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Micellar carriers based on block copolymers of poly(ε-caprolactone) and poly(ethylene glycol) for doxorubicin delivery

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Abstract

Diblock copolymers of poly(ε-caprolactone) (PCL) and monomethoxy poly(ethylene glycol) (MPEG) with various compositions were synthesized. The amphiphilic block copolymers self-assembled into nanoscopic micelles and their hydrophobic cores encapsulated doxorubicin (DOX) in aqueous solutions. The micelle diameter increased from 22.9 to 104.9 nm with the increasing PCL block length (2.5–24.7 kDa) in the copolymer composition. Hemolytic studies showed that free DOX caused 11% hemolysis at 200 μg ml^{-1}, while no hemolysis was detected with DOX-loaded micelles at the same drug concentration. An in vitro study at 37 °C demonstrated that DOX-release from micelles at pH 5.0 was much faster than that at pH 7.4. Confocal laser scanning microscopy (CLSM) demonstrated that DOX-loaded micelles accumulated mostly in cytoplasm instead of cell nuclei, in contrast to free DOX. Consistent with the in vitro release and CLSM results, a cytotoxicity study demonstrated that DOX-loaded micelles exhibited time-delayed cytotoxicity in human MCF-7 breast cancer cells.

Keywords: Polymer micelles; Drug delivery

1. Introduction

Polymeric micelles from amphiphilic block copolymers [1,2] are supramolecular core-shell-type assemblies of tens of nanometers in diameter, which can mimic naturally occurring biological transport systems such as lipoproteins and viruses [3]. Recently, polymeric micelles as carriers of hydrophobic drugs have drawn increasing research interests, due to their various advantages in drug delivery applications. First, polymeric micelles are highly stable in aqueous solution because of their intrinsic low critical micelle concentration (cmc), which prevents the drug-entrapped micelles from dissociation upon dilution in the blood stream after intravenous injection. Furthermore, the nano size of polymeric micelles can facilitate their extravasations at tumor sites while avoiding renal clearance and non-specific reticuloendothelial uptake. In these micellar delivery systems, the hydrophobic

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core of the micelles is a carrier compartment that accommodates anti-tumor drugs, and the shell consists of a brush-like protective corona that stabilizes the nanoparticles in aqueous solution [3–5]. The micelle cores are usually constructed with biodegradable polymers such as aliphatic polyesters and polypeptides, and water soluble poly(ethylene glycol) is most frequently used to build the micelle corona because it can effectively stabilize the nanoparticles in blood compartments and reduce the uptake at the reticuloendothelial sites (e.g. liver and spleen) [4–9]. By encapsulating drugs within the micelles, solubility limits for hydrophobic drugs can be exceeded.

Anti-tumor drugs, such as doxorubicin (DOX) and paclitaxel, are widely used in cancer chemotherapy. Besides their low water solubility, major drawbacks of these drugs are the acute toxicity to normal tissue and inherent multi-drug resistance effect. To reduce the acute toxicity of the free drugs and improve their therapeutic efficacy, various liposome [10,11] and polymeric micelle systems were designed as delivery vehicles. The use of polymeric micelles as carriers of hydrophobic anticancer drugs has advanced greatly by the work of Kataoka group and others [12–20]. Hydrophobic drugs can be incorporated into the micelle inner core by both chemical conjugation and physical entrapment [4], depending on the chemical structure of drugs. For instances, paclitaxel was encapsulated into micelle cores usually by physical entrapment driven by hydrophobic interactions between the drug and the hydrophobic components of polymers. In contrast, DOX can also be chemically bound to the core of polymeric micelles through amidation of DOX amino groups, yielding high loading content. By this way, Kataoka and coworkers have achieved an efficient DOX delivery system based on doxorubicin-conjugated poly(ethylene glycol)–poly (aspartic acid) block copolymer (PEG–PAsp-(DOX)) [14]. The conjugation with DOX converted the hydrophilic poly(aspartic acid) into hydrophobic blocks that formed the hydrophobic micelle core and physically entrapped free DOX as well. Notable micellar accumulation [21] in solid tumors which eventually led to the complete tumor regression [22] in mice was achieved by the prolonged circulation in blood as well as the enhanced permeability and retention (EPR) effect [23]. However, the authors reported that DOX *conjugated* to poly(aspartic acid) played no direct role in anti-tumor activity in the murine tumor model, and instead the unconjugated DOX entrapped in micelle cores exerted the anti-tumor effects [14]. Very recently, DOX conjugation to the micelle cores through acid-cleavable linkage, such as a hydrazone bond, was reported to be an effective way to enhance the bioavailability of the chemically bound DOX [24,25]. The hydrazone linkage was cleaved in the endosomes/lysosomes (pH around 5) to yield free DOX molecules, which then functioned as the physically entrapped DOX. 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, and enhanced drug bioavailability. Although several micellar systems based on non-ionic amphiphilic block polymers such as PEO–PPO–PEO [15] and PEG-b-PBLA [26] have been reported, physically entrapped DOX delivery with polymeric micelles based on the well-known block copolymers of poly(ethylene glycol) and biodegradable polyesters is still very limited.

In the present work, diblock copolymers of MPEG and PCL were synthesized with various compositions for DOX delivery. Micelles formed with this class of copolymers were efficient carriers of paclitaxel, as reported by Kissel and coworkers [27]. Here, we investigated the relationships between the copolymer composition and the DOX-loading content (DLC) as well as the physicochemical properties of these DOX-loaded micelles, including the micelle size and DOX-release profiles. Furthermore, we evaluated the potential of these micelles as efficient DOX carriers by examining their blood compatibility, cellular internalization efficiency, and cytotoxicity against the cultured MCF-7 tumor cells.

