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pH-Sensitive Amphiphilic Copolymer Brush Chol-*g*-P(HEMA-*co*-DEAEMA)-*b*-PPEGMA: Synthesis and Self-Assembled Micelles for Controlled Anti-Cancer Drug Release

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Abstract:

A typical cholesterol modified amphiphilic copolymers poly((hydroxyethyl methylacrylate)-*co*-(2-(diethylamino)ethyl methacrylate))-*b*-poly(poly(ethylene glycol) methyl ether methacrylate) (Chol-*g*-P(HEMA-*co*-DEAEMA)-*b*-PPEGMA) with specific pH-sensitive/hydrophilic/hydrophobic structure containing different ratios of pH-sensitive PDEAEMA segments were designed and synthesized *via* the combination of activators regenerated by electron transfer atom transfer radical polymerization (ARGET ATRP) and alcoholysis reaction, and their self-assembled three-layered micelles were used as doxorubicin (DOX) delivery carriers. The structures of the polymers were determined by ^{1}H NMR and GPC. The critical micelle concentrations (CMC) of the polymers at different pH values were confirmed by fluorescence spectroscopy, resulting in 9.33 mg/L and 13.18 mg/L for two polymers even at weakly acidic conditions (pH 6.0). The pK_b values, particle sizes and zeta potentials of the polymers in the solutions with different pH values were studied in order to investigate the pH-sensitivity of the polymers. The morphological shapes of the polymers were detected by transmission electron microscopy (TEM). As the pH decreasing, the sizes and zeta potentials of the polymeric micelles increased markedly.

DOX was loaded in the micelles by dialysis method, and the *in vitro* release rate was enhanced sharply in the solution pH of 6.0 when compared to pH of 7.4 for both of the polymers. The cytotoxic effects for HepG2 cells were measured and compared with free DOX, resulting in low and high cytotoxicity for polymers and DOX-loaded micelles, respectively. All the results demonstrated that these pH-sensitive micelles could be used as the potential anti-cancer drug carriers.

Keywords: amphiphilic, pH-sensitivity, drug delivery, ARGET ATRP, cholesterol, anti-cancer

1. Introduction:

In the last few years, drug delivery system (DDS) which is used as an effective method to treat many diseases, especially cancer, is attracting more and more attention[1-5]. However, there are still many key technical issues, such as poor solubility of the drugs, low bioavailability, side-effects, poor therapeutic effect and serious toxicities, to limit the further study and clinical applications[6-10]. In order to address these challenges, more and more polymeric vectors, such as nanoparticles[11-13], liposomes[14-16] and nano-scale carriers[17-19], have been developed by many researchers around the world. Among these novel drug vehicles, polymeric micelles which are generally self-assembled by amphiphilic polymers with hydrophilic and hydrophobic segments show many advantages, including high drug loading capacity, small particle size (<200 nm), controlled release behavior and easily modified[20-23]. The typical core-shell micelles structure could load drugs in the core and protect the system effectively in the long-time circulation to reduce reticuloendothelial systems (RES) clearance and renal filtration[24]. During the circulation process in the body, drug-loaded micelles could be accumulated at the tumor cells site because of different pressure and retention effect (EPR). Furthermore, the extracellular pH around tumor cells are slightly lower and weakly acidic (6.5-7.0), compared with normal physiological pH (7.4), and the endosomal and lysomomal pH circumstance are much lower (5.0-6.5)[25-27]. Therefore, the anticancer drug carrier should keep and protect the drug at normal pH value and release them at the weakly acidic circumstance. Based

on the above preconditions, many different kinds of pH stimulus-response polymeric micelles have been extensively designed and developed as effective drug delivery carriers[28-31].

A potential and successful drug delivery vector which could be investigated further and applied in practice should satisfy these conditions at least: a) Release the delivered drug only in the diseased tissues efficiently as much as possible, in order to enhance the therapeutic efficacy and reduce the side effects; b) The drug release time should be well controlled (long-time or short-time release for different purposes); c) The polymer used as drug delivery carrier, possessing high drug loading capacity, good biocompatibility and barely cytotoxic, should be synthesized and modified easily with convenient and controlled synthetic process. For example, PDEAEMA and PAE showed a pH-sensitive range of 6.0-7.2[32-35], indicating that the solubility could be changed sharply between water-soluble and non-water-soluble according to the protonated and deprotonated of the amine groups on the side depending on the pH values. Shen's team designed and synthesized a kind of pH-responsive nanoparticles from hierarchical self-assembly of triblock polymer brush containing pH sensitive PDEAEMA segment modified by hydrophobic PCL and hydrophilic PEG segments. Camptothecin (CPT) selected as a model drug was loaded in the core, and the pH-dependent cellular and controlled release were well investigated[36]. Huang et al sucessfully synthesized a novel gold nanoparticle with well size-conrollable and high stability based on the pH sensitive PDEAEMA segments[37]. Pan's group developed a fabrication of PDEAEMA-coated mesoporous silica nanoparicles with pH-sensitive shell and MSN core. The controlled release behavior was also studied, demonstrating rapid release rate at acidic pH value and much lower leakage at alkaline condition[38]. Lee group developed a pH-sensitive drug carrier using an amphiphilic graft polymer PAEGAE-*g*-PCL, and DOX was selected as the model drug. Even at pH of 5.0, the drug cumulative release was less than 70 % for 7 days[27], demonstrating the long-time drug controlled releasee. In our previous work[39], a novel amphiphilic copolymer with random pH-sensitive/hydrophobic structure was designed and used as DOX delivery

vector. After 140 h, at pH of 5.0, the drug cumulative release was about 80 %-90 %, which was also for the long-time release.

