J Mater Sci: Mater Med (2010) 21:567–574
DOI 10.1007/s10856-009-3887-x

Micellar carrier based on methoxy poly(ethylene glycol)-block-poly(ε-caprolactone) block copolymers bearing ketone groups on the polyester block for doxorubicin delivery

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Received: 29 June 2009 / Accepted: 30 September 2009 / Published online: 15 October 2009

Abstract Block copolymers of Methoxy poly(ethylene glycol)-block-poly(ε-caprolactone) bearing ketone groups (MPEG-*b*-P(CL-*co*-OPD)) are synthesized and evaluated for its potential to form micelles containing doxorubicin (DOX), a representative anticancer drug, by using an in vitro method based on membrane dialysis to emulate drug release in vivo. The ^{1}H NMR spectra of the prepared block copolymers in D_2O solution exhibit peaks due to the P(OPD-*co*-CL) in decreased intensity, indicates that the polymers form micelle particles containing the hydrophilic segments in their external parts. The CMC of the copolymer decrease with an increase in the content of ketone groups in the hydrophobic chain. Drug-free and drug-loaded solutions of structurally related copolymers indicate the polymeric aggregation into micellar-type constructs. The size of the drug-loaded micelles is found to be larger than corresponding drug-free micelles. The release rate of MPEG-*b*-PCL micelles is faster than MPEG-*b*-P(OPD-*co*-CL) micelles in pH 7.4 buffered solution and they have a similar release rate in pH 5.0 buffered solution. This study, therefore, confirms the potential of a novel functional block copolymers, Methoxy poly(ethylene glycol)-block-poly(ε-caprolactone) bearing ketone Groups, for the formation of polymeric micelles for drug delivery.

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1 Introduction

Over the past decades, polymeric micelles from amphiphilic block copolymers have drawn considerable interests as promising delivery systems for drug targeting and diagnostic imaging applications [1–3], due to their ability to manipulate the structure of particles with functional derivatives, the low cytotoxicity in circulation, and the prolonged circulation time associated with a highly water-soluble structure, the escaping the reticuloendothelial cell system (RES) and renal extraction because of their prosmall particle size [4, 5]. In these micellar delivery systems, the hydrophobic core of the micelles is usually constructed with biodegradable polymers such as aliphatic polyesters, and the shell-forming block is frequently built with poly(ethylene glycol) (PEG), this is because they are biocompatible which makes them safe for human administration. At a long term, aliphatic polyesters have a history of safe application in human, but the lack of functional groups on the polymeric backbone restricts their extensive application of the medical field [6, 7].