2. Experimental section

2.1. Materials

ε-Caprolactone was purchased from Aldrich (Saint Louis, MO) and purified by vacuum distillation over calcium hydride (CaH_2). Toluene (from Aldrich) was dried by refluxing over sodium and distilled under dry argon. Monomethoxy poly(ethylene glycol) (MPEG, from Aldrich) was first purified

by precipitation from tetrahydrofuran (THF, from Aldrich) into hexane (from Aldrich), and then the vacuum-dried precipitates were further dehydrated by azeotropic distillation with dry toluene. Stannous(II) octoate ($Sn(Oct)_2$, from Aldrich) was used as received. DOX in aqueous solution (DOX–HCl, 2 mg ml^{-1}) was purchased from the Bedford Laboratories (Bedford, OH), and was deprotonated at pH 9.6 to obtain the hydrophobic DOX. Phosphate buffered saline (PBS, pH 7.4) and sodium acetate buffered solution (pH 5.0) were prepared. All organic solvents are of analytic grade. Human MCF-7 breast tumor cells were kindly provided by Dr. David Boothman's lab (Radiation Oncology Department, Case Western Reserve University).

2.2. Syntheses of diblock copolymers of PCL and MPEG

Detailed synthetic method for this class of block copolymers was described in a recent publication [28]. Briefly, the diblock copolymers (MPEG-b-PCL), with yields >95%, were synthesized by ring-opening polymerization of ε-caprolactone at 115 °C using MPEG as a macro-initiator and $Sn(Oct)_2$ as a catalyst. The degree of polymerization of the PCL block was calculated by comparing integrals of characteristic peaks of the PCL block at ~2.31 ppm (*triplet*, $-C(=O)-CH_2-$) and PEG block at 3.39 ppm (*singlet*, $-OCH_3$) in the ^1H NMR spectrum [28].

2.3. Characterization of copolymers

Fourier transform infrared (FTIR) spectral studies were carried out with a BIO-RAD FTS-575C FTIR spectrometer in the range between 4000 and 750 cm^{-1}, with a resolution of 2 cm^{-1}. Powdery samples were compressed into KBr pellets for the FTIR measurements. ^1H NMR spectra were recorded on a Varian 600-MHz NMR spectrometer in deuterated water (D_2O) or chloroform ($CDCl_3$) at room temperature.

Gel permeation chromatography (GPC) was employed to determine the molecular weight and the molecular weight distribution. GPC analysis was carried out using a PLgel 5 μm Mixed-D 300 × 7.5 mm column (Polymer Laboratories) with THF as an eluent (1 ml min^{-1}) and polystyrene standards for column calibration. Twenty microliter samples were injected. The eluent was analyzed with a Perkin–Elmer Series 200 differential refractive index (RI) detector.

2.4. Determination of critical micelle concentration

The cmc of copolymers was measured using a surface tension method as reported previously [28]. Surface tension of polymer solutions with different micelle concentrations was recorded with a manual digital tensiometer (Sigma 703, KSV Instruments, Finland) at 25 °C.

2.5. Preparation of DOX-loaded micelles

Polymeric micelles containing DOX were prepared as follows: MPEG-b-PCL copolymer (10 mg) and DOX (2 mg) were dissolved in THF (2 ml) in a glass vial. Afterwards, the solution was added dropwise to pure water (20 ml) under vigorous ultrasonic agitation using a Type 60 Sonic Dismembrator (Fisher Scientific) at a power level of 10. The beaker was then opened to air overnight, allowing slow evaporation of THF and formation of micelles. The residual THF was completely removed by vacuum distillation with a rotary evaporator. The micelle solution concentrated to 5 ml was filtered with a syringe filter (pore size: 0.45 μm) to eliminate the polymer and DOX aggregates, and then filtered through a MILLIPORE Centrifugal Filter Device (Mw cut-off: 100,000 Da) to remove free DOX dissolved in the micelle solution.

The micelles thus obtained were characterized with photon correlation spectroscopy. Measurements were performed at 25 °C on a 90 Plus Particle Size Analyzer from Brookhaven Instruments. Scattered light was detected at 90° angle and collected on an autocorrelator. For each sample, data obtained from five measurements were averaged to yield the size and size distribution.

2.6. Determination of DOX-loading content (DLC)

The DLC was defined as the weight percentage of DOX in the micelle. DLC was quantified by determining the absorbance at 485 nm using a Perkin–Elmer Lambda 20 UV–Vis spectrophotometer. First, the micelle solutions were frozen and lyophilized to

yield the solid micelle samples. Then the dried samples were redissolved in a mixture of chloroform and DMSO (1:1, v/v) for the UV–Vis measurement. DOX solutions of various concentrations were prepared, and the absorbance at 485 nm was measured to generate a calibration curve for the DLC calculations from various micelles.

2.7. In vitro release of DOX from polymer micelles

Freeze-dried micelle samples (15 mg each) were re-suspended in PBS or acetate buffered solutions and transferred into a dialysis tubing (Mw cut-off: 50,000 Da, supplied by Spectrum Laboratories, USA). The tubing was placed into 50 ml PBS or acetate buffered solutions. Release study was performed at 37 °C in a New Brunswick Scientific C24 Incubator Shaker. At selected time intervals, buffered solution outside the dialysis bag was removed for UV–Vis analysis and replaced with fresh buffer solution. DOX concentration was calculated based on the absorbance intensity at 485 nm.

