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Folate-conjugated amphiphilic block copolymers for targeted and efficient delivery of doxorubicin

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ABSTRACT

In this paper, novel biodegradable amphiphilic block copolymers based on folate-conjugated poly(ethylene glycol)-*b*-copolycarbonates (FA-PEG-*b*-P(MAC-*co*-DTC)) and methoxy poly(ethylene glycol)-*b*-copolycarbonates (mPEG-*b*-P(MAC-*co*-DTC)) were successfully synthesized for targeted and efficient delivery of doxorubicin (DOX) to cancer cells. Immobilized *porcine pancreas* lipase (IPPL) was employed as the catalyst to perform the ring-opening copolymerization in bulk, while the folate-conjugated poly(ethylene glycol) (FA-PEG) or methoxy poly(ethylene glycol) (mPEG) was used as the initiator. The resulting copolymers, characterized by ^{1}H NMR and GPC, could self-assemble to form nano-sized micelles in aqueous solution by dialysis method. P(MAC-*co*-DTC) acted as the hydrophobic core, thereby aggregating hydrophilic PEG chains as the outer shell with FA as targeting ligand located at the surface of the polymeric micelles. Transmission electron microscopy (TEM) observation showed that the micelles dispersed in spherical shape with nano-size before and after DOX loading. Both the FA-conjugated and non-conjugated block copolymers showed low cellular cytotoxicity. Furthermore, as compared to the non-conjugated copolymers, much more efficient cellular uptake of the FA-conjugated copolymers *via* FA-receptor-mediated endocytosis could be observed by confocal laser scanning microscopy (CLSM), while MTT assays also demonstrated highly potent cytotoxic activity against HeLa cells.

1. Introduction

Drug delivery system (DDS), which is driven by the need to maximize therapeutic activity while minimizing negative side effects, is the key to the clinical success of many potent drugs [1,2]. As one of the most important kind of DDS, polymeric micelles have received significant attention over the past decades, which are formed by self-assembling of amphiphilic block copolymers in aqueous solution with a core and shell structure [3,4]. The hydrophobic inner core could encapsulate the poorly water-soluble drug, whereas the hydrophilic outer shell or corona of amphiphilic block copolymers could protect the drug from the aqueous environment and stabilize the polymeric micelles against recognition *in vivo* by the reticuloendothelial system (RES) [5]. PEG is the most popular choice of hydrophilic segment [6], while biodegradable aliphatic polycarbonates have been used as the hydrophobic core of polymeric micelles due to their low toxicity, favorable mechanical properties and biodegradability [7–9]. Aliphatic polycarbonates are generally synthesized by ring-opening polymerization (ROP) of cyclic carbonates [8]. In our previous studies, immobilized *porcine pancreas* lipase on silica particles (IPPL) has proven to be a powerful catalyst for the ROP of cyclic carbonates such as trimethylene carbonate (TMC), dimethyltrimethylene carbonate (DTC), 5-methyl-5-allyloxycarbonyl-1,3-dioxan-2-one (MAC) and also their copolymers with other kinds of cyclic monomers [10–13]. Enzymes have remarkable properties such as natural kinds of protein without toxicity, high catalytic and high selectivity under mild reaction conditions, while immobilized enzyme (such as IPPL) present promising stability and recyclability which will be good for its applications in the polymer synthesis systems [11–13]. Furthermore, it is very interesting that the control synthesis of aliphatic polycarbonates with functional pendent groups by enzymatic methods would offer a wide range of opportunities for further modification and functionalization.

On the other hand, the drug in the blood circulation usual leads to drug degradation and loss upon administration, and subsequently decreased drug bioavailability and lower drug accumulation in the pathological zone [14]. To address this challenge, various drug targeting systems have been developed in the past decades. One of the well-known receptor mediated targeting moieties is folic acid (FA), which has been widely exploited for the tumor-targeting DDS because FA-receptors are frequently overexpressed on the surface of human cancer cells [15,16]. Polymers