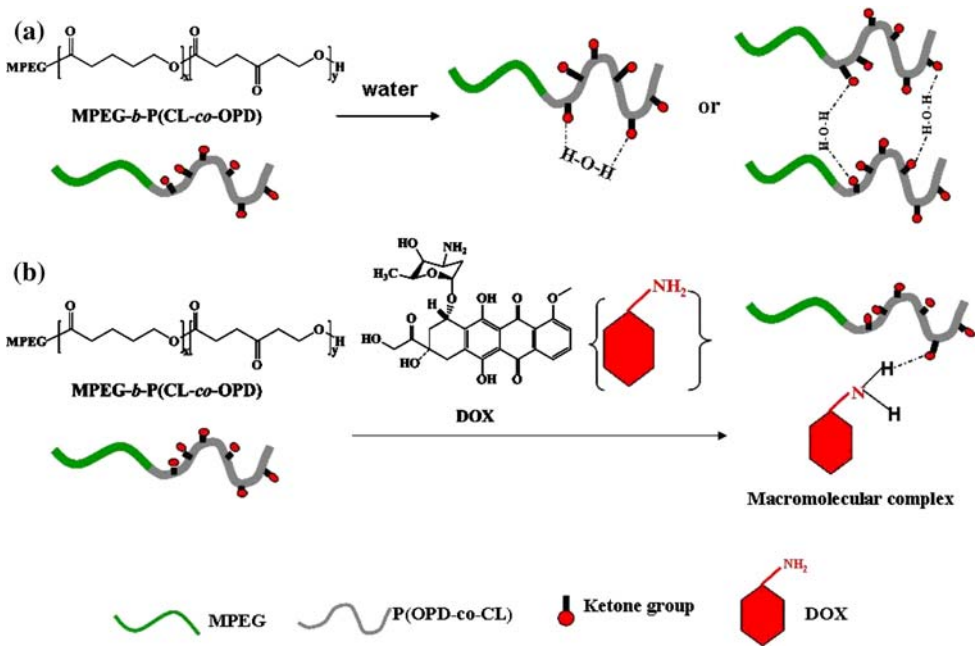

Fig. 1 **a** Interaction of MPEG-*b*-P (OPD-*co*-CL) and water during the preparation of micelles. **b** Interaction of MPEG-*b*-P (OPD-*co*-CL) and DOX during the preparation of drug-loading micelle

Recently, many amphiphilic aliphatic polyesters bearing reactive groups have been reported in the literature [8–13]. The introduction of functional groups to the polyester segment of PEG-*b*-polyester block copolymer has various advantages in drug delivery applications. First, the structure of core and shell in the polymeric micelles can be chemically manipulated to achieve the required micellar stability, which changes their thermodynamic and kinetic stability by attaching different functional groups such aromatic, hydroxyl, carboxyl and hydrophobic drug on the polyester chain [8–13]. Furthermore, the encapsulation and release properties of core-functionalized micelles can be controlled for certain drugs through formation of π–π interaction [14], hydrogen [15], electrostatic complexation [16], or some chemical reaction between the core forming block and drug [12, 13], so they have the potential for use as a pH-triggered drug release delivery system.

It is well known that doxorubicin (DOX) is a widely used anticancer drug in the treatment of many types of cancer [17, 18]. However, its water solubility is very low, and also it is the acute toxicity to normal tissue and inherent multi-drug resistance effect [19, 20]. To reduce the acute toxicity of the free drugs and alleviate the multi-drug resistance effect, polymeric micelle systems have been designed as delivery vehicles of DOX by most study groups [21–23]. Because of hydrophobic and chemical structure of DOX, it can be incorporated into the micelle inner core by both physical entrapment [14, 24–26] and chemical conjugation [12, 13, 27–29]. Lavasanifar and coworkers [13] reported a DOX delivery system based on doxorubicin-conjugated poly (ethylene oxide)-block-poly(ε-caprolactone) (PEO-*b*-PCL) block copolymers. Micelle-forming DOX conjugate didn't show any signs of DOX release at 37°C within 72 h of incubation at pH 7.4 and 5.0, but revealed signs of Polyester core degradation at pH 5.0. In their further study, PEO-*b*-PCL micelles bearing benzyl, carboxyl or DOX groups in the core were also used as micellar nano-containers for the physical encapsulation of DOX, where maximum level of drug-loading and controlled over the rate of DOX release were achieved by polymeric micelles containing benzyl groups in the core. Compared to the chemical conjugation strategy, physical entrapment of drugs in the micelle cores may be advantageous in terms of easy polymer preparation, simple micelle fabrication, high drug bioavailability (drug conjugated to polymer may affect the activity of drugs [30]) and dual-drug delivery.

In this paper we report on the self-assemble behavior and drug releasing behavior of MPEG-*b*-PCL block copolymers bearing ketone groups. The self-assemble micelle has functional inner core whose structure can increase the stability of micelles and change the encapsulation and release properties for DOX through formation of hydrogen bonds (Fig. 1), which lead to the development of pH-sensitive micelles with triggered drug release at basic pHs.