2.8. Hemolysis study

Blood was freshly obtained from a male beagle dog and collected in heparin-coated tubes. Blood was washed three times with PBS and collected by centrifugation at 2800 rpm for 5 min. DOX-loaded micelle solutions were prepared at different micelle concentrations varying from 29.3 μg ml^{-1} to 5.46 mg ml^{-1} for PEG5k-b-PCL5k micelles (cmc: ~ 20 μg ml^{-1}), and 45.4 μg ml^{-1} to 4.14 mg ml^{-1} for PEG5k-b-PCL24.7k micelles (cmc: ~ 12 μg ml^{-1}) in the PBS buffer. Accordingly, polymer concentration range is from 28.1 μg ml^{-1} to 5.24 mg ml^{-1} for PEG5k-b-PCL5k micelles, and from 43.4 μg ml^{-1} to 3.96 mg ml^{-1} for PEG5k-b-PCL24.7k micelles, calculated based on the DLCs of these two micelles. DOX concentrations from the micelle solutions were also calculated based on the DLCs of micelles (Table 1). Typically, 100 μl of the erythrocyte suspension were added to 900 μl of micelle solutions. The samples were incubated for 60 min at 37 °C in a New Brunswick Scientific C24 Incubator Shaker. The release of hemoglobin was measured by UV–Vis analysis of the supernatant at 540 nm after centrifugation at 12,000 × *g* for 60 min. The complete hemolysis was achieved by incubating the same amount of erythrocytes with 0.2% Triton X-100, and all hemolysis data points are presented as the percentage of the complete hemolysis. The DOX concentrations of the measured micelle solutions were obtained based on their DLCs (Table 1), and were plotted versus hemolysis percentage.

Table 1
Influence of copolymer compositions on micellar properties

Micelles	Copolymers	Micelle size (nm)		Loading content (%)	IC_{50} (μM)
		DOX-free	DOX-loaded		
MD1	MPEG2k-b-PCL2k	17.0 ± 0.1	25.4 ± 0.2	3.29	0.035
MD2	MPEG5k-b-PCL2.5k	29.7 ± 0.2	22.9 ± 0.2	3.10	0.037
MD3	MPEG5k-b-PCL5k	41.0 ± 0.2	37.3 ± 0.2	4.03	0.033
MD4	MPEG5k-b-PCL8.5k	56.9 ± 0.3	84.0 ± 0.3	4.09	0.035
MD5	MPEG5k-b-PCL24.7k	86.3 ± 0.2	104.9 ± 0.2	4.30	0.048

2.9. Confocal laser scanning microscopy (CLSM)

Free DOX (1 μM) and DOX-containing micelles (DOX concentration: 1 μM) were incubated in MCF-7 cell culture for 2 and 24 h before confocal laser scanning microscopy (CLSM) examination. To identify the micelle location, cell nuclei were stained with Hoechst 33342 (Molecular Probes) and culture media were replaced with PBS during microscopy. Samples were examined by CLSM using a Zeiss LSM 510 (Zurich, Switzerland) with a confocal plane of 300 nm. Hoechst 33342 and DOX were excited at 352 and 485 nm with emissions at 455 and 595 nm, respectively.

2.10. In vitro cytotoxicity study against MCF-7 breast tumor cells

Human MCF-7 breast cancer cells were seeded onto 48-well plates with a seeding density of 7000 cells per well. Cells were maintained in Roswell Park Memorial Institute (RPMI-1640, Sigma) media supplemented with 5% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 5000 units ml^{-1} peni-

cillin, 5 mg ml^{-1} streptomycin, 0.1 mg ml^{-1} gentamicin sulfate and amphotericin-B, 25 mM KCl, 25 mM D-glucose, and incubated for 1 day at 37 °C in a humidified atmosphere with 5% CO_2. Then the cells were incubated in the free DOX or DOX-loaded-micelle containing media. For all micelle samples tested, a culture media containing 10 μM of DOX was first prepared by suspending a certain amount of micelle powder in the culture media. It was then diluted to obtain the other four DOX concentrations at 1, 0.1, 0.01, and 0.001 μM. The amount of micelle powder needed to prepare the culture media containing 10 μM of DOX was calculated based on the DLCs (Table 1) in these micelles. After 1 or 5 days, cells were harvested and DNA levels were analyzed through a DNA assay with a Perkin–Elmer HTS 7000 Bioassay Reader. The cytotoxicity of DOX and DOX-containing micelles in aqueous solution was analyzed and compared.

3. Results and discussion

3.1. Characterization of diblock copolymers

In the ^1H NMR spectra of block copolymers dissolved in $CDCl_3$, the characteristic chemical shifts corresponding to both PCL (1.38, 1.65, 2.31, and 4.06 ppm) and MPEG (3.39 and 3.64 ppm) were observed. The lengths of PCL blocks were calculated from the integral values of characteristic peaks of PEG (e.g. CH_3O- at ~3.39 ppm) and PCL (e.g. $-C(=O)-CH_2-$ at ~2.31 ppm), using the known molecular weights of MPEGs. For all block copolymers, a unimodal distribution was 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 ^1H NMR data. Molecular weights determined by ^1H NMR measurements are adopted to describe the molecular compositions of these block copolymers. Five copolymers were synthesized in this study, as listed in Table 1. These copolymers have different PCL or PEG lengths, and thus allow us to investigate the effect of copolymer compositions on the micelle properties.