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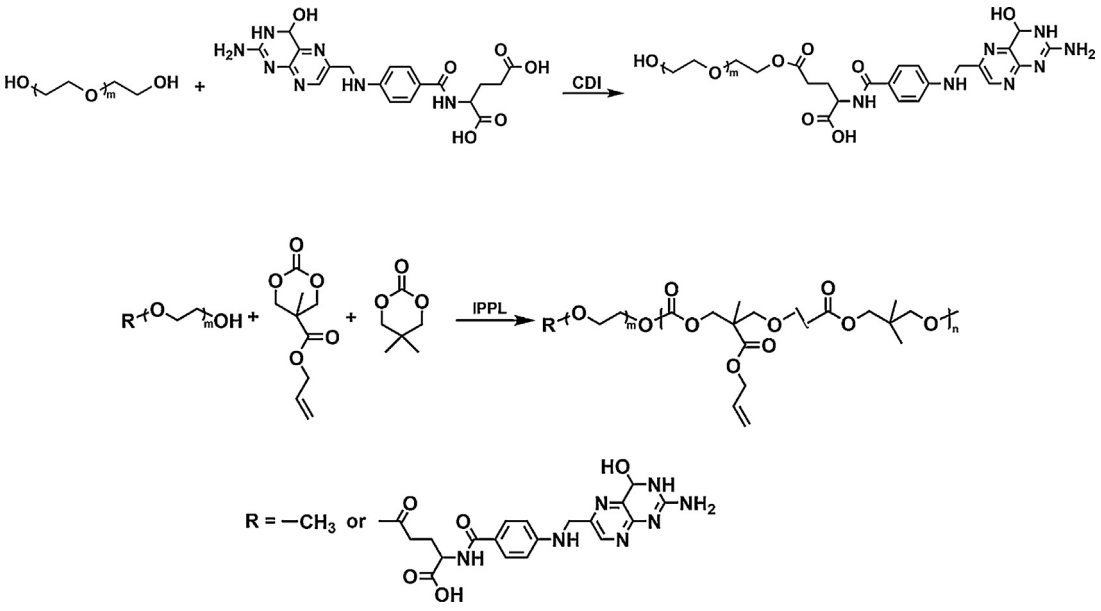

Scheme 1. Synthesis of folate-conjugated poly(ethylene glycol) (FA-PEG), folate-conjugated poly(ethylene glycol)-*b*-copolycarbonates (FA-PEG-*b*-P(MAC-*co*-DTC)) and methoxy poly(ethylene glycol)-*b*-copolycarbonates (mPEG-*b*-P(MAC-*co*-DTC)).

conjugated with FA could be efficiently taken up by malignant cells *via* FA receptor-mediated endocytosis. Many reports have focused on the FA-conjugated drug carriers based on polymeric micelles and nanoparticles [17,18].

In this paper, novel biodegradable amphiphilic block copolymers were successfully synthesized using IPPL as the catalyst. As shown in Scheme 1, the biodegradable copolymers of MAC with DTC acted as the hydrophobic core, while the hydrophilic long-circulating PEG block was employed as the outer shell with FA as targeting ligand located at the surface of the polymeric micelles. Considering the tumor-targeting properties of FA, anticancer drug of doxorubicin (DOX) was used as the model drug. The FA-conjugated amphiphilic block copolymers were proposed as a novel targeting DDS for efficient delivery of DOX to cancer cells.

2. Materials and methods

2.1. Materials

Methoxy poly(ethylene glycol) (mPEG, M_n = 2000 and 5000) were purchased from Acros and poly(ethylene glycol) (PEG, M_n = 2000 and 4000) were purchased from Shanghai Chemical Co., China. DOX hydrochloride (DOX·HCl) was obtained from Dalian Meilun Biology Technology Co. Ltd. MAC and DTC were synthesized according to the literature [19,20]. IPPL was prepared according to He et al. [21]. Folic acid (FA), 1,1-carbonyldiimidazole (CDI) were obtained from J&K Chemical Ltd. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), Dulbecco's phosphate buffered saline (PBS), 3-Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen Corp. HeLa cells were incubated in DMEM containing 10% FBS and 1% antibiotics (penicillin–streptomycin, 10,000 U/mL) at 37 °C and a humidified atmosphere containing 5% CO_2. Other reagents were of analytical grade and purified by general methods.

2.2. Synthesis of FA-PEG-b-P(MAC-co-DTC) and mPEG-b-P(MAC-co-DTC)

2.2.1. Synthesis of folate-conjugated polyethylene glycol (FA-PEG)

FA-PEG was synthesized *via* CDI mediated ester formation [22]. At first, 2 mmol (0.883 g) of FA was dissolved in 40 ml of dried dimethylsulfoxide (DMSO) followed by adding 2.2 mmol (0.358 g) of CDI and reacted for 4 h at room temperature in the dark. Then, 0.5 mmol of PEG (2000 and 4000) was added into the above solution. The reaction was further carried out in a dark condition for 24 h at room temperature. The reaction mixture was transferred into a dialysis tube (MWCO: 3500 or 14,000 Da) and dialyzed for 48 h against distilled water, which was changed every 4 h. The resulting solution was filtered and then lyophilized to obtain FA-PEG, which was kept in a dry box for future use.

