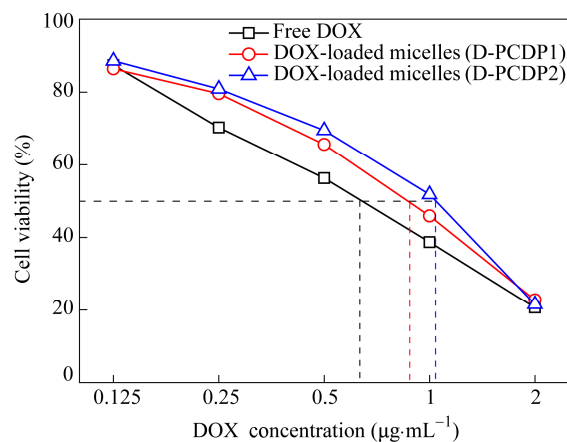


**Fig. 8** Cytotoxicity studies of polymer micelles and free DOX against HeLa cells after incubation for 48 h

#### *In Vitro Release of DOX from Micelles*

In order to evaluate the effects of pH-dependent behavior on controlled drug delivery, the *in vitro* drug release profiles of DOX-loaded polymer micelles were determined under physiological condition (pH 7.4) or in a slightly acidic environment (pH 5.0) (to simulate the pH of the endosomal and lysosomal microenvironments), as shown in Fig. 9. It was observed that the release rate at pH 5.0 was significantly higher than that at pH 7.4. At pH 5.0, about 80% of DOX was gradually released into the medium within 15 h. In contrast, nearly 40% of DOX was rapidly released within 15 h at the pH 7.4, and the cumulative release curve almost leveled off after 15 h. These results could be caused by the swollen drug-loaded micelles attributing to the protonation of amino groups in PDEAEMA segments at acidic conditions, and the micelles started to associate due to the protonation of P(DMAEMA-*co*-PEGMA) corona. Herein, the copolymers with specific random structure could satisfy the requirements of fast, short-time and efficient drug release for special occasions. Meanwhile, the DOX molecules were not only encapsulated inside the micellar core, but absorbed by the P(DMAEMA-*co*-PEGMA) shell due to

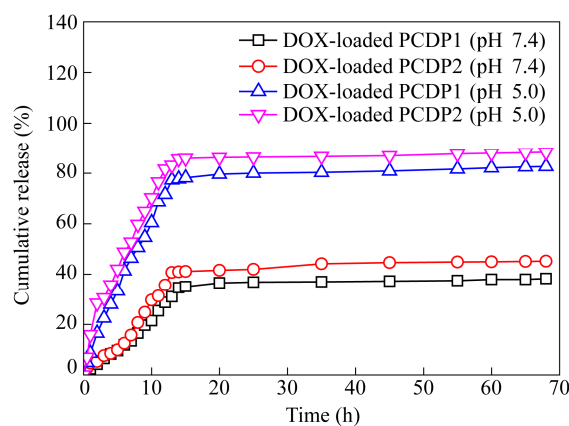


**Fig. 9** *In vitro* cumulative drug release profiles of DOX-loaded polymeric micelles (D-PCDP1 and D-PCDP2) dispersed in PBS at pH = 7.4 and pH = 5.0 at 37 °C

the electric action. Since only the loaded ones *via* hydrophobic effect could be released comparatively faster, it might take extended period to achieve complete release. As may be concluded from the discussion above, these PCDP1 and PCDP2 micelles were just like “on-off” switching nanocarriers in release kinetics by changing pH values. Therefore, they were highly promising for intracellular anti-cancer drug delivery.

