Synthesis and Characterization of PEG–PCL–PEG Triblock Copolymers as Carriers of Doxorubicin for the Treatment of Breast Cancer

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ABSTRACT: Triblock copolymers of monomethoxy poly(ethylene glycol) (mPEG) and ε-caprolactone (CL) were prepared with varying lengths of poly(ε-caprolactone) (PCL) compositions and a fixed length of mPEG segment. The molecular characteristics of triblock copolymers were characterized by 1H NMR, gel permeation chromatography (GPC), Fourier transform infrared spectroscopy (FT-IR), X-ray diffraction (XRD), and differential scanning calorimetry (DSC). These amphiphilic linear block copolymers based on PCL hydrophobic chain and hydrophilic mPEG ending, which can self-assemble into nanoscopic micelles with their hydrophobic cores, encapsulated doxorubicin (DOX) in an aqueous solution. The particle size of prepared micelles was around 40–92 nm. The DOX loading content and DOX loading efficiency were from 3.7–7.4% to 26–49%, respectively. DOX-released profile was pH-dependent and faster at pH 5.4 than pH 7.4. Additionally, the cytotoxicity of DOX-loaded micelles was found to be similar with free DOX in drug-resistant cells (MCF-7/adr). The great amounts of DOX and fast uptake accumulated into the MCF-7/adr cells from DOX-loaded micelles suggest a potential application in cancer chemotherapy.

INTRODUCTION

Biodegradable polymeric nanoparticles such as micelles and vesicles have been extensively employed as carriers to enhance the efficacy of encapsulated drugs by optimizing their release profile in the human body. Poly(ε-caprolactone) (PCL) is a biodegradable, biocompatible, and nontoxic thermoplastic polyester. Additionally, PCL is an inexpensive and a highly hydrophobic polyester with degradation time could be tailored to fulfill targeted therapeutic applications.1–3 Polymerization via ring opening of ε-caprolactone (CL) by using polyethylene glycol (PEG) as a macro-initiator and stannous octoate (Sn(Oct)2) as catalyst, respectively, has been well investigated recently.4–6 Remarkably, the amphiphilic block copolymers can self-assemble to form polymeric micelles in an aqueous solution, and have been used as delivery carriers for hydrophobic drugs. In particular, polymeric micelles of a diameter less than 100 nm have demonstrated great potential as carriers for the delivery of anti-cancer agents and have been shown to possess an enhanced vascular permeability.7–9 Polymeric micelles are composed of a hydrophilic outer shell (PEG) and a hydrophobic inner core (PCL). PEG is a common constituent for the hydrophilic outer shell. Its high water-solubility and low cytotoxicity makes PEG a widely used material for medical applications including drug carriers. Additionally, PEG segments on the outer shell are known to reduce the adhesion of plasma proteins. Further, drugs that are encapsulated by small-sized polymeric micelles with a hydrophilic outer shell can potentially increase the circulation time of drugs and can prevent recognition by macrophages of the reticuloendothelial system (RES) after intravenous injection.10,11

Doxorubicin (DOX), an anthracycline drug, has been found very effective for the treatment of breast cancers as well as tumors of ovarian, prostate, brain, cervix, and lung. Nevertheless, drawbacks such as cardiac toxicity, short half-life, and low solubility in aqueous solution have hindered its applications.
Also, multidrug resistance (MDR) has been found in some tumor cells and has been attributed to the P-glycoprotein (P-gp) efflux pump on the plasma membrane. To overcome these obstacles, strategies such as encapsulating DOX into the core of a polymeric micelle by chemical conjugation or physical entrapment have been attempted.\textsuperscript{12,13} The employment of polymeric shells also provides an opportunity to tailor the release profiles of drugs that are encapsulated.

1,6-Diisocyanatohexane (HMDI) is a common coupling agent used in polymer synthesis. However, its toxicity has raised a serious issue for its application in the synthesis of biomaterials. Owing to this undesirable toxicity, additional procedures are required for synthesis that involves HMDI. The extra procedures for the elimination of excessive HMDI, one-side products and by-products (self-polymerization of isocyanates) at the end of the synthesis are obviously disadvantageous.\textsuperscript{14} Further, the incomplete elimination of the residual toxic materials could still be a concern of causing harmful effects to healthy normal tissues if the residuals are introduced into the human body accompanying drugs.