3.2. Micelle properties

As shown in Table 1, micelle size depends much on the copolymer composition and is mostly less than 100 nm in diameter. In general, copolymers with longer PCL blocks formed larger micelle particles. For example, for the same PEG length (5 kDa), increasing PCL length from 2.5 (MD2) to 24.7 kDa (MD5) led to an increased micelle diameter from 22.9 to 104.9 nm. Effect of DOX-loading on the micelle size is less obvious and depends on the copolymer composition. DOX-loading caused size increases for MD1, MD4 and MD5 micelles while a slight size decrease was observed in the other two micelle formulations (Table 1). In addition, DOX-loading generally did not obviously change the narrow size distributions of micelles (Fig. 1). Similar results were also obtained with the paclitaxel-loaded MPEG-b-PCL micelles, as demonstrated by Kissel and coworkers [27].

Encapsulation of DOX inside the micelle cores was demonstrated by comparing the ^1H NMR spectra of the micelle samples in $CDCl_3$ and D_2O (Fig. 2). In $CDCl_3$, prominent resonance peaks (~ 0.8, 1.2, and 7.1 ppm) of DOX were observed in addition to those of PCL and MPEG blocks, indicating that the micelle contains both copolymer and DOX. In contrast, only the MPEG resonance peaks were detected in D_2O while both the PCL and DOX resonance peaks were hardly observed. These results clearly suggest the core-shell structure of DOX-loaded micelles, consistent with literature reports based on similar ^1H or ^{13}C NMR data [29–33]. The micelle shells consisting of PEG blocks were well solvated in D_2O and therefore showed clear ^1H NMR signal. In contrast, when DOX was encapsulated inside the micelle cores, resonance peaks of PCL blocks and DOX were not observed due to their insufficient chain motion in D_2O.

3.3. Dependence of drug loading on copolymer compositions

Unlike the paclitaxel-loaded MPEG-b-PCL micelles in which the drug-loading content increased significantly with the increase of the PCL length [27], the DLC in the MPEG-b-PCL micelles seems to be much less affected by the copolymer composition. DLC increased only by 1.3% from 3.1% to 4.4%

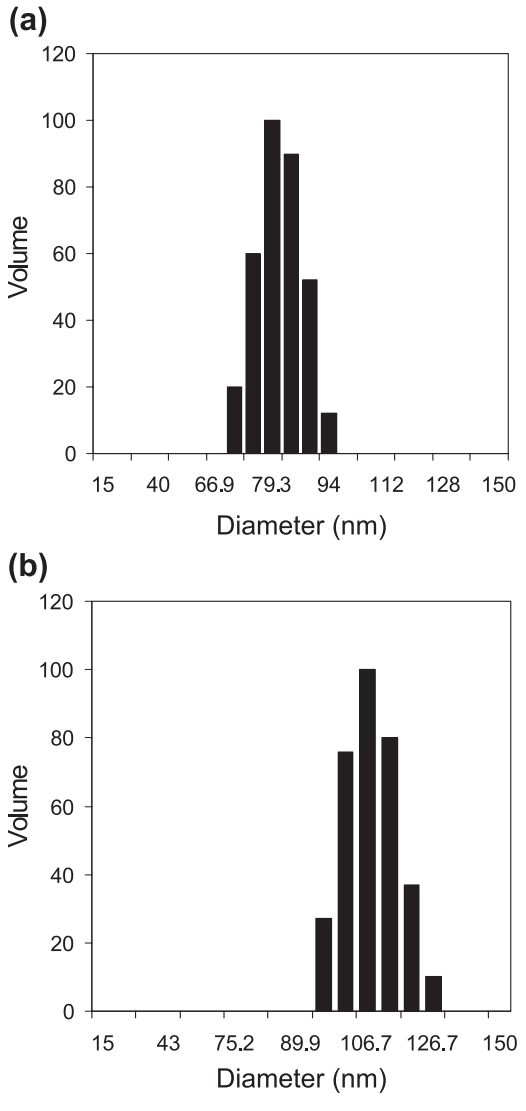

Fig. 1. Size distribution of DOX-free (a) and DOX-loaded (b) micelles based on MPEG5k-b-PCL24.7k.

when the PCL molecular weight increased from 2.5 to 25 kDa in the MPEG5k-b-PCL micelles. The DLC in the MPEG2k-b-PCL2k micelle (3.29%) is very close to that in the MPEG5k-b-PCL2.5k micelle (3.10%), indicating that the MPEG length has even less effect on the DLC than the PCL length.