2.2.2. Synthesis of FA-PEG-b-P(MAC-co-DTC)

FA-PEG-*b*-P(MAC-*co*-DTC) was synthesized in bulk by IPPL-catalyzed ring-opening copolymerization. The vessel containing FA-PEG, MAC, DTC and IPPL (3 wt‰ of MAC and DTC) with a magnetic stirring bar was dried *in vacuo* with anhydrous phosphorus pentoxide at room temperature for 24 h. Then the vessel was sealed *in vacuo* and immersed into an oil bath at 100 °C for 48 h. The reaction mixture was dissolved in DMSO and the insoluble IPPL was removed by filtration. Then the solvent was dialyzed in distilled water (MWCO: 3500 or 14,000 Da) for 48 h at room temperature. The distilled water was refreshed every 4 h. The resulting solution was lyophilized to obtain FA-PEG-*b*-P(MAC-*co*-DTC).

2.2.3. Synthesis of mPEG-b-P(MAC-co-DTC)

In comparison to FA-PEG-*b*-P(MAC-*co*-DTC), mPEG-*b*-P(MAC-*co*-DTC) was also synthesized by enzymatic methods simultaneously. Typically, mPEG (M_n = 2000 and 5000), MAC, DTC and IPPL (3 wt‰ of MAC and DTC) were copolymerized *in vacuo* at 90 °C for 8 h. The other procedures correspond to those described in the synthesis of FA-PEG-*b*-P(MAC-*co*-DTC).

2.3. Measurement

^{1}H NMR spectra were performed on a Mercury VX-300 spectrometer using tetramethylsilane (TMS) as an internal reference and $CDCl_3$ or DMSO-d_6 as the solvent. GPC analysis was performed on a Waters HPLC system equipped with a model 2690D separation module and a 2410 refractive index detector. DMF was used as the eluent at a flow rate of 0.3 ml/min. 20 μl of 1.0% (w/v) sample solutions was injected for each analysis. Waters Millennium module software was used to calculate molecular

weights based on a universal calibration curve generated by narrow molecular weight distribution polymethylmethacrylate standards. Fluorescence spectra were recorded using a RF-5301/PC (Shimadzu) spectrofluorometer (slit widths: 5 nm). Transmission electron microscope (TEM) observations were conducted on a JEM-2100 (HR) electron microscope at an acceleration voltage of 200 kV. The samples were prepared by dropping materials solution (0.1 mg/ml) onto the copper grid with Formvar film and dried at air. Nano-ZS 3600 (Malvern Instruments, UK) was used to measure the size distribution of the micelle solution. The micelle aqueous solution (0.1 mg/ml) was passed through a 0.45 μm pore-sized syringe filter before measurements. An average value was determined by three repeated measurements at 25 °C for each sample.

2.4. Loading DOX into the polymeric micelles

The DOX-loaded polymeric micelles were prepared by dialysis method, while the whole procedure was performed in the dark. The copolymers (40 mg), DOX·HCl (4 mg) and triethylamine (20 μl) were dissolved in 8 ml of DMSO, and then gently stirred for about 1 h. The solutions were transferred into a dialysis tube (MWCO: 3500 Da) and dialyzed against distilled water for 24 h. The distilled water was refreshed every 4 h to remove the residual DOX. The obtained solution was then filtered and lyophilized. DOX-loading content was analyzed by dissolving the DOX-loaded micelles in DMF and then measured by a UV spectrophotometer at 485 nm. Drug-loading content (DLC) and entrapment efficiency (EE) were calculated as follows:

$$\text{DLC}\,(\text{wt}\%) = \left(\frac{\text{weight of loaded drug}}{\text{weight of drug-loaded micelles}}\right) \times 100$$

$$\text{EE}\,(\%) = \left(\frac{\text{weight of loaded drug}}{\text{weight of drug in feed}}\right) \times 100$$

2.5. DOX release from the polymeric micelles

The release of DOX from the polymeric micelles was also investigated using dialysis method. 10 mg of the drug-loaded micelles was dispersed in 3 ml of phosphate buffered solution (PBS, 0.1 M, pH = 7.4) and transferred in a dialysis tube (MWCO: 3500 Da), which was then dialyzed against 25 ml of the same medium at 37 °C. At desired time intervals, 3 ml of release media was taken out and an equal volume of fresh media was then complemented. The amount of DOX released was determined by fluorometry (excitation at 480 nm and emission recorded at 555 nm). The release experiments were conducted in triplicate and the results were presented as the average values.