Here, we report on a new approach to synthesize an ABA-type triblock copolymer aiming for its application as a carrier for the delivery of anti-cancer drugs. The triblock copolymer is composed of two identical hydrophilic segments (A: mPEG) and one hydrophobic segment (B: PCL). Triblock copolymers were synthesized by coupling mPEG–PCL–OH and mPEG–COOH in a mild condition by using dicyclohexylcarbodiimide (DCC) and 4-dimethylamino pyridine (DMAP). Most significantly, the new approach eliminates the requirement of using toxic reagents such as HMDI and thus prevents the undesirable cytotoxic effect to normal cells resulting from the residual toxic reagents. The structures of the synthesized triblock copolymers were systematically characterized with proton nuclear magnetic resonance (\(^1\)H NMR), gel permeation chromatography (GPC), Fourier transform infrared spectroscopy (FT-IR), X-ray diffraction (XRD), and differential scanning calorimetry (DSC), respectively. The self-assembly and morphology of the polymeric micelles were also studied in an aqueous solution. To demonstrate the pharmacological application of the triblock copolymers as drug carriers, DOX-loaded nanoparticles composed of triblock copolymers were prepared. The release profile and the cell uptake of DOX and its cytotoxic effect against drug-sensitive (MCF-7) and drug-resistant (MCF-7/adr) breast cancer cell lines were also investigated, respectively.

**MATERIALS AND METHODS**

**Materials**

Monomethoxy poly(ethylene glycol) (mPEG, \(M_n = 5000\)), \(\varepsilon\)-caprolactone, doxorubicin hydrochloride (DOX-HCl), dicyclohexylcarbodiimide (DCC), 4-dimethylamino pyridine (DMAP), succinic anhydride, 2-diphenyl-1,3,5-hexatriene (DPH), and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (St.Louis, MO). Stannous 2-ethyl hexanoate (stannous octoate, Sn(Oct)_2) was obtained from MP Biomedicals (Solon, OH). The mPEG was purified by recrystallization on the dichloromethane/diethyl ether system, and \(\varepsilon\)-caprolactone was dried using CaH\(_2\) and distilled under a reduced pressure. Triethylamine (TEA), 1,4-dioxane, dichloromethane (DCM), diethyl ether, tetrahydrofuran (THF), and other chemicals (purchased from ECHO, Miaoli, Taiwan) were all reagent-grade and were used without further purification.

Human breast cancer cell lines (MCF-7 and MCF-7/adr) were kindly provided by Dr. Y. H. Chen of the School of Pharmacy, College of Medicine, National Taiwan University (Taipei, Taiwan). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St.Louis, MO). Dulbecco’s modified eagle’s medium (DMEM) and antibiotic/antimycotic were purchased from Invitrogen (NY). The fetal bovine serum (FBS) was obtained from HyClone (Logan, Utah).