2 Materials and methods

2.1 Materials

Methoxy poly(ethylene glycol) (MPEG, $Mn = 5.0 \times 10^3$ g/mol, J&K Chemical) was purified by azeotropic

distillation with dried toluene three times, then freeze dried. ε-Caprolactone (Aldrich) was dried over CaH_2 for 3 days at room temperature and distilled under reduced pressure, prior to use. 2-Oxepane-1,5-dione (OPD) was synthesized by the Baeyer–Villiger oxidation of 1,4-cyclohexanedione following the method of Jérôme and coworkers [31]. Doxorubicin hydrochloride was supplied by Zhongshan Hospital, Shanghai, china, and was magnetically stirred in PBS (pH 9.18) for 1/2 h to neutralize hydrochloride, centrifuged (8000 rpm, 10 min), and then was washed with de-ionized water by such the same method. Toluene was purified by refluxing over a benzophenone-Na complex and distilled under nitrogen. *N*,*N*-dimethylformamide(DMF) was dried over anhydrous $MgSO_4$ for 1 night at room temperature and distilled under reduced pressure. Stannous octanoate (Sn(II)Oct, Aldrich) and methanol (AR grade, Nanjing Chemical Reagent Co, China) were used as received.

2.2 Methods

2.2.1 *Synthesis of Methoxy poly(ethylene glycol)-block-poly (ε-caprolactone) bearing ketone groups (MPEG-b-P(OPD-co-CL))*

The synthesis of block copolymers of MPEG-*b*-P(OPD-*co*-CL) with different contents of OPD were reported in detail in our recent publication [32]. Briefly, MPEG (0.5 g, 0.1 mmol), OPD (0.477 g, 3.73 mmol) and ε-CL (1.323 g, 11.61 mmol) were weighed in a glovebox, placed in a flame-dried flask, followed by addition of dry toluene (15 ml). Sn(II)Oct (0.153, 1 mol% of comonomer) was added to the comonomer mixture, that was maintained under N_2 and stirring in an oil bath at 90°C for 21 h. The resulting solution was added into excess cold methanol in a dropwise manner to precipitate MPEG-*b*-P(CL-*co*-OPD), filtered and dried in vacuum at room temperature until a constant weight was obtained. MPEG-*b*-P (CL-*co*-OPD)s with different OPD contents were obtained by varying the feed ratio of MPEG, OPD and ε-caprolactone monomer.

2.2.2 *Characterizations of block copolymers*

The ^1H spectra recorded on a Bruker AVANCE 500 spectrometer in deuterated water (D_2O) or chloroform ($CDCl_3$) at room temperature. ^1H measurements were made at frequencies of 500 MHz, and calibrated with respect to the solvent signal with tetramethylsilane as standard. The gel permeation chromatography (GPC) measurements were carried out with *N*,*N*-dimethylformamide (DMF) as the eluent (1.0 ml/min) with a Water 2414 HPLC pump, three Ultrastyragel columns (2 × 105, 105, and 5 × 104 Å) in series, and refractive index detector. The sample concentration is 10–15 mg/ml of DMF. The columns were calibrated with polystyrene standards with a narrow molecular weight distribution.

2.2.3 *Preparation of micelles*

Blank and DOX-loaded micelles were prepared by a membrane dialysis method. For blank micelles, the polymer (40 mg) was dissolved in 2 ml DMF, then was slowly added dropwise (~1 drop/15 s) into 20 ml Ultrapure water under moderate stirring at room temperature. The obtained solution was dialysed against Ultrapure water for 2 days using a dialysis membrane with a molecular weight cut-off 14,000. The water was replaced hourly for the first 3 h. For DOX-loaded micelles, DOX (5 mg) and block copolymers (20 mg) were both dissolved in 2 ml of DMF, followed by dropwise addition (~1 drop/15 s) of polymer solutions to Ultrapure water (20 ml) under moderate stirring at room temperature. The solution of DOX and polymer was dialysed against Ultrapure water 2 days to remove organic solvents and unencapsulated DOX dissolved in aqueous solution (M_w cut-off: 14,000 Da). After dialysis, the solution in the dialysis bag was collected and filtered with 0.45 μm syringe filter and freeze-dried for 2 days. To determine DOX loading level, a known amount of DOX-loaded nanoparticles was dissolved in 5 ml of DMF. The DOX concentration was estimated by using the UV–Vis spectrophotometer at 485 nm. The drug loading was calculated according to the standard curve obtained from DOX in DMF. The results of % DOX loading content (DLC) and encapsulation efficiency (DEE) were calculated using Eqs. 1 and 2.