In this study, the primary objective was to design a novel kind of amphiphilic copolymers containing pH-sensitive segment conjugated by hydrophobic and hydrophilic segments: cholesterol grafted poly((hydroxyethyl methylacrylate)-*co*-(2-(diethylamino)ethyl methacrylate))-*b*-poly(poly(ethylene glycol) methyl ether methacrylate) (Chol-*g*-P(HEMA-*co*-DEAEMA)-*b*-PPEGMA) which exhibited specific random pH-sensitive/hydrophilic/hydrophobic structure. The synthetic process was trying to simplify *via* the combination of activators regenerated by one-step electron transfer atom transfer radical polymerization (ARGET ATRP) and alcoholysis reaction. The hydrophilic brush PPEGEMA with short side chains was selected to block on the terminal of the precursors P(HEMA-*co*-DEAEMA) with random pH-sensitive/hydrophilic structure This whole synthetic process was hoped to finish by one-step ATRP reaction. Then in order to increase the drug loading capacity and reduce the initial burst, the high biocompatibility and hydrophobic cholesterol[40-42] was conjugated on the terminal of hydrophilic HEMA segment. During the process of micellization, cholesterol formed the core of the polymeric micelle, random pH-sensitive PDEAEMA/hydrophilic PHEMA comprised the middle layer, and the PPEGEMA distributed and stretched dendritically on the surface of the polymeric micelles as the shell on the surface because of nonimmunogenicity, nonantigenicity and nontoxicity[43, 44], creating a three-layered onion-like-structured nanoparticles. Scheme 1 presented the micellization and structural changes in the PBS solution with different values of pH (7.4 and 6.0). Doxorubicine (DOX), with known a wide range of applications in tumor therapy, was selected as the small molecule hydrophobic model drug. It was supposed that this special structure could enhance the drug loading capacity, reduce particle size and initial burst effect, optimize pH-responsibility and drug release behavior as well as short-time drug release time. The self-assembled DOX-loaded micelles kept compact and tight structure, entrapped drug in the core at normal physiological conditions (pH 7.4). Nevertheless, in the weakly acidic environment, the drug-loaded micelles were swollen and loose because of the

protonation of amine groups on the side of the PDEAEMA moieties, resulting in drug released from the micelles, as shown in Scheme 1. The cytotoxicity, particle size, zeta potential and other physicochemical properties also should be studied by different methods.

2. Materials and methods

2.1. Materials

Hydroxyethyl methylacrylate (HEMA，99%，Aldrich) and 2-(Diethylamino)ethyl methacrylate (DEAEMA, TCI-EP) were purified prior to use, and stored under argon at -20 ^{o}C. Triethylamine (TEA), tetrahydrofuran (THF), dichloromethane (DCM) and toluene were further purified and distilled before used. Cholesteryl chloroformate (Chol, 99%, Alfa Aesar), 1,1,4,7,10,10-hexamethyltriethylenetetramine (HMTETA, 99%, Aldrich), Poly(ethylene glycol) methyl ether methacrylate (PEGMA, M_n = 475 Da, 99%, Aldrich). Doxorubicin hydrochloride, Phosphate-buffered saline (PBS) buffers, methylthiazoltetrazolium (MTT), Dulbecco's modified eagle media (DMEM) growth media, fetal bovine serum (FBS), and other biological materials were purchased as before[44]. Pyrene (99%, Aldrich), dimethyl formamide (DMF), stannous octoate ($Sn(Oct)_2$), and all other reagents were used as received.

2.2. Synthesis of P(HEMA-*co*-DEAEMA)-*b*-PPEGMA

$CuBr_2$ (22.34 mg) was placed in a flame-dried 50 mL Schlenk flask with a magnetic stirring bar, and the flask was evacuated and flushed with nitrogen for thrice. HEMA (1.95 g), DEAEMA (4.63 g) and HMTETA (0.27 mL) were respectively dissolved in 10 mL anhydrous methylbenzene and added into the flask. And $Sn(Oct)_2$ (0.40 g) was dissolved in 2 mL anhydrous methylbenzene and added dropwise into the flask. After the resulting heterogeneous mixture was stirred for 15 min, EBriB (0.15 mL) was added into the reaction. The reaction was carried out for 5-7 h at 70 ℃ under nitrogen. The process was monitored by gel permeation chromatography (GPC). PPEGMA (4.99 g) was dissolved in 5 mL anhydrous methylbenzene and added dropwise. The reaction was sequentially carried out for 24 h. Cooled it to room temperature, the received mixture was solved in THF (50 mL) and was purified by passing through a

column filled with neutral alumina. After rotary evaporation, the product was recovered by being precipitated into 10-fold excess of *n*-hexane, filtered, and finally dried under vacuum for 24 h.

2.3. Synthesis of Chol-*g*-P(HEMA-*co*-DEAEMA)-*b*-PPEGMA

P(HEMA-*co*-DEAEMA)-*b*-PPEGMA (2.92 g) was dissolved in anhydrous DCM (20 mL) and placed in a flame-dried 50 mL Schlenk flask with a magnetic stirring bar, and the flask was evacuated and flushed with nitrogen for thrice. Anhydrous TEA (0.87 mL) and cholesterol (1.68 g) were dissolved and added into the flask orderly. The reaction was carried out at 0 °C for 2 h, allowed to attain room temperature, and then stirred for a further 24 h. After filtration, the product was recovered by being precipitated into 10-fold excess of *n*-hexane, filtered, and finally dried under vacuum for 24 h.

2.4 Characterization

The molecular weight (M_n) was determined by gel permeation chromatography (GPC) (Agilent 1200, RI detector) using HPLC grade THF as mobile phase with a flow rate of 1.0 mL/min at 30 °C.

^{1}H NMR spectra measurements were recorded on a Bruker AVANCE III 400 (Switzerland) spectrometer operating at 400 MHz at room temperature in deuterated chloroform ($CDCl_3$-*d*) containing tetramethylsilane (TMS) as solvent.

Dynamic light scattering (DLS) test was executed on Malvern Zetasizer Nano S (UK) in a 1.0 mL quartz cuvette, using a diode laser of 800 nm at 25 °C and the scattering angle was fixed at 90°, after the samples were purified by 0.45 μm pore size filter.

Morphological shapes of samples were studied by transmission electron microscopy (TEM, Hitachi H-7650, Japan) operating at 80 kV.

2.5 Potentiometric titration

The base dissociation constant (pK_b) values of the copolymers were obtained by potentiometric titrations. In briefly, the polymer was dissolved in deionized water at a concentration of 1 mg/mL, and the pH was adjusted to 3 by HCl. The solution was titrated by 0.1 M NaOH aqueous solution at an increment of 100 μL. The pH

increases of the solution were monitored with an automatic titration titrator (Hanon T-860, Jinan, China) at room temperature. The pK_b value was defined as the solution pH at 50 % neutralization of tertiary amine groups.

2.6 CMC measurement

The CMC of Chol-*g*-P(HEMA-*co*-DEAEMA)-*b*-PPEGMA was determined by the fluorescence probe technique using pyrene as a fluorescence probe[45]. Pyrene was preferentially entrapped into the core after micellization of the polymers, leading to environment of pyrene changed from polar to non-polar. In briefly, the pyrene solution was prepared firstly. A series of polymer solutions at different concentrations (0.0001 to 0.1 mg/mL) were received and mixture with pyrene solution (6×10^{-7} M). The resulted solutions were equilibrated at room temperature in dark for 24 h. Fluorescence spectra were determined by a fluorescence spectrophotometer (F-4500, Hitachi, Japan, emission wavelength 373 nm and bandwidth 0.2 nm) scanning samples from 300 to 350 nm at 20 °C. The relationship between the concentrations of polymers and the intensity ratios of I_{339} to I_{336} were further studied to confirm the CMC values of the polymers.