**Scheme 1** illustrates the procedures for the synthesis of copolymers reported here. The synthesis of mPEG–PCL–OH diblock copolymer has been reported elsewhere.\textsuperscript{9,15,16} In brief, the diblock copolymers were synthesized by ring opening polymerization of \(\varepsilon\)-caprolactone in the presence of mPEG–OH as a macroinitiator with Sn(Oct)_2 serving as a catalyst. A predetermined amount of mPEG–OH, \(\varepsilon\)-caprolactone, and Sn(Oct)_2 were introduced into a 250 mL three-necked flask under a nitrogen atmosphere and mechanical stirring. The resulting mixture was refluxed for 4 h at 130°C. After reaction was completed, the mixture was dissolved in DCM and precipitated in diethyl ether : hexane (9 : 1, v/v). The product was filtered and dried in vacuum for 24 h to deliver mPEG–PCL–OH diblock copolymer. mPEG–PCL–OH (\(M_n,_{PCL} = 3600, 8000, \) and 22,000) were designated and synthesized in the present study. To synthesize triblock copolymers, the hydroxyl group of mPEG was modified to a carboxylic acid group and was connected with mPEG–PCL–OH diblock copolymers. mPEG (1 mmol) was dissolved in dry 1,4-dioxane then an excess of succinic anhydride (2 mmol) and DMAP (2 mmol) were added to this solution. The resulting solution was stirred at room temperature for 24 h. The solution was precipitated in diethyl ether : hexane (1 : 1, v/v). The white powder was dried in vacuum for 24 h.
The dried material was dissolved in DCM and stirred at room temperature for 30 min. Then DCC and DMAP were added sequentially. The mPEG–PCL–OH was dissolved in DCM and introduced to the reaction mixture. The resulting mixture was stirred for 24 h at room temperature under an atmosphere of nitrogen. The product was obtained by precipitation in a mixture of diethyl ether and hexane (1:1, v/v). The powder was dialyzed against water to remove unreacted residual mPEG–COOH and the final product, white solid, was obtained by lyophilization. The obtained triblock copolymers were abbreviated EC36E, EC80E, and EC220E, respectively.

**Characterizations of triblock copolymers**

The formations of ABA-type triblock copolymers were confirmed by $^1$H NMR, GPC, FT-IR, DSC, and XRD, respectively. The molecular weight of the triblock copolymers was estimated by calculating the integration area of the mPEG and PCL segments. The sum of $M_{\text{PEG}}$ and $M_{\text{PCL}}$ was obtained to represent the average molecular weight of the synthesized polymer.$^{15,16}$ The vibrational spectra were measured using an FT-IR spectrometer (FT-IR 410, Jasco, Tokyo, Japan) in the range of 4000 to 400 cm$^{-1}$. The powdery block copolymers were compressed into a KBr disk for FT-IR measurements. $^1$H NMR spectra of the block copolymers were recorded by using a Bruker spectrometer operating at 500 MHz, using deuterated chloroform as solvent or D$_2$O. The weight average molecular weight ($M_w$) and the polydispersity ($M_w/M_n$) of the ABA-type triblock copolymers were determined by GPC measured on LabAlliance GPC with a refractive index detector RI 2000 using a Laboratories PLgel 5 mm Mixed-D column. THF was used as an eluent. The molecular weight was calculated using standard polystyrene samples as a reference. The DSC experiments were performed using Jade DSC (PerkinElmer, Waltham, MA). The samples of block copolymers were heated and cooled in a temperature range of 20°C–110°C under an atmosphere of nitrogen at a heating and cooling rate of 10°C/min. X-ray diffraction was carried out in X`Pert Pro MRD (PANalytical, Netherlands) at room temperature with Cu-K$_\alpha$ radiation ($\lambda$ = 0.154 nm) and an incident angle (2$\theta$), ramping from 2 to 30$^\circ$ at a rate of 50 sec/step.

**Determination of critical micelle concentration**

The critical micelle concentration (CMC) of copolymers was determined with UV-Vis spectroscopy using 1,2-diphenyl-1,3,5-hexatriene as the fluorescent probe. Samples for UV-Vis measurements were prepared according to procedures that have been described in previous literature.$^{14}$ The concentration of the aqueous copolymer solution was between 0.01 mg/mL and 10$^{-6}$ mg/mL. A 2.0 mL copolymer
solution was added to a 20 μL DPH solution (0.4 mM in MeOH) to give a $4 \times 10^{-6}$ M DPH/copolymer solution. The resultant solution was incubated in dark place for 5 h. The UV-Vis absorption of incubated solution was measured in a range of 250–500 nm. The absorbance at 359 nm was selected to determine the CMC.

**Preparation and characterization of micelles**

For the DOX-unloaded micelles, the micelle solution was prepared by dissolving 20 mg of ABA block copolymer in 2.0 mL THF, and then 1.0 mL double distilled water was added under stirring. The resulting solution was placed at room temperature for 12 h, then was transferred to a dialysis bag and dialyzed against double distilled water for 24 h (MWCO: 50,000 Da, Spectrum Laboratories, CA).