### *In Vitro Cellular Uptake Studies*

CLSM images were used to investigate and compare the cell uptake behavior and intracellular distributions of the DOX-loaded micelles (D-PCDP2) with that of free DOX by HeLa cells. The CLSM images of HeLa cells after 0.5, 4, and 24 h of incubation with free DOX and D-PCDP2 are presented in Fig. 10. After incubation for 0.5 h, stained with DAPI, the nuclei and cytoplasm of pretreated cells were observed by CLSM. Compared with Fig. 10(b), the observation revealed that free DOX was slightly accumulated in the cell nuclei of HeLa cells in Fig. 10(a). After incubation for 4 and 24 h, D-PCDP2 was largely accumulated than free DOX, and while DOX released from DOX-loaded micelles (D-PCDP2) was mainly located in the cytoplasm, DOX was released into the cytoplasm and nuclei of cells under acid conditions in lysosomes. The schematic illustration of this pH-responsive degradation process and the efficient intracellular release of anti-cancer drugs is presented in Scheme 2. After 24 h of incubation with DOX-loaded micelles (D-PCDP2), it is indicated that the nuclei of HeLa cells were dissociated by DOX-loaded micelles (D-PCDP2), which exhibited a higher inhibition of the proliferation of HeLa cells. In addition, these results also suggested that free DOX was taken up by diffusion through the cell membrane while the DOX-loaded micelles by the nuclei of cells *via* endocytosis process<sup>[16, 43, 46]</sup>. Therefore, polymeric micelles with excellent biocompatibility could be a safe material for efficient intracellular drug delivery.

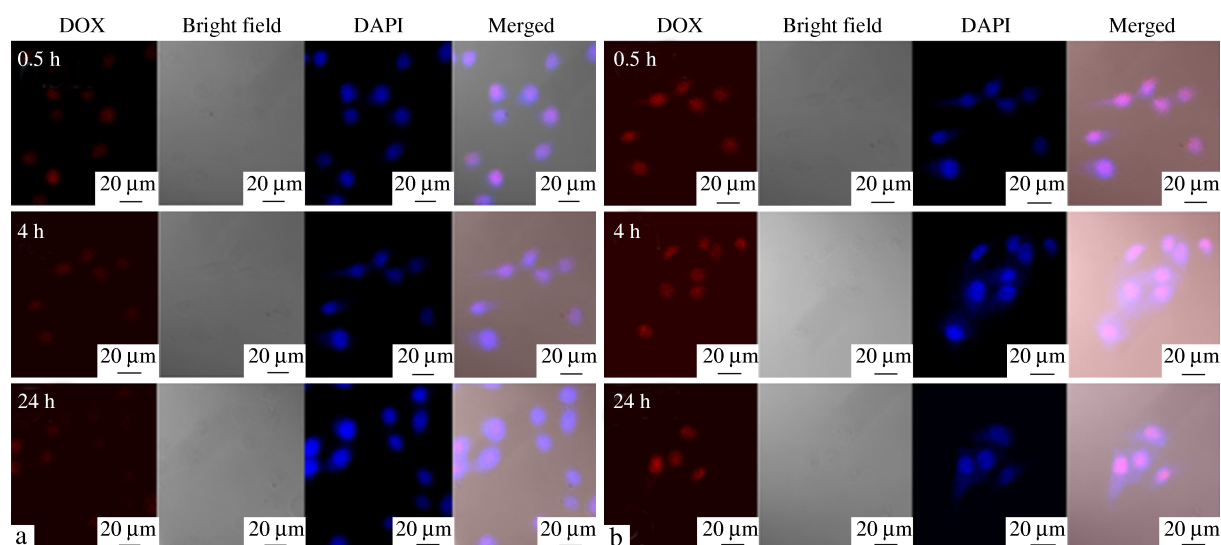


**Fig. 10** Confocal laser scanning microscopy images of HeLa cells incubated with free DOX (a) and DOX-loaded (D-PCDP2) (b) for different time

The DOX dosage was  $5 \mu\text{g}\cdot\text{mL}^{-1}$ . For each panel, images from left to right show cell nuclei stained by DOX fluorescence in cells (red), bright field of cells, DAPI (blue), and overlays of two images. (The online version is colorful.)