$$\%\ \text{Drug loading content} = \left(\frac{\text{Amount of drug in beads}}{\text{Amount of beads}}\right) \times 100 \quad (1)$$

$$\%\ \text{Encapsulation efficiency} = \left(\frac{\text{Actual loading}}{\text{Theoretical loading}}\right) \times 100 \quad (2)$$

2.2.4 *Determination of critical micelle concentration*

The critical micelle concentration (CMC) of copolymers was measured using a surface tension method as reported by Fairley et al. [33]. Surface tension of polymer solutions with different micelle concentrations was recorded with a manual digital tensiometer (BZY-1, Shanghai equity Instruments, China) at 25°C.

2.2.5 Size and morphology of micelles

Average diameter (intensity mean) and size distribution of prepared micelles were estimated by dynamic light scattering (DLS) using a Malvern Nano-ZS at a polymer concentration of 1–1.6 mg/ml in water at 25°C after filtration through 0.45 μm filter.

Morphology of self-assembled structures was investigated by transmission electron microscopy (TEM). An aqueous droplet of micellar solution (0.04 ml) with a polymer concentration of 1–1.6 mg/ml was dropped on a copper-coated grid. After 1 min, the excess fluid was removed by filter paper, and then another seven drops of micellar solution were placed on the copper-coated grid by the same method. After that, the samples were air-dried and loaded into a Hitachi H 600 transmission electron microscope. Images were obtained at a magnification of 50,000 at 75 kV.

2.2.6 In vitro release

To obtain the drug release profile, a fixed amount of pure DOX drug and DOX-loaded micelle samples were suspended in 5 ml phosphate buffer solution (pH 7.4 or 5.0), respectively, then sealed in a dialysis tube (M_w cut-off: 14,000 Da) that immersed in 45 ml buffer solution at 37°C. The system was shaken at moderate speed. At regular time intervals, buffered solution (5 ml) outside the dialysis bag was removed for UV–Vis analysis and replaced with fresh buffer solution (5 ml). DOX concentration was calculated based on the absorbance intensity at 485 nm.

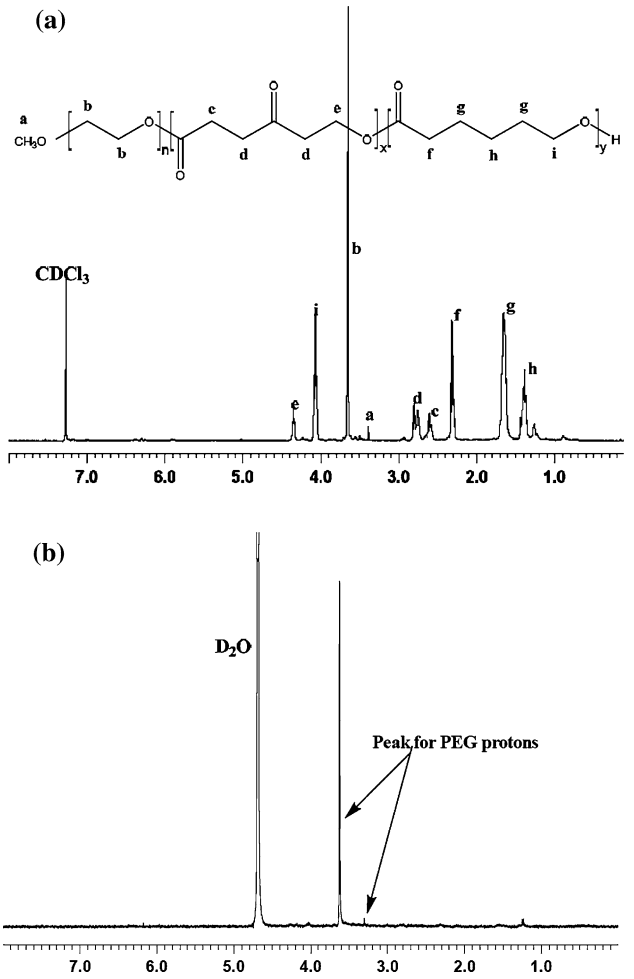

Fig. 2 ^{1}H NMR spectrum of MPEG-*b*-P (OPD-*co*-CL) in **a** $CDCl_3$, and **b** in D_2O