2.6. In vitro cytotoxicity assay

The cytotoxicity assay was performed with HeLa cells by MTT assay. Cells were seeded in a 96-well culture plate at a density of 6000 cells/well in 100 μl DMEM containing 10% FBS at 37 °C in a 5% CO_2 atmosphere for 24 h, and were exposed to polymeric micelle or DOX-loaded micelle solutions at a series of concentration for another 44 h. Then, 20 μl of MTT solution (5 mg/ml) was added to each well and further incubated for 4 h. After that, the media were exchanged by 150 μl DMSO to dissolve the formazan crystals formed by proliferating cell. The concentration of the proliferating cells in each well was confirmed by the absorbance of solvent at 570 nm using a microplate reader (Bio-Rad, Model 550, USA). The relative cell viability was calculated according to the following equation:

$$\text{Cell viability}\,(\%) = \frac{(A_{samples} - A_0)}{(A_{control} - A_0)} \times 100$$

where $A_{samples}$ was obtained in the presence of micelles extract solutions and A_0 was obtained with complete DMEM. The results were presented as the average values of four runs.

2.7. Confocal laser scanning microscopy

Live cell confocal microscopy was used to image the polymeric micelles uptake into HeLa cells. Hoechst 33342 water stock solution (blue molecular probe) was used to stain nucleus. HeLa cells were seeded at a density of 1×10^5 cells/well into biohousing chamber slide dishes loaded with a 25 mm diameter slide on cover-glass slides. After incubation for 24 h, the FA-conjugated or non-conjugated polymeric micelle solutions (with 5.1 mg/l DOX concentration) were added to each well and further incubated for 4 h. The medium was removed and the cells were washed three times with PBS. Then the nuclei were stained with 10 μl (2 μg/μl) of Hoechst 33342 at 37 °C for 15 min. Finally, the cells were washed three times again with PBS and incubated with 200 μl DMEM. The fluorescence was observed with a confocal laser scanning microscope (NOL-LSM 710, Germany) and the fluorescence signals of DOX and Hoechst 33342 staining were excited at 488 nm and 405 nm, respectively.

3. Results and discussion

3.1. Synthesis of FA-PEG-b-P(MAC-co-DTC) and mPEG-b-P(MAC-co-DTC)

In this study, the FA-conjugated biodegradable amphiphilic block copolymers FA-PEG-*b*-P(MAC-*co*-DTC) were developed for receptor-mediated delivery of DOX. For comparison, the non-conjugated copolymers mPEG-*b*-P(MAC-*co*-DTC) were also synthesized by the same method.

As shown in Scheme 1, FA-PEG was prepared according to the reported method [22]. 4 molar equivalent of FA was employed to perform the conjugated reaction completely, which was confirmed by ^{1}H NMR. Although the double esterified product FA-PEG-FA might also be obtained, it could not further reacted with MAC and DTC to yield the amphiphilic block copolymers.

Then FA-PEG-*b*-P(MAC-*co*-DTC) and mPEG-*b*-P(MAC-*co*-DTC) were synthesized in bulk by IPPL-catalyzed ring-opening polymerization using FA-PEG or mPEG as the macro-initiator. The results were confirmed by the ^{1}H NMR shown in Fig. 1. For both FA-PEG-*b*-P(MAC-*co*-DTC) and mPEG-*b*-P(MAC-*co*-DTC), 1.2–1.3 ppm, 4.2–4.3 ppm and 4.5–4.6 ppm were assigned to —C***H***$_3$, —OCOC***H***$_2$C— and —OC***H***$_2$CH=CH$_2$ of MAC respectively, while the characteristic allyl peaks (5.8–5.9 and 5.2–5.3 ppm) were also clearly shown. In addition to the characteristic signals in the region 0.9–1.0 and 3.9–4.0 ppm belonging to the protons in DTC segment, the characteristic proton signal of mPEG was observed at 3.65 ppm in $CDCl_3$. For mPEG-*b*-P(MAC-*co*-DTC), 3.24 ppm were assigned to the terminal —C***H***$_3$ protons of mPEG, while the FA-PEG-*b*-P(MAC-*co*-DTC) ^{1}H NMR spectrum clearly showed characteristic signals of FA at 2.3 ppm (γ-CH_2, glutamic acid), 6.6 and 7.6 ppm (aromatic protons), and 8.6 ppm (pteridine proton). The EG:MAC:DTC molar ratio in the copolymers was calculated from the integration of PEG signals at 3.65 ppm, MAC signals at 1.2–1.3 ppm and DTC signals at 0.9–1.0 ppm. As shown in Table 1, in the case of PEG initiator, the resulting block copolymer was relevant to the monomer feed ratio (Polymer-1 and Polymer-2). However, low initiating activity was

Table 1
Characterization of FA-PEG-*b*-P(MAC-*co*-DTC) and mPEG-*b*-P(MAC-*co*-DTC).