For the DOX-loaded micelles, DOX was first neutralized before micelle preparation. DOX (3.0 mg) was neutralized with an excess mount of TEA in 1.0 mL THF. The DOX solution was then added into the 2.0 mL THF solution of ABA copolymer (20 mg). This solution was added to 1.5 mL double distilled water under stirring for 6 h. To remove un-trapped DOX and TEA, the mixture was next transferred for dialysis against double distilled water for 24 h to produce DOX-loaded micelles. The water was replaced hourly for the first 3 h.

The drug loading efficiency (DLE) was defined as the weight percentage of DOX in micelles relative to the initial feeding amount of DOX. The drug loading content (DLC) was calculated from the mass of incorporated DOX divided by the weight of polymer. The amount of DOX loaded in micelles was determined by the absorption at 485 nm using UV-Vis spectrometry (UV-530, Jasco, Tokyo, Japan). The DOX solutions of various concentrations were prepared, and the absorptions of the solutions were measured to obtain a calibration curve.\(^{16,17}\)

The particle size and its polydispersity were determined by dynamic light scattering (DLS) at 25°C using a Zetasizer 3000HSA (Malvern Instruments, UK) with an excitation of 633 nm illuminated at a fixed angle of 90°. The micellar solutions, prepared as described earlier, were diluted into a final concentration of 0.2 mg/mL and then filtered through a Millipore 0.2 μm filter prior to measurements. The average diameter was calculated by a CONTIN analytical method. The sample for the AFM measurement was prepared by placing a drop of the micellar solution (0.2 mg/mL) on a freshly cleaved mica slide and was left to dry at room temperature to remove residual water. The morphological images were obtained using a commercial AFM (NanoWizard\textsuperscript{TM} Atomic Force Microscope, JPK Instruments AG, Berlin, Germany) operated under a tapping mode in air using a cantilever with a nominal spring constant of 25 N/m and a resonance frequency around 172 kHz. The images were acquired at a pixel density of 512 × 512 and a frame rate between 0.5 Hz and 0.8 Hz. The particle size was calculated by “SPM” and “Imaging Processing” JPK’s software.

**In vitro DOX release study**

The experimental procedures were adapted from an earlier study.\(^{18}\) In brief, 1.5 mL of DOX-loaded micellar solution was dissolved in 0.5 mL PBS (0.1 M, pH 7.4) and acetate buffer solution (0.1 M, pH 5.4) and was transferred into a dialysis tube (MWCO: 50,000 Da). The tube was immersed into a buffer solution of 15 mL and was kept at 37°C. At several time intervals, 1.0 mL of the buffer solution outside the dialysis bag was withdrawn for UV-Vis analysis and replaced with fresh buffer solution. DOX concentration was calculated based on the absorbance at 485 nm as described before.

**In vitro cytotoxicity test**

The in vitro cytotoxicity of free DOX and DOX-loaded micelles was tested against two human breast cancer cell lines: MCF-7 and MCF-7/adr. The cell culture medium was composed of DMEM with 10% fetal bovine serum and antibiotic/antimycotic. The cell viability was determined by tetrazolium dye (MTT) assay. MCF-7 and MCF-7/adr cells were seeded in 48-well plates at a density of $1 \times 10^5$ cells/well and were incubated at 37°C under a humidified atmosphere containing 5% CO₂ for 24 h before assay. After that, the cells were further incubated in media containing either free DOX or DOX-loaded micelles of various concentrations. A solution of media with unloaded micelles (placebo) was used as a control to be compared with results obtained from DOX-loaded micelles. For the control experiments of free DOX, the cells were incubated in a DOX-free medium. After 96 h, MTT solution was added to each well followed by 4 h of incubation at 37°C. Subsequently, the medium was removed and violet crystals were solubilized with DMSO. After shaking slowly twice for 5 sec, the absorbance of each well was determined using a Multiskan Spectrum spectrophotometer (Thermo Electron Corporation, Waltham, MA) at 570 nm and 630 nm. The cell viability (%) was calculated as the ratio of the number of surviving cells in drug-treated samples to that of control.