2.7 Preparation and characterization of blank and DOX-loaded micelles

The micelles of Chol-*g*-P(HEMA-*co*-DEAEMA)-*b*-PPEGMA were formed by the dialysis method. Briefly, different amount of DOX-HCl (0, 5, 10 and 20 mg) and polymer (40 mg) were dissolved in DMF (40 mL). Overdose of TEA was dropped in the mixture to remove hydrochloride with stirring for 20 min. The resulted solution was moved into a cellulose membrane bag (MWCO 3500-4000 Da) and dialyzed against 1 L of deionized water for 48 h at room temperature. The deionized water was replaced at the desired time. Subsequently, after lyophilization, the blank polymeric micelles, DOX-loaded polymeric micelles (PMs-1 and PMS-2 for the Polymer-1 and Polymer-2, respectively) were received stored at -20 °C until further experiments.

DOX loading content (LC) and entrapment efficiency (EE) were confirmed as: briefly, DOX-loaded micelle (1 mg) was solved completely in DMF (10 mL). The samples were measured by UV-vis spectrophotometer (UV-2450, Shimadzu, Japan) at 480 nm.

The concentration of DOX was attained according to a standard curve of pure DOX/ DMF solution. The LC and EE were calculated based on the following equations:

$$\mathrm{LC}(\%) = \frac{\text{Weight of loaded drug}}{\text{Weight of drug loaded micelle}} \times 100\% \quad (1)$$

$$\mathrm{EE}(\%) = \frac{\text{Weight of loaded drug}}{\text{Weight of drug in feed}} \times 100\% \quad (2)$$

2.8 *In vitro* release of DOX from polymeric micelles

The *in vitro* controlled release behavior of DOX-loaded Chol-*g*-P(HEMA-*co*-DEAEMA)-*b*-PPEGMA micelles at different PBS buffer solutions was studied by Dissolution Tester (RCZ-8B, TDTF, China). In a typical experiment, DOX-loaded micelles (4 mg) was dissolved in the PBS buffer solutions (4 mL, V_e) with different pH values (7.4 and 6.0) at a concentration of 1 mg/mL. The resulted solutions were moved to dialysis bag and placed in the beakers containing 40 mL PBS buffer (pH 6.0 and 7.4, respectively). The experiment was carried out in a water bath at 37 °C with stirring 110 rpm in triplicate. At the desired time, the quantitative samples were received and detected by UV-vis spectrophotometry at 480 nm. The cumulative drug release percent (E_r) was as following:

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

where, m_{DOX} represents the amount of DOX in the micelle, V_0 is the volume of the solution in the beaker (V_0 = 44 mL), and C_i represents the concentration of DOX in the i_{th} sample.

2.9 Cytotoxicity test

The cytotoxic effects of polymers, free DOX or DOX-loaded Chol-*g*-P(HEMA-*co*-DEAEMA)-*b*-PPEGMA micelles was evaluated against HepG2 cells by the standard MTT[46]. The HepG2 cells were cultured and prepared as previous work[39]. To perform cytotoxicity assay, HepG2 cells were seeded at a density of 1 × 10^4 cells/well on a 96-well plate and cultured foe 24 h. The samples were prepared at a series of desired concentrations. Every experimental well was treated with the samples for 24h or 48 h, and others were added with fresh medium as control. MTT

PBS buffer solution was placed in the plates, which was shaken for 10 min at 150 rpm. And then it was incubated for another 4 h. In order to dissolve the internalized purple formazan crystals, the medium in the well was replaced by DMSO. After the plate was agitated for 15 min, microplate reader (Multiskan Spectrum, Thermo Scientific, Finland) was used to detect the samples at 490 nm. The relative cell viability (%) was received based on the following equation:

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

where, A_{control} and A_{sample} are the absorbance of the wells with different samples treatment, and A_{blank} is the absorbance of wells without cells. The cytotoxicity test was performed in replicates of six wells.

2.10 Statistical analysis

The experimental data were presented with an average values, expressed as the mean ± standard deviation (S.D.). Statistical significance was determined by Student's t-test (Excel, 2007) and considered to be significant when the p values were less than 0.05 ($p < 0.05$).

3. Results and Discussion

3.1 Synthesis and characterization of the Chol-*g*-P(HEMA-*co*-DEAEMA)-*b*-PPEGMA copolymer

The amphiphilic polymers P(HEMA-*co*-DEAEMA)-*b*-PPEGMA were synthesized by one-step ARGET ATRP of HEMA, DEAEMA and PPEGMA segments, and then modified by cholesterol segment, resulting in triblock pH-sensitive brush copolymers Chol-*g*-P(HEMA-*co*-DEAEMA)-*b*-PPEGMA with specific random pH-sensitive/hydrophilic/hydrophobic structure, as shown in Fig. 1. Firstly, the P(HEMA-*co*-DEAEMA) segment was synthesized by using $CuBr_2$/HMTETA and $Sn(Oct)_2$ as catalyst system and redactor, respectively. The process was monitored by on-line infrared detector. Subsequently, PPEGMA segment was conjugated under the same circumstance. The reaction was carried out for more than 30 h. The whole synthetic process could be finished by one step. The different copolymers could be

synthesized and modified easily by adjusting the ratios of blocks. Finally, the cholesterol segment was grafted on the terminal hydroxyl of the HEMA segment in the main chain. The final triblock copolymers were acquired after precipitation in the cool *n*-hexane three times and dried. Two kinds of precursors and block copolymers were synthesized by changing the mole ratio of HEMA and DEAEMA in the first step, and confirmed by GPC and ^{1}H-NMR, as shown in Table 1. In our previous work, the brush shape PPEGMA segment was introduced as the hydrophilic part, distributing on the surface of the micelle, and acting as a protecting group[44]. With the purpose of controlled drug release, PDEAEMA segment showed pH-sensitivity because of terminal amines, and its pK_b value was about 6.5-7.2[47]. Cholesterol segment introduced as the hydrophobic and biocompatible part in order to increase the drug loading capacity and reduce the cytotoxicity.