		EG:MAC:DTC		Molecular weight[b]	
		Feed	^{1}H NMR[a]	M_n	M_w/M_n
Polymer-1	$mPEG_{2K}$-*b*-P(MAC-*co*-DTC)	3.00:0.14:0.06	3.00:0.17:0.06	8100	1.16
Polymer-2	$mPEG_{5K}$ -*b*-P(MAC-*co*-DTC)	8.00:0.21:0.09	8.00:0.29:0.10	18,000	1.23
Polymer-3	FA-PEG_{2K}-*b*-P(MAC-*co*-DTC)	3.00:0.70:0.30	3.00:0.19:0.03	7600	1.07
Polymer-4	FA-PEG_{4K}-*b*-P(MAC-*co*-DTC)	6.40:1.75:0.75	6.40:0.25:0.09	11,200	1.10

[a] Calculated by ^{1}H NMR.
[b] Measured by GPC.

found for FA-PEG, resulting in much lower MAC and DTC contents in Polymer-3 and Polymer-4.

Furthermore, the molecular weight of FA-PEG-*b*-P(MAC-*co*-DTC) and mPEG-*b*-P(MAC-*co*-DTC) was also measured by GPC analysis. The results showed symmetric and narrow molecular weight distributions (Table 1).

3.2. Characterization of the polymeric micelles

The polymeric micelles were prepared by a dialysis method. FA-PEG-*b*-P(MAC-*co*-DTC) and mPEG-*b*-P(MAC-*co*-DTC) copolymers were all capable of self-assembling to nano-sized micelles in aqueous solution with FA-PEG or mPEG shells and hydrophobic P(MAC-*co*-DTC) cores.

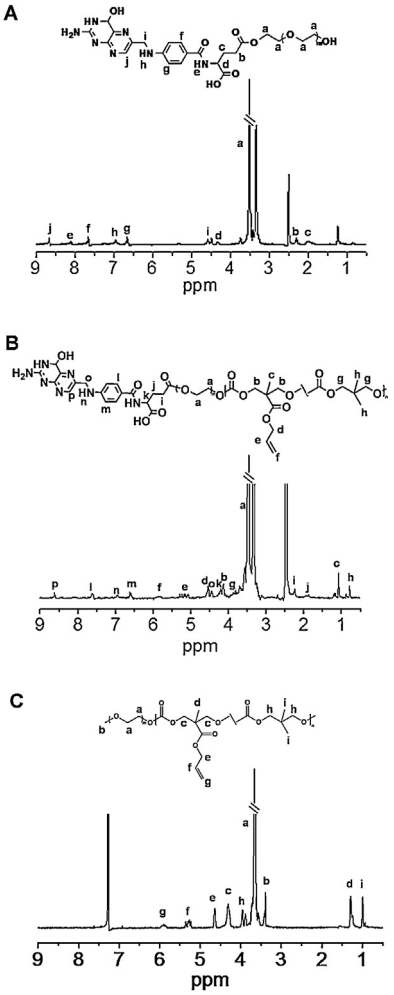

Fig. 1. ^{1}H NMR (300 MHz, $CDCl_3$/DMSO-d_6) spectra of FA-PEG (A), FA-PEG-*b*-P(MAC-*co*-DTC) (B) and mPEG-*b*-P(MAC-*co*-DTC) (C).

The critical micelle concentration (CMC) was determined using pyrene as a fluorescence probe [23], and was calculated from the plot of the excitation intensity ratio I_{338}/I_{335} as a function of the concentration of the polymer. It is reported that both a larger hydrophobic block and a smaller hydrophilic block would result in a higher overall hydrophobicity and thereby reducing the CMC of the amphiphilic block copolymers [24,25]. As shown in Table 2, the CMC values of Polymer-1 and Polymer-2 were 36.4 and 31.6 mg/l, while the CMC values of Polymer-3 and Polymer-4 were 28.4 and 23.2 mg/l, respectively. Compared with Polymer-1 and Polymer-3, Polymer-2 and Polymer-4 with higher hydrophilic/hydrophobic ratio showed the lower CMC values. Furthermore, although with almost the same total hydrophilic/hydrophobic ratio, the FA-conjugated Polymer-3 could self-assemble more easily to form micelles than the non-conjugated Polymer-1, which was consistent with previous report [26].

Dynamic light scattering (DLS) and TEM were used to characterize size and distribution of the resulting polymeric micelles. As shown in Table 2, the average diameters of Polymer-1–4 were 152.0, 109.1, 120.3 and 104.3 nm, respectively. In addition, TEM observation (Fig. 2) also showed the micelles dispersed in spherical shape with nano-size. Due to the collapse of the free hydrophilic segments of the polymer as well as the possible dehydration of the polymer chains [27], the average diameters of Polymer-1–4 estimated on the bases of the TEM images were about 23–33 nm, which were smaller than the values measured by DLS.