3 Results and discussion

3.1 Characterizations of block copolymers

In the ^{1}H NMR spectra of block copolymers dissolved in $CDCl_3$ (Fig. 2a), the characteristic chemical shifts corresponding to PCL (1.38, 1.65, 2.31, and 4.08 ppm), POPD(2.59, 2.77, 4.35 ppm)and MPEG (3.61 ppm) are observed. The lengths of P(CL-*co*-OPD) blocks and the content of OPD unit in polymer chain are calculated from the integral values of characteristic peaks of PEG (e.g., $–CH_2CH_2–$ at ~3.61 ppm), PCL (e.g., $–C(=O)–O–CH_2–$ at ~4.08 ppm) and POPD (e.g., $–C(=O)–O–CH_2–$ at ~4.35 ppm), using the known molecular weights of MPEGs. For all block copolymers, a unimodal distribution is observed in the GPC chromatograms (not shown). In addition, molecular weights detected by GPC are mostly in reasonable agreement with those calculated based on the ^{1}H NMR data. Molecular weights determined by ^{1}H NMR measurements are adopted to describe the molecular compositions of these block copolymers. Four copolymers were synthesized in this study, as listed in Table 1. These copolymers have different contents of OPD units in polymer chain, and thus allow us to investigate the effect of copolymer compositions on the micelle properties.

3.2 Chemical structure of micelles

Since the P(OPD-*co*-CL) block of the MPEG-*b*-P(OPD-*co*-CL) are hydrophobic in aqueous solution, self-assembly occurs in water, leading to micelles in water with P(OPD-*co*-CL) block as cores and MPEG block as shell. To prove the formation of core/shell structures of micelles in an aqueous environment, a certain amount of freeze-dried blank micelles was scattered in D_2O by ultrasound and characterized by ^{1}H NMR spectroscopy. As shown in Fig. 2, the spectra for MPEG-*b*-P(OPD-*co*-CL) block copolymers in $CDCl_3$ (Fig. 2a) and micelles in D_2O (Fig. 2b) are compared. By comparison of Fig. 2a and b, one can see, once MPEG-*b*-P(OPD-*co*-CL) micelles

Table 1 Composition and characterization of four copolymers

Copolymer	f^{a}_{opd}	Mn of hydrophilic segement[b] ($\times 10^3$g/mol)	Mn of hydrophobic segement[b] ($\times 10^3$g/mol)	PDI[c]	Yield (%)	CMC[d] (mg/ml)
P1	0.000	5.0	17.5	1.56	95.2	0.139
P2	0.070	5.0	17.1	1.56	87.4	0.298
P3	0.137	5.0	15.0	1.50	94.3	0.094
P4	0.215	5.0	16.8	1.62	88.6	0.051

[a] Molor fraction of OPD units in the copolymer is determined by ^{1}H-NMR spectroscopy

[b] Mn of hydrophobic segment is calculated from ^{1}H-NMR determined

[c] Polydispersity index is determined from GPC

[d] CMC value is determined from surface tension

formed (see Fig. 2b), the intensity of the signals due to the core-forming block relative to the one in $CDCl_3$ are reduced dramatically. These results indicate that the polymer molecules are dissolved in $CDCl_3$ where the formation of micelle is not expected, and that the molecules are not dissolved in D_2O to aggregate forming nano-particles.