### CONCLUSIONS

In summary, the pH-responsive star-shaped PCL-*g*- $\beta$ -CD-*g*-P(DMAEMA-*co*-PEGMA) copolymers were synthesized by ROP and “click” chemistry. The chemical structures of the polymers were confirmed by  $^1\text{H}$ -NMR and GPC analyses. These nanoscale copolymer micelles could self-assemble into spherical micelles with an average diameter of about 60 nm in aqueous solution, which were determined by TEM and DLS and could be used for simultaneous loading of hydrophobic DOX. The DOX-loaded polymeric micelles showed

much lower toxicity than free DOX for the HeLa cells. Further, the DOX-loaded micelles were taken up by the nuclei of cells *via* the endocytosis process by CLSM, which exhibited inhibition of the proliferation of HeLa cells. Furthermore, the applicability of these micelles in response to cellular components toward tumor-targeting delivery applications *in vivo* was an exploratory research area. In addition, the polymeric micelles could be accelerated for cellular uptake by a manner of endocytosis. This research provides a facile way to prepare a multifunctional polymeric micelles system, which is promising for the targeted intracellular drug release.

## REFERENCES

- 1 Liu, F. and Urban, M.W., *Prog. Polym. Sci.*, 2010, 35: 3
- 2 Urban, M.W., *Prog. Polym. Sci.*, 2009, 34: 679
- 3 Mura, S., Nicolas, J. and Couvreur, P., *Nat. Mater.*, 2013, 12: 991
- 4 Aloorkar, N.H., Kulkarni, A.S., Patil, R.A. and Ingale, D.J., *Int. J. Pharm. Sci. Nanotech.*, 2012, 5: 1675
- 5 Zhang, Q., Ko, N.R. and Oh, J.K., *Chem. Commun.*, 2012, 48: 7542
- 6 Li, L., Lu, B.B., Fan, Q.K., Wei, L.L., Wu, J.N., Hou, J., Guo, X.H. and Liu, Z.Y., *RSC Adv.*, 2016, 6: 27102
- 7 Zhang, Y.J., Li, P., Pan, H., Liu, L.L., Ji, M.Y., Sheng, N., Wang, C., Cai, L.T. and Ma, Y.F., *Biomaterials*, 2016, 83: 219
- 8 Lu, B.B., Li, L., Wei, L.L., Guo, X.H., Hou, J. and Liu, Z.Y., *RSC Adv.*, 2016, 6: 50993
- 9 Hervault, A., Lim, M., Boyer, C., Dunn, A.E., Mott, D., Maenosono, S. and Thanh, N.T.K., *Nanoscale*, 2016, 8: 12152
- 10 Zhang, H.T., Tian, W., Suo, R.T., Yue, Y., Fan, X.D., Yang, Z., Li, H., Zhang, W.B. and Bai, Y., *J. Mater. Chem. B*, 2015, 3: 8528
- 11 Guo, Z.R., Feng, Y.J., Zhu, D.W., He, S., Liu, H.B., Shi, X.R., Sun, J. and Qu, M.Z., *Adv. Funct. Mater.*, 2013, 23: 5010
- 12 Wang, W., Liu, H.B., Mu, M., Yin, H.Y. and Feng, Y.J., *Polym. Chem.*, 2015, 6: 2900
- 13 Yin, H.Y., Feng, Y.J., Liu, H.B., Mu, M. and Fei, C.H., *Langmuir*, 2014, 30: 9911
- 14 Lee, S., Saito, K., Lee, H.R., Lee, M.J., Shibasaki, Y., Oishi, Y. and Kim, B.S., *Biomacromolecules*, 2012, 13: 1190
- 15 Liu, X., Ni, P.H., He, J.L. and Zhang, M.Z., *Macromolecules*, 2010, 43: 4771
- 16 Ulbrich, K., Holá, K., Šubr, V., Bakandritsos, A., Tuček, J. and Zbořil, R., *Chem. Rev.*, 2016, 116: 5338
- 17 Durmaz, Y.Y., Lin, Y.L. and ElSayed, M.E.H., *Adv. Funct. Mater.*, 2013, 23: 3885
- 18 Ren, J.M., McKenzie, T.G., Fu, Q., Wong, E.H.H., Xu, J.T. and An, Z.S., Shanmugam, S., Davis, T.P., Boyer, C. and Qiao, G.G., *Chem. Rev.*, 2016, 116: 6743
- 19 Altintas, O., Vogt, A.P., Kowollik, C.B. and Tunca, U., *Polym. Chem.*, 2012, 3: 34
- 20 Wang, J.J., Zhang, J.L., Yu, S.L., Wu, W. and Jiang, X.Q., *ACS Macro Lett.*, 2013, 2: 82
- 21 Cameron, D.J.A. and Shaver, M.P., *Chem. Soc. Rev.*, 2011, 40: 1761
- 22 Ren, K., Zhang, M.Z., He, J.L., Wu, Y.X. and Ni, P.H., *ACS Appl. Mater. Interfaces*, 2015, 7: 11263
- 23 Boyer, C., Corrigan, N.A., Jung, K., Nguyen, D., Nguyen, T.K., Adnan, N.N.M., Oliver, S., Shanmugam, S. and Yeow, J., *Chem. Rev.*, 2016, 116: 1803
- 24 Higashihara, T., Hayashib, M. and Hirao, A., *Prog. Polym. Sci.*, 2011, 36: 323
- 25 Boyer, C., Derveaux, A., Zetterlund, P.B. and Whittaker, M.R., *Polym. Chem.*, 2012, 3: 117
- 26 Hao, Y., He, J.L., Li, S., Liu, J., Zhang, M.Z. and Ni, P.H., *J. Mater. Chem. B*, 2014, 2: 4237
- 27 Warren, N.J. and Armes, S.P., *J. Am. Chem. Soc.*, 2014, 136: 10174
- 28 Shi, P.F., Li, Q.L., He, X., Li, S.T., Sun, P.C. and Zhang, W.Q., *Macromolecules*, 2014, 47: 7442
- 29 Golas, P.L. and Matyjaszewski, K., *Chem. Soc. Rev.*, 2010, 39: 1338
- 30 Kempe, K., Krieg, A., Becer, C.R. and Schubert, U.S., *Chem. Soc. Rev.*, 2012, 41: 176
- 31 Yuan, W.Z., Li, X.F., Gao, S.Y., Cao, A. and Ren, J., *Polymer*, 2011, 52: 658
- 32 Manakker, F., Vermonden, T., Nostrum, C.F. and Hennink, W.E., *Biomacromolecules*, 2009, 10: 3157
- 33 Chen, G.S. and Jiang, M., *Chem. Soc. Rev.*, 2011, 40: 2254
- 34 Zhang, J.X. and Ma, P.X., *Adv. Drug Deliver. Rev.*, 2013, 65: 1215
- 35 Wei, H. and Yu, C.Y., *Biomater. Sci.*, 2015, 3: 1050