The synthesized copolymers and precursors were determined by ^{1}H-NMR, as shown in Fig. 2. With regard to precursor P(HEMA-*co*-DEAEMA)-*b*-PPEGMA (Fig. 2A), the signals at 1.20 ppm (a) were ascribed to -CCH_3 of the HEMA, DEAEMA and PEGMA units. The signals at 1.85 ppm (e), 4.18 ppm (b), and 3.67 ppm (g) were ascribed to -CCH_2C-, -CH_2OCO-, -CH_2CH_2O- of the HEMA, DEAEMA and PEGMA units, respectively. The characteristic HEMA peaks of block copolymers at 3.85 (c) and was due to -CH_2CH_2OH protons on the side chain. The signal at 2.55 (f) and 2.65 (d) ppm were the characteristic peaks of $CH_3CH_2NCH_2$- and $CH_3CH_2NCH_2$- in the DEAEMA unit on the side chain, respectively. The signal at 3.42 ppm (h) was the characteristic of CH_3O- in the PEGMA unit. After cholesterol grafted (Fig. 2B), the signals around 0.5-1.7 ppm (a) were due to the cholesterol group. The signals at 1.8-2.1 ppm (e) were ascribed to the protons of -CH_2- in the HEMA units. And there was a separated peak (5.40 ppm, i) which is the characteristic of the cholesterol segment. With the purposes of confirming the ratios of the block segments and the grafting percentage of the cholesterol, the areas of the above characteristic peaks were calculated. The results were shown in the Table 1.

3.2 Micelle formation

The CMC values of the novel two copolymers were measured by fluorescence spectroscopy using pyrene as a probe at different values of pH (7.0 and 6.0), in order to evaluate the formation and the pH-sensitivity of micelles self-assembled from cholesterol modified polymers, as shown in Fig. 3. The CMCs of the synthesized polymers were 5.10 mg/L and 6.92 mg/L for Polymer-1 and Polymer-2 in pH 7.4 PBS buffer, respectively. When the pH decreased from 7.4 to 6.0, the CMC values showed an increase trend (9.33 mg/L and 13.18 mg/L for Polymer-1 and Polymer-2, respectively) for both of the copolymers. In the weakly acidic environment, more and more pendant tertiary amine groups of PDEAEMA segment were ionized, leading to this block was transformed to be hydrophilic. Compared to the polymer solution with pH of 7.4, the amount of hydrophilic segments were increased, and the amount of hydrophobic segments (cholesterol) were unchanged, therefore, it required a greater driving force for micellar formation of polymer solution with pH of 6.0. Furthermore, greater hydrophobic interaction was needed to counteract the greater electrostatic repulsive force (increased zeta potential at pH 6.0) from the hydrophilic blocks of polymer molecules, resulting in a higher CMC of polymer with decrease of pH values. As the above detections, compared to Polymer-1, Polymer-2 had more pH-sensitive PDEAEMA segments, resulting in more hydrophilic segments and higher repulsive forces when tertiary amine groups were ionized, leading to a much higher CMC value at pH of 6.0.

3.3 Characterization of blank and DOX-loaded micelles

The pH sensitivity of the synthesized novel polymers were confirmed by acid-base titration (Fig. 4A) and DLS test (Fig. 4B). As exhibited in Fig .4A, both of the polymers displayed a buffering pH region at pH 5.5 to 7.2, attributed to the protonation of amine groups in PDEAEMA segment. The two copolymers displayed different pK_b values because of different amount of ionizable amine groups in the structure, demonstrating that the pK_b value of the copolymer could be adjusted by changing the ratio of the PDEAEMA segment. Fig. 4B showed the average particle sizes and zeta potentials of the polymers, respectively. When the pH of the polymer solution was higher than 7.4, there were no obvious changes for particle sizes (125 nm

and 160 nm, respectively) and zeta potentials (5 mV and 10 mV, respectively), either Polymer-1 or Polymer-2. The reason may be that the pendant tertiary amine groups in PDEAEMA segments were deprotonated and hydrophobic, which composed the core with grafted cholesterol, leading to the compact polymeric micelles. Compared to the previous work[39], the hydrodynamic diameter of the polymeric micelles with or without DOX (less than 200 nm) was decreased significantly because of PPEGEMA with short side chains and the small core formed by hydrophobic cholesterol, facilitating the EPR effect to accumulate the delivered drug at the tumor site. With regard to zeta potential, the reason could be that electronegative hydroxyls were adsorbed on the surface of the nanoparticles and neutralized with the positive in the basic solution (7.4-9.2), leading to the lower the zeta potential. When the pH decreased sequentially, the particle sizes (from 130 nm to 188 nm for Polymer-1, from 160 nm to 215 nm for Polymer-2, respectively) and zeta potentials (from 5 mV to 33 mV for Polymer-1, from 10 mV to 55 mV for Polymer-2, respectively) of the polymeric micelles increased sharply. The reason was that the tertiary amine groups of PDEAEMA segments were gradually protonated, indicating the pH-sensitive block was transformed to be water-soluble, resulting in the higher hydrophilicity of polymer. The increased hydrophilic segments made the micelles swollen and loose, leading to the increase of particle sizes. The ionized amine moieties of the PDEAEMA units also caused the significant changes in the zeta potentials. Furthermore, the higher electrostatic repulsion among the protonated and hydrophilic PDEAEMA chains aggravated the increase of particles. Polymer-2 showed higher particle sizes and zeta potentials when compared to Polymer-1 because of much more pH-response PDEAEMA segments. All the above results demonstrated that both of the synthesized copolymers showed well pH-sensitivity and the sensitive range around 6.0-7.2.

Fig. 5 presented the TEM images of the polymeric micelles at pH of 7.4 and 6.0 (Polymer-2). It can be observed that the micelles displayed a spherical morphology. The results showed there were some differences between DLS and TEM data. This can be explained that the micelles were compressed in the alkaline solution prior to DLS test. In addition, as we known that DLS showed the intensity average.

DOX was loaded into the polymeric micelles by membrane dialysis using PBS buffer solution with pH 7.4. As the feed amount of DOX increased, the LC and EE also increased. For the both of the polymers (40 mg), when the DOX was fed 10 mg, the EE was the highest, and the drug loading contents are about 8.7 % and 10.8 % in weight for Polymer-1 and Polymer-2, respectively, which were also higher than those of previous copolymers (4.5 %)[39]. At the ratio of DOX/polymer 20/40 mg, the LC was increased, but the EE was reduced because of the aggregation of the drugs in the solution. Since, the two polymers containing same grafted cholesterol segment, which was hydrophobic to enhance the drug loading capacity, on the HEMA terminal. The slightly increase of LC for the Polymer-2 could be explained that it possessed much more pH sensitive PDEAEMA block, which was deprotonated and hydrophobic at the pH of 7.4, compressed on the surface of the core with cholesterol to offer more drug loading space, compared with Polymer-1. With regard to sizes, the particle sizes of the DOX-loaded micelles increased moderately than those of blank polymeric micelles, either Polymer-1 or Polymer-2, because of entrapped drug in the core and adsorbed drug on the surface. It could also be found that the zeta potentials of the drug-loaded micelles were slightly lower than those of blank micelles for both of the polymers, resulting from decreased charge density because of larger particle sizes. The characteristic properties of blank and DOX-loaded self-assembled micelles were showed in Table 2.