3.3 CMC determination

The use of block copolymer micelles as long circulating drug delivery vehicles relies heavily on their thermodynamic stability which is determined by the CMC of the copolymer material. In this study, the aqueous association of the block copolymers was investigated by surface tension. A typical change of surface tension with copolymer concentration is shown in Fig. 3. The surface tension of this copolymer solution first is level, then diminishes with increasing concentration and starts to level off at a copolymer concentration of 0.0512 mg/ml. Therefore, we assume that 0.0512 mg/ml is the CMC of this copolymer [33]. The CMC values are collected in Table 1.

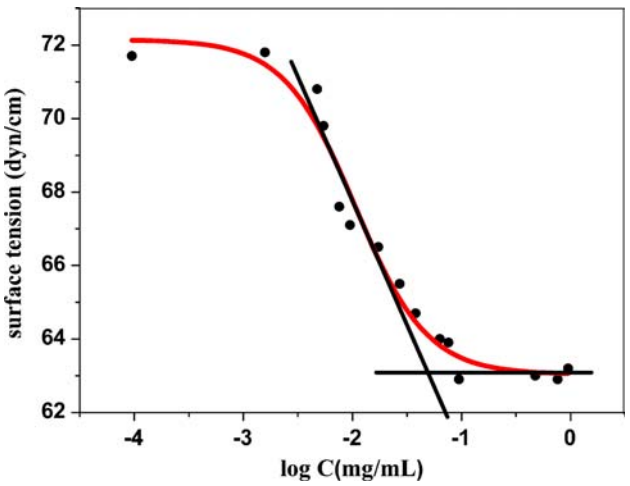

Fig. 3 Surface tension versus concentration of MPEG-*b*-P(OPD-*co*-CL) ($f_{opd} = 0.215$) in water

Interestingly, there is a trend observed when comparing the three copolymers (P2, P3, P4) with different ketone contents, the CMC values decrease with an increase in the content of ketone groups in the block copolymer, this is because that the presence of ketone groups in the block copolymers increase the intramolecular and intermolecular associations by hydrogen bond of water [34–36] (Fig. 1a). In other words, the presence of ketone groups on PCL block is favorable to increase the thermodynamic stability of micelles.

3.4 Morphology and size of blank and DOX-loaded micelles

The morphology and size distribution of blank and DOX-loaded micelles were investigated by TEM measurement and dynamic light scattering (DLS). It can be seen from TEM pictures (Fig. 4a, b) that the self-assembled micelles are well dispersed as individual nanoparticles with regularly spherical shape, which confirms that the micellization does take place. Furthermore, the diameters of the blank and DOX-loaded micelles are around 10–50 and 70–95 nm, respectively. The data are more likely to support the opinion that the micellization takes place as a result of molecular association, rather than aggregation of smaller micelles. The PDI of corresponding micelles, relatively low about 0.29 and 0.12 determined by DLS also reinforce this opinion, although the micelles exhibit larger average diameter of 75.1 ± 0.6 and 114.5 ± 0.5 nm, respectively, determined by DLS (Fig. 4c, d). This difference in micelles size measured by TEM and DLS should be attributed to that the latter is the hydrodynamic diameter of micelles in water, whereas the former reveals the morphology size of the micelles in solid state. Similar difference in size as a result of different measuring techniques was also reported in other studies [9].

The size of the copolymers with different ketone contents is listed in Table 2. From the Table 2, it can be see that the size of copolymer micelles is increasing with the