3.3. DOX loading

The potential of FA-PEG-*b*-P(MAC-*co*-DTC) and mPEG-*b*-P(MAC-*co*-DTC) as delivery carrier was evaluated using an anti-cancer chemotherapy drug DOX as the model drug. DOX was physically

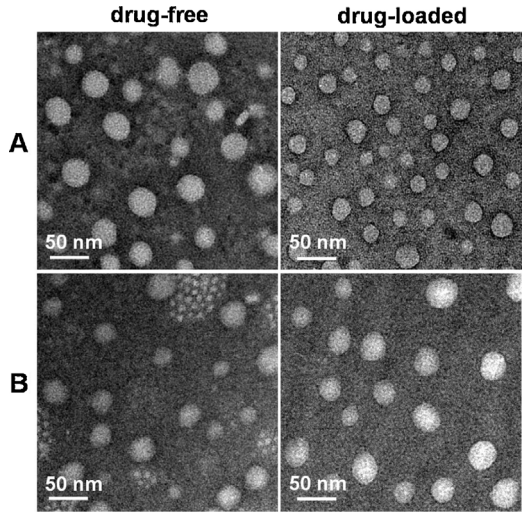

Fig. 2. Transmission electron microscope of drug-free and drug-loaded polymeric micelles based on Polymer-2 (A) and Polymer-4 (B).

Table 2
Properties of blank/DOX-loaded copolymer micelles.

	CMC (mg/l)	Diameter (nm)		PDI by DLS[b]	DLC[d] (wt%)	EE[d] (%)
		By DLS[a]	By TEM[c]			
Polymer-1	36.4	152.0/126.5	30/21	0.113/0.286	0.5	5.5
Polymer-2	31.6	109.1/100.9	33/27	0.184/0.113	1.7	19.0
Polymer-3	28.4	120.3/148.1	32/38	0.228/0.165	1.5	16.4
Polymer-4	23.2	104.3/128.8	23/32	0.233/0.134	4.0	43.6

[a] Hydrodynamic diameter of blank micelles/DOX-loaded micelles in water.
[b] Polydispersity index of blank micelles/DOX-loaded micelles obtained from DLS measurements.
[c] Estimated by TEM.
[d] Measured by a UV spectrophotometer at 485 nm.

loaded into FA-PEG-*b*-P(MAC-*co*-DTC) and mPEG-*b*-P(MAC-*co*-DTC) micelles by dialysis method. DOX loading was performed at 10% feed weight ratio, while the results were shown in Table 2. Compared with Polymer-1 and Polymer-3, the longer hydrophobic P(MAC-co-DTC) chain in Polymer-2 and Polymer-4 could enhance the interaction of polymer chains with drug molecules and then improve the DLC and EE [28]. In addition, the FA-conjugated block copolymers showed obviously higher DLC and EE values than the non-conjugated copolymers [29], in good agreement with CMC studies. The conjugation of FA led to the decrease of the CMC values [26], indicating the higher micelle stability of FA-PEG-*b*-P(MAC-*co*-DTC) and further suggesting in favor of DOX encapsulation into these micelles.

Furthermore, it is reported the increase in particle size after drug loading [30]. In this study, DLS and TEM results also showed that the DOX-loaded FA-conjugated micelles were slightly larger than that of corresponding blank micelles. And Fig. 2B displayed the morphology of DOX-free and DOX-loaded polymeric self-assemblies which formed spherical micelles with an average diameter of about 23 nm and 32 nm respectively. However, in the case of the non-conjugated copolymers PEG-*b*-P(MAC-*co*-DTC), the decrease in particle size after drug loading could be found in Fig. 2A, probably due to better hydrophobic interaction *via* DOX-loaded [31].

3.4. *In vitro* and intercellular drug release

The drug release behavior was investigated in PBS (pH 7.4) by monitoring the cumulative drug amounts released from the drug-loaded micelle solution placed in a dialysis tube. Fig. 3 shows the *in vitro* release profiles of DOX from polymeric micelles based on Polymer-2 and Polymer-4. At all time intervals, the FA-conjugated Polymer-4 showed an obviously higher release rate of DOX than the non-conjugated Polymer-2. For example, about 75% and 56% of loaded DOX could be released from Polymer-4 and Polymer-2 respectively after 72 h. It has been reported that the release rates of drug depend on multiple factors such as diffusion of drug through the nanoparticles stuff in the case of incorporated drug, polymer stuff biodegradation, desorption from the surface in the case of surface-bound drug, or the combination of both of these factors [32]. In this study, the conjugation of FA might accelerate the release of DOX at a certain extent, which was in accordance with the literature [33,34].