3.4 *In vitro* release of DOX from micelles

As expected, the received polymeric micelles displayed a well pH-sensitivity. *In vitro* drug release performances of the Chol-*g*-P(HEMA-*co*-PDEA)-*b*-PPEGMA micelles were finished under physiological conditions (PBS, pH 7.4) and in a slightly acidic environment (PBS, pH 6.0), as exhibited in Fig. 6. It can be found that the drug release rates of DOX from the particles were obviously changed by pH values as well as time. With regard to pH of 7.4, the micelle stayed compact and the loaded DOX was released slowly. After 4 h, less than 20 % of DOX (17 % and 15 % for PMs-1 and PMs-2, respectively) were released, indicating the lower initial burst. Even after 24 h, only about 35 % and 33 % for PMs-1 and PMs-2, respectively, resulting from tight

structure of the micelles. These results demonstrated that the drug could be well protected and stayed stable under pH of 7.4. In contrast, at the pH 6.0, the drug release of DOX was accelerated significantly. After 24 h, the cumulative release was 84 % and 76 % for PMs-1 and PMs-2, respectively. The results were due to the swollen drug-loaded micelles, attributing to the protonation of amino groups in PDEAEMA segment at weakly acidic conditions. Compared to the previous work[39], the cumulative release was less than 40 % when the pH value was 6.5. Even though the pH value was decreased to 5.0, the cumulative release was still less than 60 %. Herein, the copolymers with specific random pH-sensitive/hydrophilic/hydrophobic structure could satisfy the requirements of fast, short-time and efficient drug release for special occasions, other than long-time drug release. In the beginning 2 h, the PMs-2 exhibited slightly higher cumulative release of DOX than PMs-1, attributing to more drug entrapped in hydrophobic PDEAEMA part as described above. When the amine groups were protonated, the PDEAEMA segment was transformed to hydrophilic and the DOX was released quickly. With regard to long-time release, the cumulative release of PMs-2 was slightly lower, compared to PMs-1, because of more protonatable amine groups in PDEAEMA units.

3.5 Cytotoxicity test

Cytotoxic effects of the polymers, free DOX or DOX-loaded micelles in HepG2 cells were determined by MTT assay, as shown in Fig. 7. Fig. 7A presented that the cell viabilities were 95.05% and 90.82 % in 48 h for the Polymer-1 and Polymer-2 at the highest polymer concentration (400 mg/L), respectively, compared to control blank sample. The results demonstrate that both of the synthesized polymers revealed no significant cytotoxicity for HepG2 cells. Fig. 7B and C showed the results of samples treated with free DOX or DOX-loaded micelles for 24 h or 48 h, respectively. The IC50 values of DOX were 1.48 mg/L, 8.51 mg/L, 4.22 mg/L for 24 h, and 0.51 mg/L, 2.05 1.33 mg/L for 48 h for free DOX, PMS-1 and PMs-2 against HepG2 cells, respectively. This slight difference between two DOX-loaded micelles could be explained that the latter containing more pH-sensitive PDEAEMA units, leading to higher drug loading level and more sensitive. With regard to Fig. 7C, at the highest

concentration (20 mg/L), the cytotoxic effects of two DOX-loaded micelles were similar to that of free DOX, suggesting the cytotoxicity of DOX was still higher and not inhibited by polymers. As can be seen from the figures, regardless of the time, free DOX showed higher and increased quickly cytotoxic effect than both of the DOX-loaded micelles, resulting from the process of DOX released from the micelles was dependent on the time and pH environment. The results were consistent with the *in vitro* experiments.

4. Conclusions

In this work, a novel pH-sensitive amphiphilic copolymers Chol-*g*-P(HEMA-*co*-DEAEMA)-*b*-PPEGMA were successfully synthesized and their self-assembled micelles were used as the drug delivery carrier for anti-cancer therapy. The results of titration and DLS experiments showed that the copolymers possessed well pH-sensitivity and suitable particle sizes and zeta potentials. Both of the polymers displayed low CMC values in the solutions with pH of 7.4 and 6.0, demonstrating that the polymers could self-assemble into core/shell structure and keep high stability, which could enhance the drug bioavailability. The polymer containing more pH-sensitive PDEAEMA segments showed moderately higher drug loading capacity and slightly larger particle size. Both of the *in vitro* DOX release rates from the micelles in the PBS buffer of pH 6.0 were much higher than those in the PBS buffer of pH 7.4. The novel copolymer could satisfy the requirements of fast, short-time and efficient drug release In the first 2 h, both of cumulative releases were less than 35 % even at weakly acidic environment, indicating the reduced initial burst. The blank polymers revealed bare toxicity for the HepG2 cells and the DOX-loaded micelles showed much higher toxic effect which was similar to free DOX. Therefore, the cholesterol modified pH-sensitive copolymers could be used as the promising anti-tumor drug carriers.

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Captions of Figures and Tables

Scheme 1 Scheme of drug entrapping and pH-dependent release from the polymeric micelles.

Figures

Fig. 1 The synthetic route of the Chol-*g*-P(HEMA-*co*-DEA)-*b*-PPEGMA.

Fig. 2 ^{1}H NMR spectra of P(HEMA$_{10}$-*co*-DEA$_{25}$)-b-PPEGMA$_{10}$ (A) and Chol-P(HEMA$_{10}$-*co*-DEA$_{25}$)-*b*-PPEGMA$_{10}$ (B) in *d*-$CDCl_3$.

Fig. 3 pH-dependent CMC of micelle self-assembled from polymer-1 and polymer-2.

Fig. 4 Titration curves of two synthesized copolymers (A), micelle size and zeta potentials (B) of polymeric micelles as a function of pH.

Fig. 5 Typical TEM images of micelles in different PBS buffer solutions.