It is known that physical entrapment of hydrophobic drugs in polymeric micelles is driven by the hydrophobic interactions between the drug and hydrophobic segments of polymers. In general, the drug hydrophobicity plays a decisive role in the drug-loading process. Compared to paclitaxel, DOX is inherently less hydrophobic due to its polar hydroxyl and amino groups (Fig. 3), and thus is less likely to be entrapped in the hydrophobic micellar core. In addition to the hydrophobic interactions between PCL and DOX, two other factors, i.e. the PCL crystallinity and hydrogen-bonding interactions between DOX and PCL, are hypothesized to affect the DLC. Higher PCL crystallinity in the micelle cores will decrease the DLC because only the amorphous PCL phase is likely to accommodate drug molecules. For semi-crystalline polymers such as PCL, higher molecular weight usually leads to higher crystallinity. Although the micelle cores constructed with longer PCL segments are generally

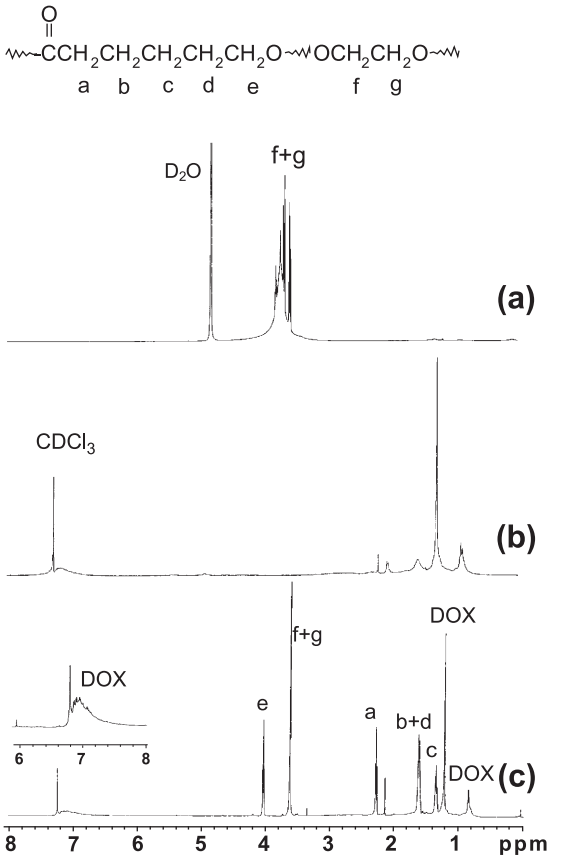

Fig. 2. ^{1}H NMR spectra of DOX-loaded MPEG5k-b-PCL5k micelles in D_2O (a) and $CDCl_3$ (c), in comparison with the spectrum of DOX in $CDCl_3$ (b).

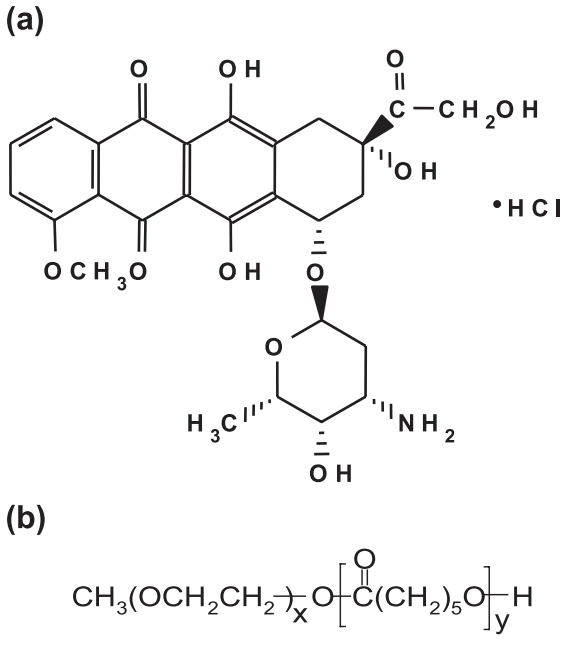

Fig. 3. Chemical structures of DOX (a) and diblock copolymer MPEG-b-PCL (b).

bigger in size and may encapsulate more drug molecules, the higher PCL crystallinity may lead to less DOX-loading in the micelles. Meanwhile, the deprotonated DOX contains hydroxyl and amino groups, which may form hydrogen-bonds with the carbonyl groups of the amorphous PCL. For shorter PCL segments that have lower crystallinity, the H-bonding interactions can enhance the DOX-loading in the micelle cores.

FTIR was employed in the present study to investigate the PCL crystallinity and intermolecular hydrogen-bonding between PCL and DOX. It is known that the carbonyl band of PCL is well resolved into two bands corresponding to crystalline (~ 1725 cm^{-1}) and amorphous (~ 1735 cm^{-1}) absorptions [34,35]. The FTIR spectra of two freeze-dried micelle samples are compared in Fig. 4. MPEG5k-b-PCL24.7k micelle shows strong absorption at ~ 1725 cm^{-1}, indicating the high PCL crystallinity in the core of this micelle (Fig. 4a and b). In contrast, the crystalline absorption of MPEG5k-b-PCL5k micelle is not recognizable (Fig. 4c). In addition, a weak but clear absorption of hydrogen-bonded carbonyl groups at ~ 1715 cm^{-1} was observed especially in the FTIR spectrum of MPEG5k-b-PCL5k micelle (Fig. 4c). The DLC inside the micelles should depend on the PCL/DOX hydrophobic interactions, PCL/DOX hydrogen-bonding interactions, and PCL crystallinity. Therefore, although MPEG5k-b-PCL24.7k provides stronger hydrophobic interactions with DOX, its higher PCL crystallinity and reduced hydrogen-bonding with DOX may have an opposite effect to decrease the DLC. These compromised effects may explain the less dramatic increase of DLC from MPEG5k-b-PCL2.5k (3.10%) to MPEG5k-b-PCL24.7k micelles (4.30%) (Table 1).