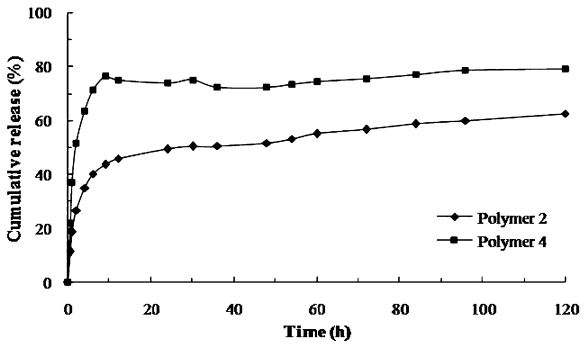

Fig. 3. *In vitro* drug release profiles from DOX-loaded micelles based on Polymer-2 and Polymer-4 in 0.1 M PBS (pH = 7.4) at 37 °C. Data represent the average values from triplicate experiments.

Furthermore, the cellular uptake of the micelles was monitored by CLSM to afford a visual inspection to track the internalization and intracellular localization of DOX (red fluorescence) in folate receptors positive HeLa cells. The nuclei of HeLa cells were stained by Hoechst 33342, and presented blue fluorescence to distinguish from the red fluorescence. As shown in Fig. 4, both the FA-conjugated Polymer-4 and the non-conjugated Polymer-2 micelles could enter into HeLa cells after 4 h of incubation. As expected, the red fluorescence intensity accumulated at the nuclei of HeLa cells internalized by the FA-conjugated polymeric micelles (Fig. 4B) was much more stronger than that by the non-conjugate micelles (Fig. 4A). This phenomenon could be attributed to the receptor-mediated targeting moieties of FA on HeLa cells [26,35].

It is reported that free DOX molecules could enter the cells and rapidly accumulate in the nuclei [36]. For the non-conjugated Polymer-2, the red fluorescence was almost uniformly distributed in the cytoplasm and nuclei (Fig. 4A). While for the FA-conjugated Polymer-4, the red fluorescence of DOX was predominantly accumulated at the nuclei of HeLa cells (Fig. 4B). The phenomenon could be due to a faster DOX-release from Polymer-4, and a lower DOX-release from Polymer-2 delaying nuclear uptake in HeLa cells [37]. These results are consistent with drug release behavior shown in Fig. 3. Considering the acidic intracellular environment, the drug delivery behavior at pH 5.0 was also performed. Compared to the medium at pH 7.4, the acidic environment accelerated the release of DOX at some extent, especially in the case of DOX-loaded FA-conjugated micelles. For example, about 93% and 60% of loaded DOX could be released from Polymer-4 and Polymer-2 respectively after 72 h, which was also in accordance with the literature [38].

3.5. *In vitro* cytotoxicity

In order to evaluate the toxicity profiles of the drug-free and drug-loaded micelles, the cell viabilities in HeLa cells were tested by MTT assay. In a wide range of concentration up to 500 mg/l, the drug-free micelles with or without FA-conjugation both displayed low cytotoxicity due to the cell-biocompatibility of polycarbonate and PEG (Fig. 5A).

In addition, the *in vitro* cytotoxicity of drug-loaded micelles based on the FA-conjugated Polymer-4 and the non-conjugated Polymer-2 was compared to that of free DOX. Fig. 5B showed the cytotoxicity results given as a function of DOX concentration from 0.16 to 10.0 mg/l. It is interesting that DOX-loaded Polymer-4 micelles in this study showed similarly potent cytotoxic activity against Hela cells in comparison with free DOX *in vitro*. At the

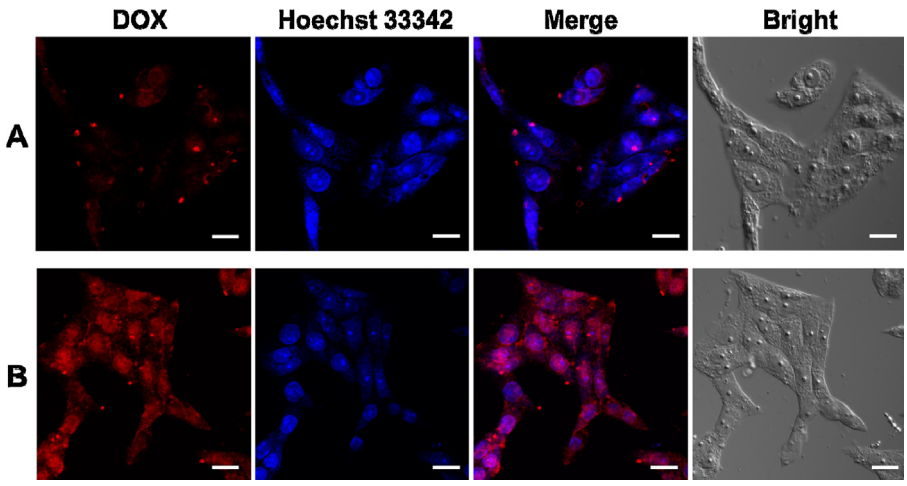

Fig. 4. Confocal microscopy images of HeLa cell lines incubated with DOX-loaded micelles based on Polymer-2 (A) and Polymer-4 (B) for 4 h at the same concentration of DOX (5.1 mg/l). Scale bar: 20 μm. For each panel, images from left to right show only the DOX fluorescence, nuclear staining by Hoechst 33342, the overlays of cells with DOX fluorescence and nuclear staining by Hoechst 33342, and bright field.