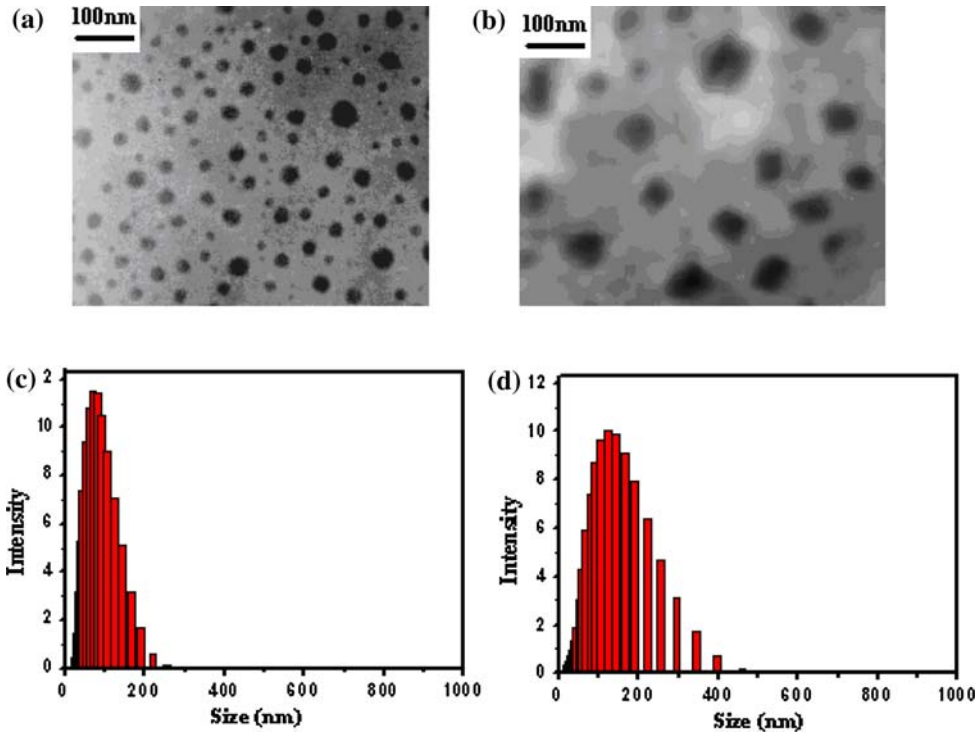

Fig. 4 TEM micropictures of blank micelles **a**, DOX-loaded micelles **b** and size distribution detected by DLS of blank micelles **c**, DOX-loaded micelles **d** in ultrapure water

Table 2 The size, DLC and DEE of the copolymers with different ketone contents

Micelles	f^a_{opd}	D^b/nm	PDI^b	D^c/nm	PDI^c	DLC/ wt%	DEE/ wt%
M1	0	47.3 ± 0.3	0.08	61.9 ± 0.8	0.13	11.2	69.4.
M2	0.070	75.1 ± 0.6	0.29	114.5 ± 0.5	0.12	9.5	40.5
M3	0.137	91.3 ± 1.5	0.23	134.0 ± 3.0	0.43	8.9	35.9
M4	0.215	120.0 ± 2.0	0.31	138.0 ± 1.0	0.17	6.0	22.3

[a] Molor fraction of OPD units in the copolymer is determined by ^{1}H-NMR spectroscopy

[b] Blank micelles

[c] DOX-loaded micelles

ketone contents increasing, and the size of the drug-loaded micelles is larger than corresponding drug-free micelles.

3.5 Drug loading and in vitro drug release

The potential application in controlled drug release of amphiphilic block poly(ε-caprolactone) bearing ketone Groups was evaluated by investigating the drug release profile from the corresponding micelles. It is known that the hydrophobic drug could be physically loaded and stabilized in the hydrophobic micellar inner core by hydrophobic interactions. In addition, the functional groups on the hydrophobic chain also play an important role in drug loading and encapsulation efficiency. The anticancer drug, DOX-loaded micelles were prepared by the dialysis method. % drug loading content (DLC) and encapsulation efficiency (DEE) of the copolymer with different ketone contents were collected in Table 2. It can be seen that both DLC and DEE decrease with an increase in the content of ketone groups on the block copolymer, this is because that the ketone groups in the hydrophobic chain may form hydrogen bonds with amino groups of DOX (Fig. 1b), this intermolecular interaction form macromolecular complex that may impede the drug physically loaded [37]. From the Table 2, we also can see that the DLC and DEE of the copolymers with different content of ketone groups are relatively high, which indicate that DOX can be effectively loaded into the micelles.