3.4. Hemolytic properties of polymer micelles

Fig. 5 showed the hemolytic activities of free DOX and two different DOX-loaded polymer micelles. Both micelle formulations with MPEG5k-b-PCL24.7k and MPEG5k-b-PCL2.5k copolymers did not show any detectable hemolytic activities on red blood cells (RBC) in the whole experimental DOX concentration range (1.2–225 μg ml^{-1}). In Fig. 5, the DOX concentrations for different micelle solutions were calculated based on the DLCs (Table 1) and micelle concentrations. It is noteworthy that the experimental

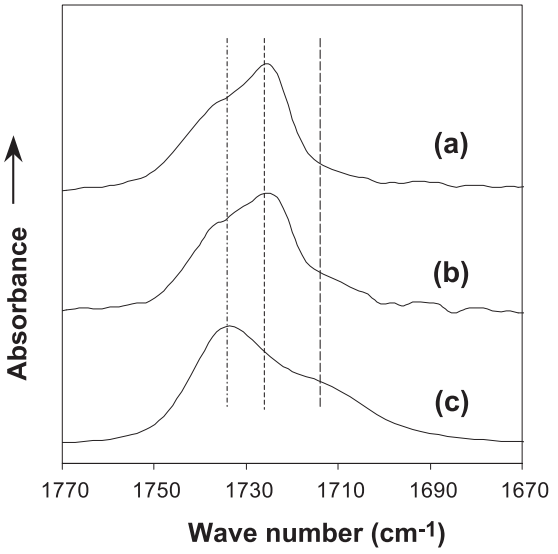

Fig. 4. FTIR spectra of the freeze-dried MPEG5k-b-PCL24.7k empty micelles (a), MPEG5k-b-PCL24.7k DOX-loaded micelles (b, 4.30% DLC), and MPEG5k-b-PCL5k DOX-loaded micelles (c, 4.03%).

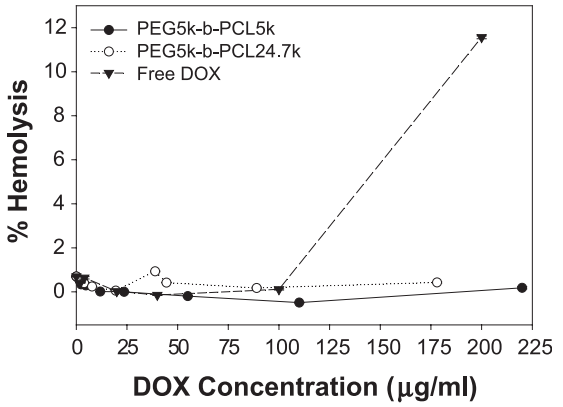

Fig. 5. Hemolytic activities of the free DOX and two DOX-loaded micelles as a function of DOX concentration. Each data was repeated three times to provide the standard deviation.

micelle concentrations of the two tested copolymers are all above their cmc values, as described in Section 2. The data demonstrate that the PCL length and micelle diameter do not affect the hemolytic activities of these micelles. The hemolytic activity of soluble DOX depends on the DOX concentration. Hemolysis was not detected at concentrations below 100 μg ml^{-1} while 11% hemolysis was detected at 200 μg ml^{-1}. It is noteworthy that some low molecular weight surfactants currently used in intravenous administration of hydrophobic drugs, such as the non-ionic Tween 80, were reported to interact with the lipid bilayer of cell membrane and cause membrane damage to normal cells [20]. Compared to these low molecular weight surfactants, MPEG-b-PCL micelles are apparently more hemocompatible for drug delivery applications.

3.5. In vitro DOX-release study

The in vitro release behaviors of DOX-loaded micelles in two different buffered solutions (pH 7.4 and 5.0) were studied and representatively shown in Fig. 6. In both solutions, a typical two-phase-release profile was observed. That is, a relatively rapid release in the first stage followed by a sustained and slow release over a prolonged time up to several weeks. In comparison with the release at pH 5.0, DOX-release from micelles at pH 7.4 is much slower. The faster release of DOX in acidic conditions was also observed by Kataoka and coworkers with the DOX-loaded PEG–PBLA copolymer micelles [26], and is likely due to the re-protonation of the amino group of DOX and faster degradation of micelle core at lower pH. This pH-dependent releasing behavior is of particular interest in achieving the tumor-targeted DOX delivery with micelles. It is expected that most DOX encapsulated in micelles will remain in the micelle cores for a considerable time period when the injected micelles stay in the plasma at normal physiological conditions (pH 7.4). However, a faster release will occur once the micelle particles reach the solid tumor site where pH value is lower than that in the normal tissue [36]. In addition, micellar particles are usually internalized inside the cells by endocytosis [37]. Therefore, a further accelerated release inside the endosome/lysosome of tumor cells may occur due to the decreased pH values.

3.6. Cell uptake study with CLSM

CLSM was used to study the internalization of DOX-loaded micelles into the breast cancer MCF-7 cells. As shown in Fig. 7, intracellular distribution of the DOX-loaded micelles is different from that of free DOX. After 2 h of cell incubation with the free DOX, strong fluorescence was observed in cell nuclei in addition to the very weak fluorescence in cytoplasm.