- 36 Davis, M.E. and Brewster, M.E., *Nat. Rev. Drug Discov.*, 2004, 3: 1023
- 37 Uekama, K., Hirayama, F. and Irie, T., *Chem. Rev.*, 1998, 98: 2045
- 38 Yuan, Y.Y., Du, Q., Wang, Y.C. and Wang, J., *Macromolecules*, 2010, 43: 1739
- 39 Chen, J.C. and Liu, M.Z., *RSC Adv.*, 2014, 4: 9684
- 40 He, Q., Wu, W., Xiu, K.M., Zhang, Q., Xu, F.J. and Li, J.S., *Int. J. Pharm.*, 2013, 443: 110
- 41 Yin, J.J., Shumyak, S.P., Burgess, C., Zhou, Z.W., He, Z.X., Zhang, X.J., Pan, S.T., Yang, T.X., Duan, W., Qiu, J.X. and Zhou, S.F., *Int. J. Pharm.*, 2015, 10: 4717
- 42 Liu, J., Xu, Y., Yang, Q., Li, C., Hennink, W.E., Zhuo, R. and Jiang, X., *Acta Biomater.*, 2013, 9: 7758
- 43 Wang, M.M., Wang, Y., Hu, K., Shao, N.M. and Cheng, Y.Y., *Biomater. Sci.*, 2015, 3: 480
- 44 Allen, T.M. and Cullis, P.R., *Science*, 2004, 303: 1818
- 45 Tungala, K., Adhikary, P. and Krishnamoorthi, S., *Carbohydr. Polym.*, 2013, 95: 295
- 46 Ge, Z.S. and Liu, S.Y., *Chem. Soc. Rev.*, 2013, 42: 7289