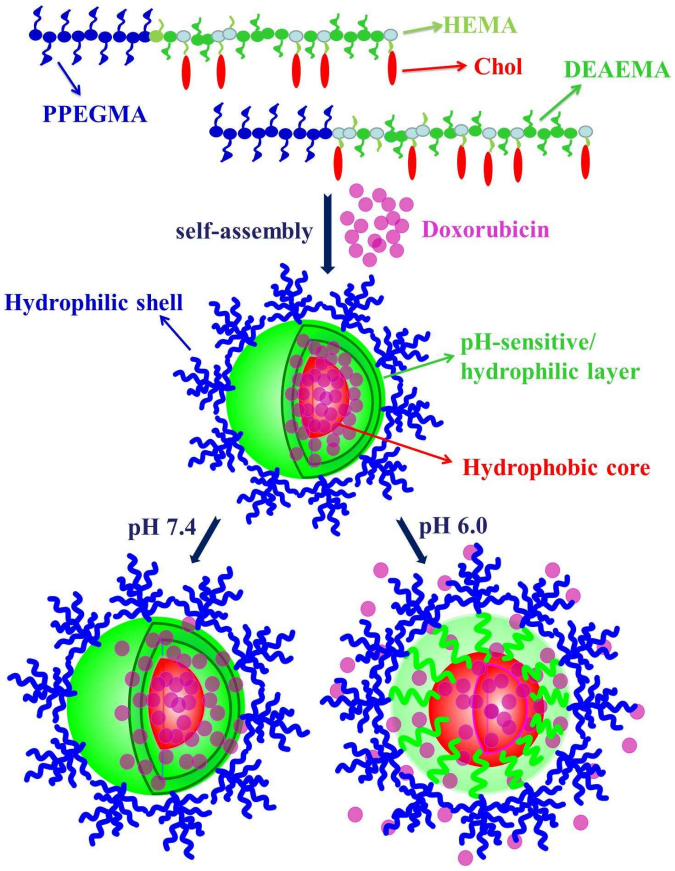
Fig. 6 *In vitro* drug release profiles of DOX-loaded micelles at pH 7.4 and pH 6.0.

Fig. 7 The cytotoxicity of polymers at the designed concentrations (A) for 48 h and free DOX and DOX-loaded micelles for 24 h (B) or 48 h (C) in concentration gradients in HepG2 cells.

Tables

Table 1 GPC and ^{1}H-NMR results of copolymer products.

Table 2 Characteristic properties of DOX-loaded assembled micelles.



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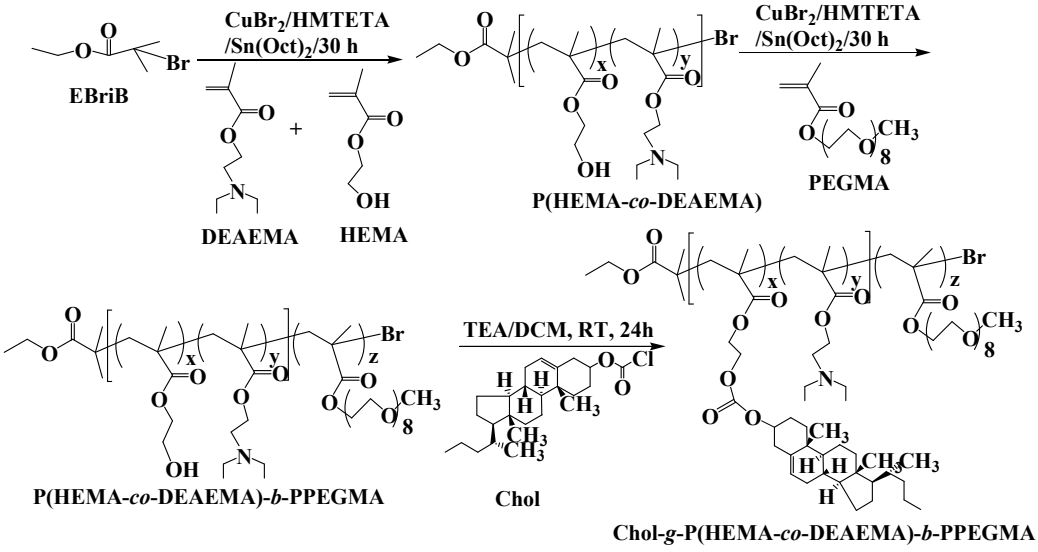

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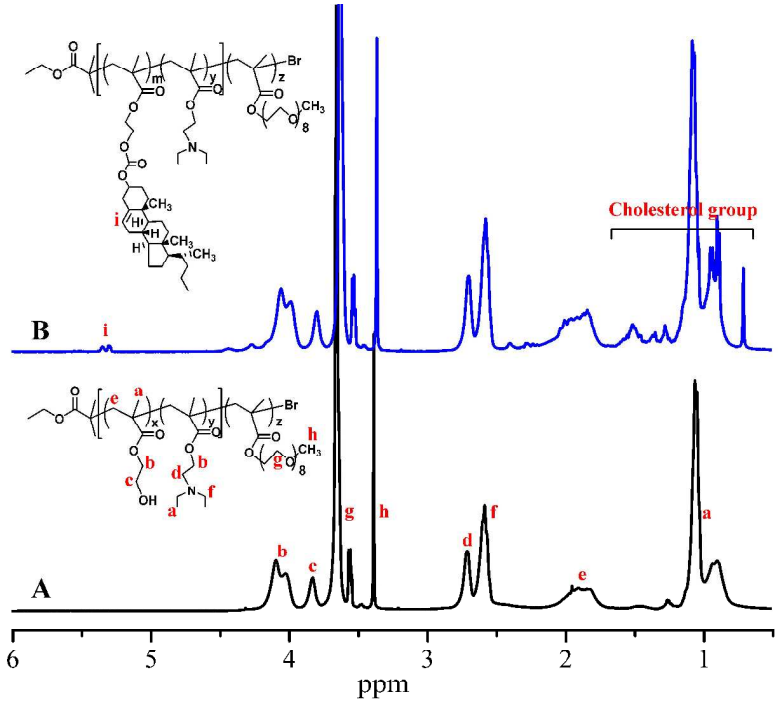