**Flow cytometry**

The intracellular uptake of doxorubicin was evaluated by flow cytometry, using a FACSCalibur flow...
cytometer (BD Biosciences, NJ). 1 × 10^5 cells were incubated in the culture medium for 24 h and were treated with free DOX and DOX-loaded micelles, respectively, in a medium containing DOX of a concentration at 10 μg/mL. Cells in 12 × 75 Falcon tubes were placed on the FACSCalibur. The fluorescence intensity of DOX was collected at a 488 nm excitation and with a 575 nm band pass filter. The WinMDI (Version 2.9, flow cytometry application) was used to analyze the cells uptake.

**Cellular uptake of DOX**

To visualize the intracellular uptake of drug, DOX-loaded micelles and free DOX were tested for human breast cancer cell lines (MCF-7 and MCF-7/adr). The cells were seeded in MatTek glass bottom dishes (1 × 10^5 cells/dish) and incubated for 24 h. The cells were then incubated with either free DOX or DOX-loaded micelles in a medium with the concentration of DOX at 10 μg/mL. After 2 and 24 h, the medium was removed and the cells were washed with cold PBS twice. The fluorescence images of cells were obtained using a confocal laser scanning microscope (FluoView FV300, Olympus, PA).

**RESULTS AND DISCUSSION**

**Synthesis and characterizations of mPEG–PCL–mPEG triblock copolymers**

mPEG–PCL block copolymers were synthesized by ring opening polymerization of ε-caprolactone with mPEG as initiator and Sn(Oct)2 as a catalyst. The mPEG–PCL was prepared with different feeding molar ratios of mPEG/ε-CL using mPEG of a molecular weight of 5000. The calculation of the degree of polymerization and the number average molecular weight (M_n) was based on the integrity ratio of the 1H NMR peaks at 4.1 ppm to methylene protons (—CH_2—) of PCL block and 3.6 ppm (—CH_2—) to PEG blocks, respectively. The detailed procedures of the synthesis are illustrated in Scheme 1. In particular, our synthetic approach for ABA-type amphiphilic triblock copolymers builds with hydrophilic mPEG segments and hydrophobic PCL segment and uses a milder coupling agent: DCC/DMAP.

The triblock copolymers were characterized by 1H NMR, GPC, FT-IR, DSC, and XRD. 1H NMR of prepared copolymers was measured in CDCl_3 and D_2O solvents. The typical signals of both mPEG and PCL were observed in the 1H NMR spectrum when CDCl_3 was used as solvent. The peak at 3.6 ppm was assigned to the methylene unit of the PEG segment, while the peaks at 4.1, 2.3, 1.6, and 1.4 ppm were assigned to the PCL block. In contrast to using CDCl_3 as a solvent, ABA triblock copolymers that were dissolved in the D_2O solvent showed only a peak at δ = 3.6 ppm, attributed to the methylene oxide segments of mPEG (—CH_2CH_2O—), and a very weak peak at δ = 1.4 ppm, assigned to the methylene protons of PCL block. Similar assignments have been reported elsewhere. The molecular weight and the molecular weight distribution of triblock copolymers were measured by GPC using THF as an eluent and monodispersed polystyrene as a standard. As a result, the molecular weights of diblock copolymers (mPEG–PCL) and triblock copolymers (PEG–PCL–PEG) increased after ring opening polymerization of ε-caprolactone and PEG conjugation, respectively. The results are summarized in Table I.