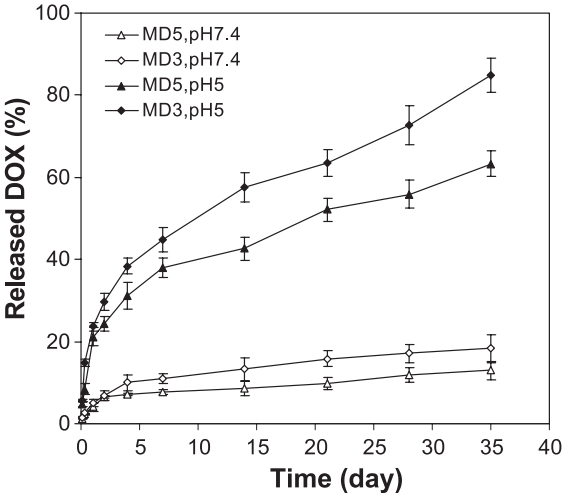

Fig. 6. In vitro DOX-release profiles from two micelle formulations (MD3 and MD5) at neutral (pH 7.4) and acidic conditions (pH 5.0) at 37 °C.

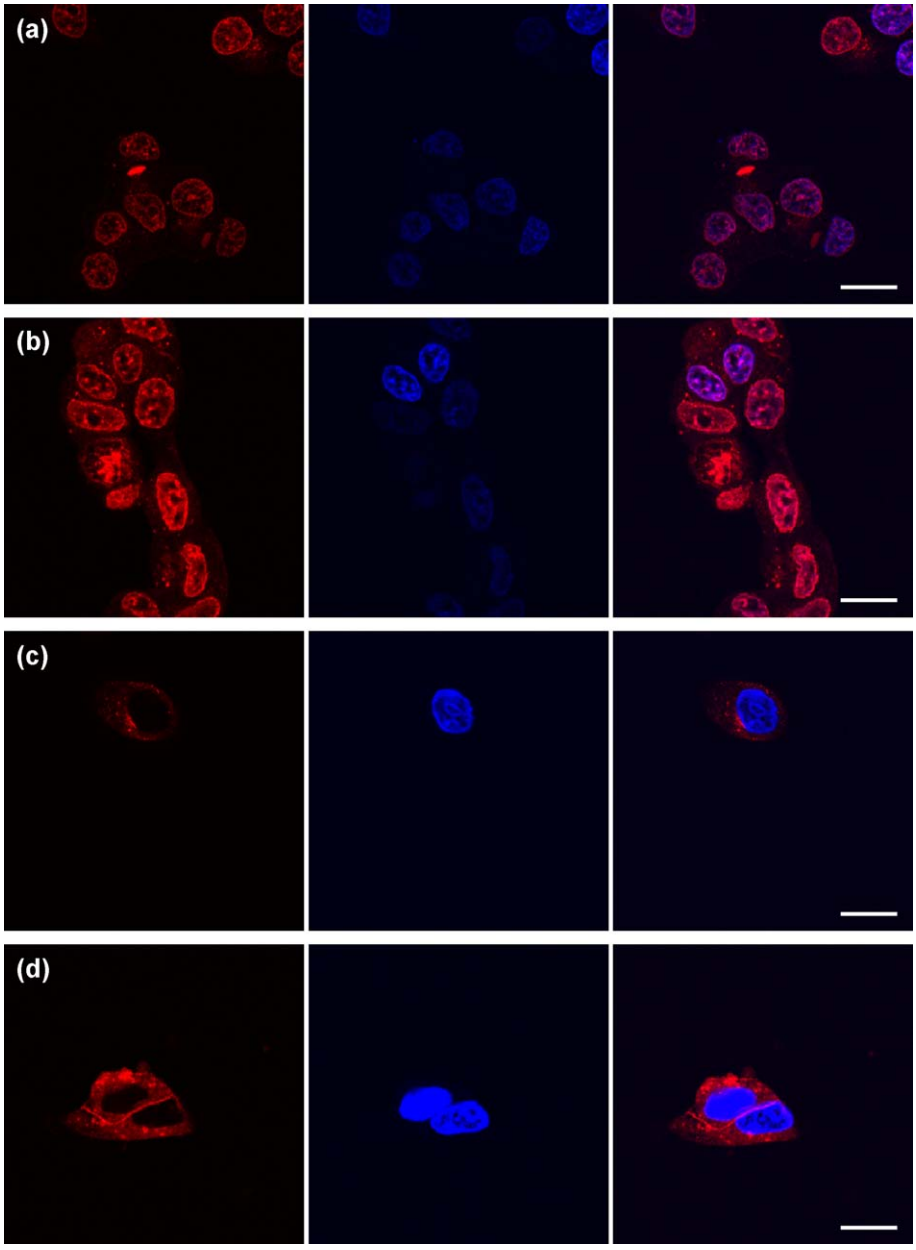

Fig. 7. CLSM-images of MCF-7 cells incubated with free DOX for 2 h (a) and 24 h (b), and with DOX-loaded MPEG5k-b-PCL24.7k micelles for 2 h (c) and 24 h (d). For each panel, images from left to right show the cells with DOX fluorescence, with nuclear staining by Hoechst 33342, and overlays of both images. Scale bars correspond to 20 μm in all the images.