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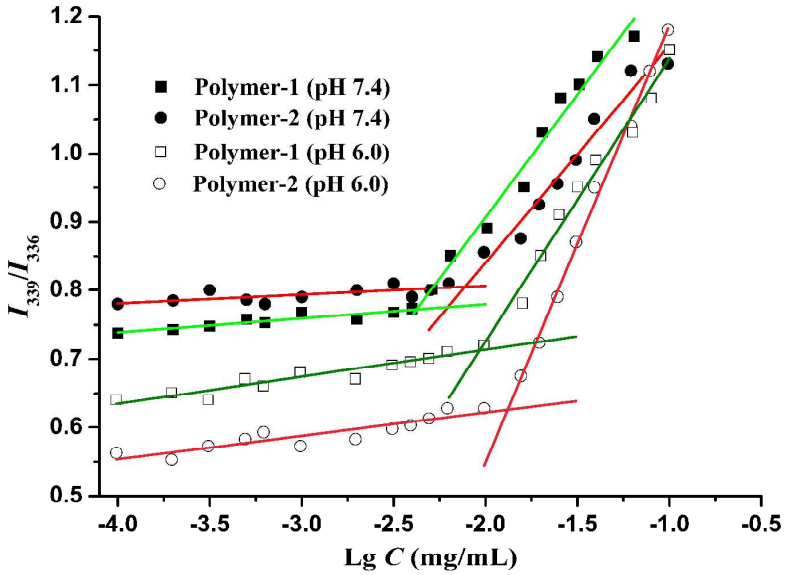

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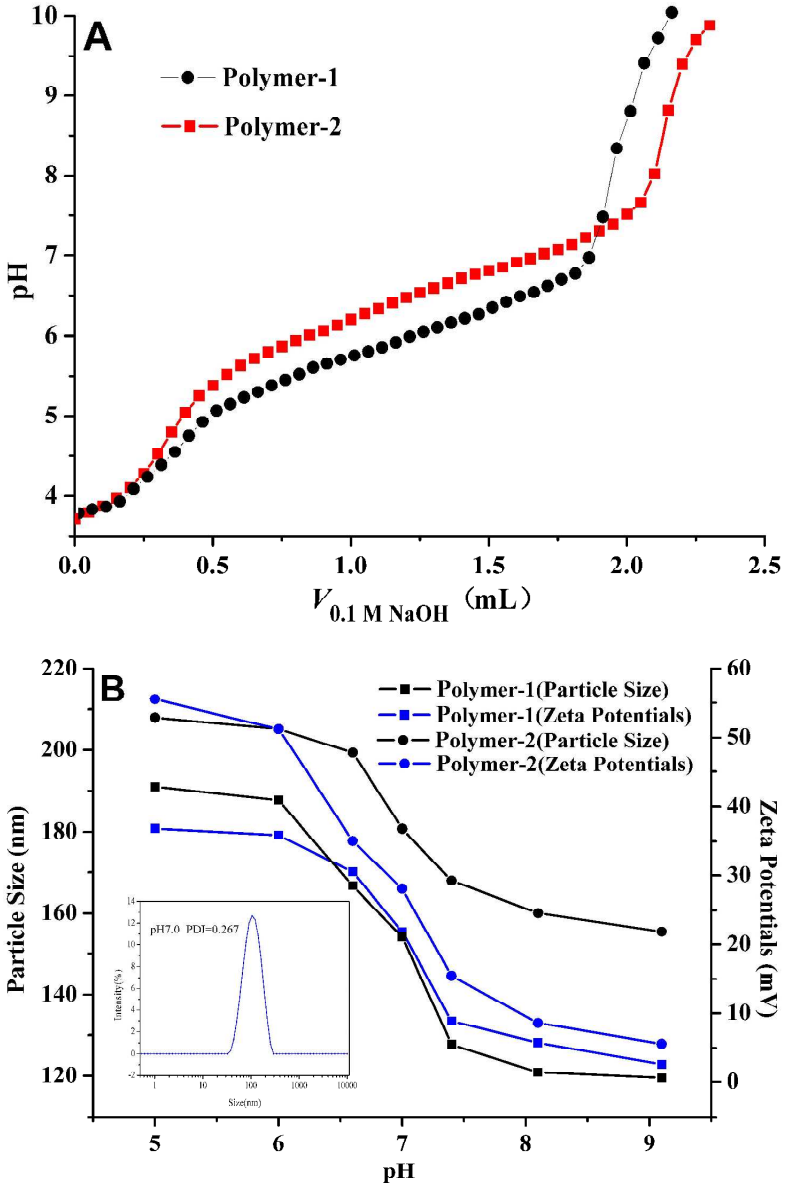

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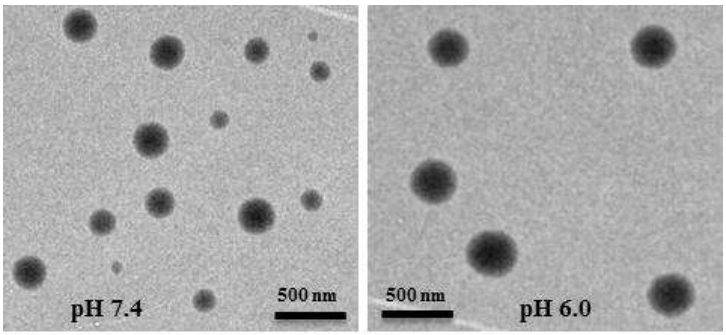

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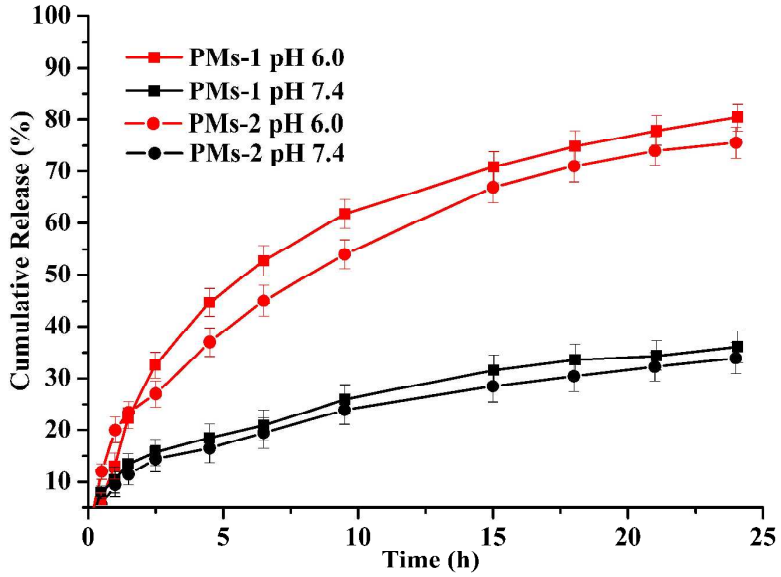