Considering the acidic nature of intracellular endocytic vesicles and tumor extracellular sites, the effect of pH on the release of drug-loaded micelles was studied and the pH-dependent release profile is shown in Fig. 5. In two different buffered solutions (pH 5.0 and 7.4), a typical two-phase-release profile is observed. That is, a relatively rapid release in the first stage followed by a sustained and slow release over a prolonged time up to 69 h. In comparison with the release at pH 7.4, DOX-release from micelles at pH 5.0 is much faster. This pH-dependent releasing behavior may benefit to reduce DOX toxicity to normal tissues and increase the cumulative release rate to tumor. Additionally, we also can see from the Fig. 5 that the release rate of MPEG-*b*-PCL micelles (M1, $f_{opd} = 0$) is faster than MPEG-*b*-P(OPD-*co*-CL) micelles (M3, $f_{opd} = 0.137$) in pH 7.4 buffered solution and they have a

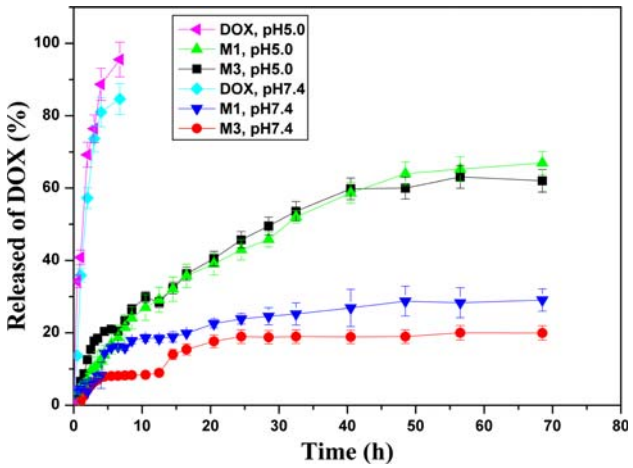

Fig. 5 Release profiles of free DOX from buffered solutions (at pH 5.0 and pH 7.5) and DOX-loaded micelles based on MPEG-*b*-PCL (M1) and MPEG-b-P(OPD-*co*-CL) (M3) incubated in phosphate-buffered saline (at pH 5.0 and 7.4) at 37°C. Data are presented as mean ± SD (n = 3)

similar release rate in pH 5.0 buffered solution. This difference of release rate in such pH solutions should be attributed to that the latter polymeric micelles may present hydrogen bonds between the ketone groups of MPEG-*b*-P(OPD-*co*-CL) and amino group of DOX at pH 7.4 buffered solution, which may be broken at pH 5.0 buffered solution. This result indicates that the MPEG-*b*-P(OPD-*co*-CL) has better performance of DOX delivery than MPEG-*b*-PCL.

4 Conclusions

Biodegradable diblock copolymers of MPEG-*b*-P(OPD-*co*-CL) with different ketone groups on PCL block were synthesized for the delivery of an anticancer drug, DOX. These amphiphilic polymers self-assembled into core–shell-structural micelles which were characterized by using ^{1}H NMR, TEM, DLS. The presence of ketone groups on PCL block is favorable to increase the thermodynamic stability of micelles. Further studies of this type of micelles loading DOX by physical entrapment leaded to the discovery that the release rate of MPEG-*b*-PCL micelles (M1, $f_{opd} = 0$) is faster than MPEG-*b*-P(OPD-*co*-CL) micelles (M3, $f_{opd} = 0.137$) in pH 7.4 buffered solution and they have a similar release rate in pH 5.0 buffered solution. This result indicates that the MPEG-*b*-P(OPD-*co*-CL) has better performance of DOX delivery than MPEG-*b*-PCL.

Acknowledgment This research was supported by the National Natural Science Foundation of China (20674019 and 20804015), "Shu Guang" Project of Shanghai Municipal Education Commission, Specialized Research Fund for the Doctoral Program of Higher Education (20060251015, 200802511021), the Natural Science Foundation of Shanghai, (08ZR1406000), Shanghai Key Laboratory Project (08DZ2230500) and Shanghai Leading Academic Discipline Project (B502).

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