In contrast, DOX fluorescence was observed only in the cytoplasm rather than the cell nuclei, when cells were incubated with the DOX-loaded MPEG5k-b-PCL24.7k micelles for 2 h. When the cells were exposed to free DOX for 24 h, more intense DOX-fluorescence was observed in the nuclei, and still no strong fluorescence was detected in the cytoplasm. In contrast, the cells incubated with DOX-loaded

micelles for 24 h emitted significantly increased fluorescence only from cytoplasm rather than the nuclei. These data not only demonstrate that MPEG-b-PCL micelle is an efficient vehicle to transport DOX into the cytoplasm, but also suggest that the internalization mechanism of micelle is different from that of free DOX. Similar results were reported by Kataoka and coworkers in the SBC-3 cell incubation with PEG-p(Asp-Hyd-DOX) micelle. In their study, dot-shaped fluorescence was observed within cytoplasm, and was considered to be micelles trapped in the endocytic vesicles. Moreover, Maysinger et al. recently detected intracellular localization of similar PCL–PEG micelles (20–45 nm) in several intracellular organelles including mitochondria, Golgi apparatus, and acidic organelles such as lysosomes in PC12 and NIH 3T3 cells. Based on these reports and above experimental results that DOX fluorescence was emitted from cytoplasm mainly in punctuated dot-shape (Fig. 7c, d), it is suggested that DOX-loaded micelles were internalized through an endocytosis pathway and were then localized in acidic endocytic compartments (i.e. endosomes and later lysosomes). For those micelles located inside endosomes/lysosomes, DOX transported with MPEG-b-PCL micelles will be released in a controlled and pH-modulated manner from the micelle particles. Because the in vitro DOX-release from the micelles was a relatively slow process even at pH 5 (Fig. 6), the DOX transported by these micelles will not enter the nuclei as quickly as the free DOX, as indicated by the LCSM measurement. DOX transported by micelles will eventually enter the nuclei where DOX is known to exert its cytotoxicity during DNA synthesis [22], as the following cytotoxicity results demonstrate that the growth of MCF-7 cells can be effectively inhibited.

3.7. In vitro cytotoxicity study

The cytotoxicity of DOX-loaded micelles compared to that of free DOX was determined by the cell growth inhibition assay. No media was changed during the 5-day study. Normal cell growth was monitored in the control group where neither DOX-loaded micelles nor DOX was added. Time-dependent cytotoxicity on MCF-7 cells was observed for both free and micelle-encapsulated DOX. Within 1 day of incubation, neither free DOX nor DOX-loaded micelles showed considerable inhibition effect on cell growth when DOX concentration was below 1 μM. Moreover, even if the DOX concentration reached 10 μM, cells maintained 80% viability when incubated with free DOX, and still no obvious cytotoxicity was detected when cells were incubated with DOX-loaded micelles. In contrast, cell viability drastically decreased after incubation for 5 days against either free DOX or DOX-loaded micelles in the DOX concentration range from 0.01 to 10 μM, as shown in Fig. 8. In particular, almost all the cells were killed when DOX concentration was above 1 μM, regardless whether DOX was in its free form or inside micelles, indicating sufficient DOX internalization inside nuclei during this relatively long incubation time. In the low DOX concentration range of 0.001–0.1 μM, free DOX was more cytotoxic than the DOX-loaded micelles, which did not show appreciable cytotoxicity when the DOX concentration is lower than 0.01 μM. The DOX concentrations that kill 50% of cells (IC_{50}) were measured as 0.01 and 0.033–0.048 μM for free DOX and DOX-loaded micelles, respectively. The lower potency of micelle-delivered DOX can be due to a time-consuming DOX-release from micelles and delayed nuclear uptake in MCF-7 cells, which are consistent with in vitro DOX-release and CLSM studies.

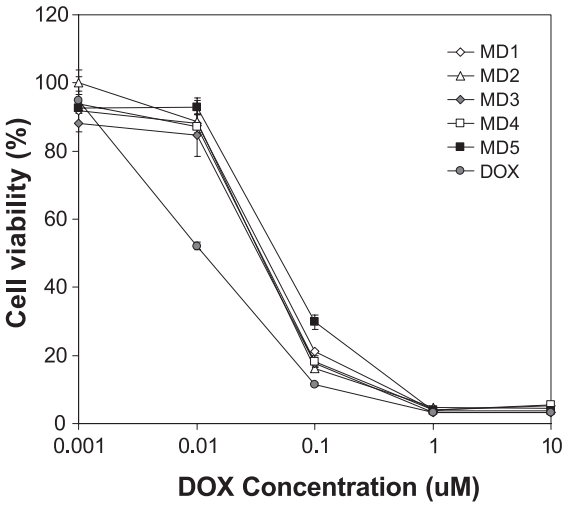

Fig. 8. Cytotoxicity study of free DOX and DOX-loaded micelles in human MCF-7 breast cancer cells after incubation for 5 days. The standard deviation for each data point was averaged over three samples ($n = 3$).

4. Conclusions

Biodegradable diblock copolymers of MPEG-b-PCL of different molecular weights and compositions were synthesized for the delivery of an anticancer drug, DOX. These amphiphilic polymers self-assembled into core-shell-structural micelles that are less than 100 nm in diameter and have hydrophobic PCL cores capable of encapsulating DOX. The PCL length rather than the DOX-loading had significant influence on the micelle size. However, the effect of PCL length on DLC was significantly less than anticipated due to the compromised molecular interactions (e.g. hydrophobic vs. hydrogen-bonding interactions, PCL crystallinity). CLSM studies showed that the patterns of cellular distribution of DOX-loaded micelles and free DOX are very different. DOX-loaded micelles accumulated mostly in cytoplasm while free DOX were localized in the cell nucleus. The in vitro release data indicate that DOX-release from micelles at pH 5.0 was much faster than that at pH 7.4. Compared to free DOX, micelle-delivered DOX showed effective but less potent cytotoxicity in the human MCF-7 tumor cells.

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