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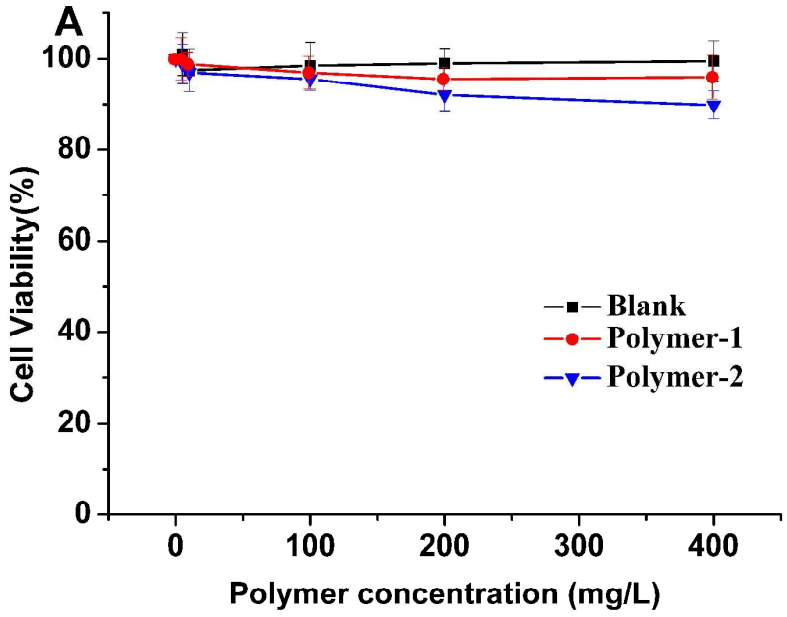

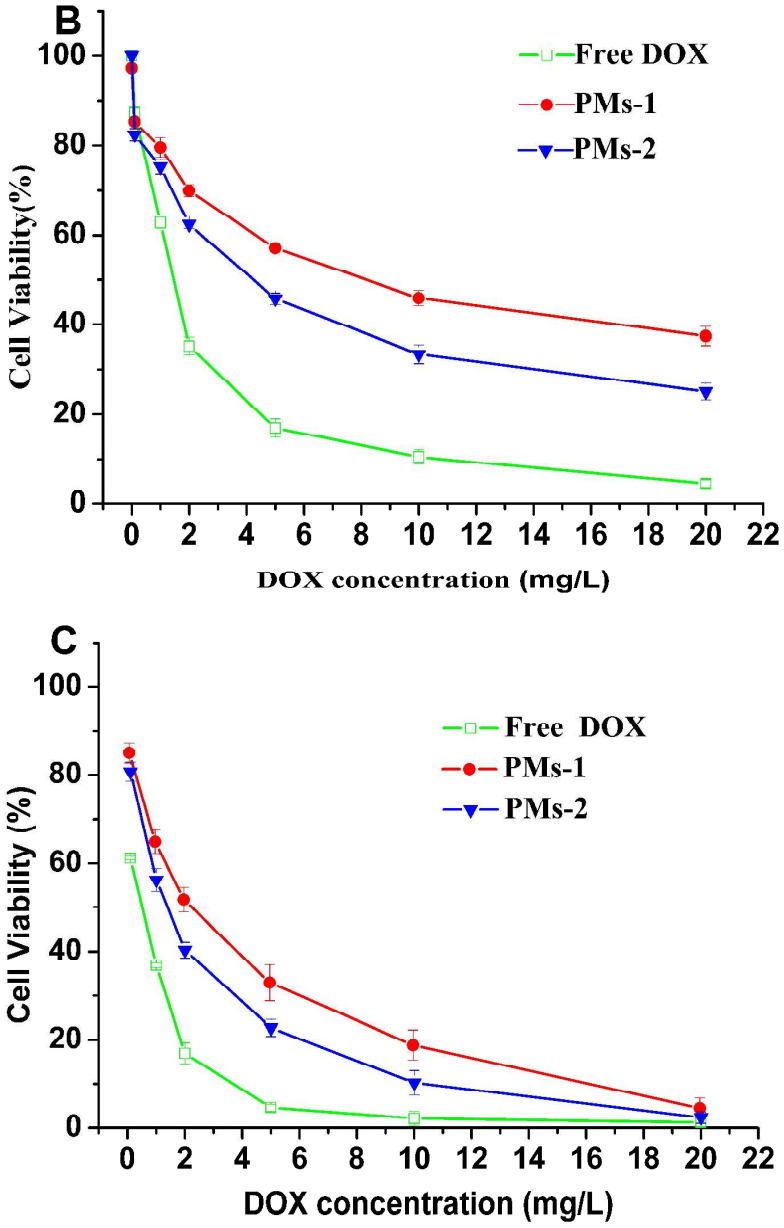

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Table 1 GPC and ^{1}H-NMR results of copolymer products.

Sample	M_n[a]	M_n[b]	M_w/M_n[b]	PHEMA/PDEAEMA/PPEGMA/Ch[c]
Precursors-1	6870	6269	1.37	10/25/0/0
Precursors-2	11625	11133	1.51	10/25/10/0
Polymer-1	14297	12501	1.47	10/25/10/2
Precursors-1’	8845	8191	1.53	10/35/0/0
Precursors-2’	13595	13367	1.60	10/35/10/0
Polymer-2	16397	14601	1.54	10/35/10/2

[a] Calculated by monomer feed ratio. [b] Measured by GPC in THF, calibrated against PS standards. [c] Determined by the integration ratio of ^{1}H NMR spectra. Precursors-1 and 1’ were, respectively, P(HEMA$_{10}$-co-DEAEMA$_{25}$)-Br and P(HEMA$_{10}$-co-DEAEMA$_{35}$)-Br. Precursors-2 and 2’ were, respectively, P(HEMA$_{10}$-*co*-DEAEMA$_{25}$)-*b*-PPEGMA$_{10}$ and P(HEMA$_{10}$-*co*-DEAEMA$_{35}$)-*b*-PPEGMA$_{10}$.

Table 2 Characteristic properties of DOX-loaded assembled micelles

Micelle	DOX (mg)	LC (%)	EE (%)	Size (nm)	PDI	Zeta (mV)
PMs-1	0	-	-	133.4	0.27	18.4
	5	4.1	20.0	145.2	0.30	17.9
	10	8.7	38.0	146.8	0.27	17.6
	20	13.1	30.0	154.3	0.32	16.5
PMs-2	0	-	-	155.6	0.29	22.1
	5	4.5	25.0	168.1	0.35	21.4
	10	10.8	48.5	174.7	0.34	20.3
	20	15.5	36.7	178.5	0.31	19.7