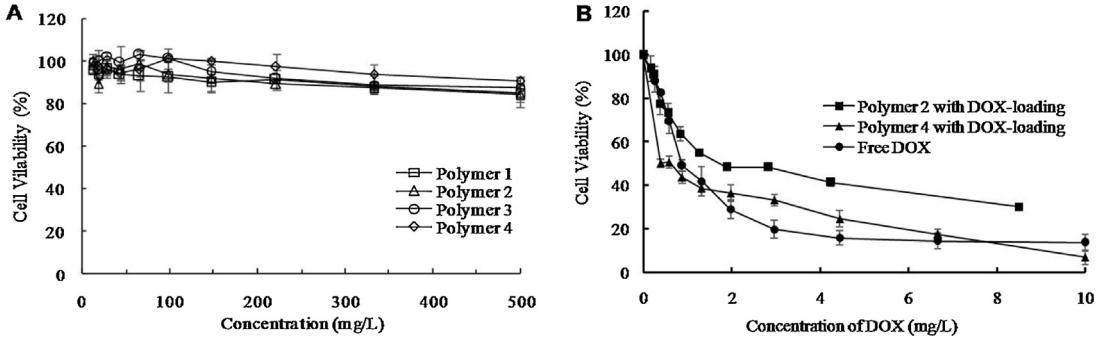

Fig. 5. Cytotoxicity of drug-free polymeric micelles (A), DOX solution and DOX-loaded polymeric micelles (B) in HeLa cells shown as mean ± S.D. ($n = 4$).

highest concentration tested (10 mg/l), free DOX and DOX-loaded Polymer-4 both exhibited lower cell viability (below 15%).

Moreover, the FA-conjugated Polymer-4 exhibited obviously higher cytotoxicity than the non-conjugated Polymer-2, while similar results have been also reported in the other cancer cells [39]. The IC50 (*i.e.*, inhibitory concentration to produce 50% cell death) values of Polymer-2 and Polymer-4 were determined to be 1.88 and 0.58 mg/l respectively for HeLa cells. As shown in Fig. 4, CLSM studies revealed a much more amount of DOX internalized by the FA-conjugated Polymer-4 than that by the non-conjugated Polymer-2. The over-expressed FA receptors on the cancer cells facilitate the effective recognition of the FA ligand distributed on the surface of micelles, leading to an increased cellular cytotoxicity originating from DOX. Moreover, also can be seen from Fig. 3, the non-conjugated Polymer-2 exhibited slower release rate and lower cumulative release, delaying cellular uptake and subsequent nuclear availability. Therefore, the higher potency of the DOX-loaded FA-conjugated Polymer-4 micelles might be attributed to its higher release rate of DOX shown in Fig. 3 and enhanced FA-receptor-mediated cell uptake by HeLa cells shown in Fig. 4. Furthermore, the higher DLS and EE of Polymer-4 as compared to that of Polymer-2 (shown in Table 2) also contributed to its highly potent cytotoxic activity, due to the benefit from a smaller amount of the DOX-loaded micelles required for cancer cells inhibition.

4. Conclusions

In this study, FA-PEG-*b*-P(MAC-*co*-DTC) and mPEG-*b*-P(MAC-*co*-DTC) were successfully synthesized by ring-opening copolymerization using IPPL as the catalyst for targeted DOX release. Both the FA conjugated and non-conjugated biodegradable amphiphilic block copolymers could self-assemble to form nano-sized micelles in aqueous solution and showed very low cellular cytotoxicity. CMC, micelle size, drug-loading capacity and entrapment efficiency, DOX release behavior as well as anti-cancer effects of DOX-loaded micelles depended on the structure of core-forming polycarbonate chains and FA-conjugated PEG chains. Compared with the non-conjugated copolymers, the FA-conjugated copolymers showed much more efficient cellular uptake and highly potent cytotoxic activity, suggesting that the FA-conjugated biodegradable amphiphilic block copolymers FA-PEG-*b*-P(MAC-co-DTC) were applicable drug carriers for targeted and efficient delivery of a hydrophobic cancer drug. Furthermore, the double bond-containing copolymers could afford facilities for further modification, which are currently underway in our laboratory.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.colsurfb.2013.11.049.

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