![Figure 1](ft-ir_spectra.png)  
**Figure 1** FT-IR spectra of ABA-type triblock copolymers.
associated with the CH stretching of PEG at 2870 cm\(^{-1}\) decreased.\(^{19}\)

The thermal properties of the block polymers were characterized by DSC. Significant differences were observed between the DSC diagrams of different copolymers (Fig. 2). For EC\(_{80}\)E (Curve b) and EC\(_{220}\)E (Curve c), the thermal curves showed double peaks, indicating that two melting temperatures (\(T_m\)) exist. The higher melting temperatures of EC\(_{80}\)E and EC\(_{220}\)E were identified at 59.7°C and 59.2°C, respectively, corresponding to the melting of mPEG blocks in the crystal phase. Lower melting temperatures at 55.9°C and 54.5°C were also observed, corresponding to the melting of PCL blocks in the crystal phase. Conversely, only a single peak at 60.2°C was observed in the DSC diagram of EC\(_{36}\)E (Curve a). The phase transition of PCL was not obvious since EC\(_{36}\)E has a long PEG block and short PCL segment. The single and bimodal phase transitions of triblock copolymers and diblock copolymers have also been reported by others.\(^{14,20}\)

To gain further insight on the crystalline state of the triblock copolymers, XRD measurements have also been employed (Fig. 3). The results reproduced typical peaks of mPEG segments (at 2\(\theta = 19.2^\circ\) and 23.4\(^{\circ}\)) and PCL segments (at 2\(\theta = 21.5^\circ\) and 23.7\(^{\circ}\)). The XRD pattern of EC\(_{220}\)E showed a lower crystallinity of PEG block. However, for EC\(_{36}\)E and EC\(_{80}\)E, the lower crystallinity of PCL block was observed. This indicated that the ratio of PEG/PCL in block copolymer affected strongly to the crystallization properties. EC\(_{220}\)E had the longest PCL segment among these triblock copolymers in this study. As a result, a great intensity was observed for PCL. Furthermore, with increased length of PCL segments, the peaks of PCL segments increased (at 2\(\theta = 21.5^\circ\), in EC\(_{36}\)E to EC\(_{80}\)E as well as EC\(_{220}\)E). This could be attributed to the interference between the PCL and mPEG segments and the PCL blocks crystallize first instead then the PEG blocks crystallize under a restricted condition and crystallinity decreased. When the mPEG content is greater (lower PCL content), the mPEG segments can prevent the PCL segments from crystallizing, while facilitating the formation of the mPEG crystallites.\(^{21,22}\)

**Preparation and characterization of micelles**

The critical micelle concentrations (CMC) of triblock copolymers with different PCL compositions were determined by fluorescence techniques using DPH as a probe. The CMC values of the ABA triblock copolymers are listed in Table II. The CMC values of EC\(_{36}\)E, EC\(_{80}\)E, and EC\(_{220}\)E were 5.6 \(\times\) 10\(^{-3}\), 1.2 \(\times\) 10\(^{-3}\), and 5.1 \(\times\) 10\(^{-4}\) mg/mL, respectively. The results showed that the length of the hydrophobic segment strongly influenced the CMC values, i.e., the longer hydrophobic segment, the lower CMC values.

The size and the size distribution of copolymeric micelles were measured by dynamic light scattering, and the results are summarized in Table II. These

![Figure 2](image1.png) The second heating DSC curves of EC\(_{36}\)E (a), EC\(_{80}\)E (b), EC\(_{220}\)E (c), and PCL (d).

![Figure 3](image2.png) XRD patterns of EC\(_{36}\)E, EC\(_{80}\)E, and EC\(_{220}\)E.

<table>
<thead>
<tr>
<th>TABLE II</th>
<th>Characteristics of Prepared ABA Triblock Copolymeric Micelles</th>
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<tr>
<td>Sample</td>
<td>CMC (mg/mL)</td>
</tr>
<tr>
<td>EC(_{36})E</td>
<td>5.6 (\times) 10(^{-3})</td>
</tr>
<tr>
<td>EC(_{80})E</td>
<td>1.2 (\times) 10(^{-3})</td>
</tr>
<tr>
<td>EC(_{220})E</td>
<td>5.1 (\times) 10(^{-4})</td>
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\(^{\text{a}}\) Micelles before DOX-loading.

\(^{\text{b}}\) Micelles after DOX-loading (the data are presented as the mean with standard deviation of ten samples).
results indicated that the particle sizes were dependent upon the block length and molecular weight of the PCL segment on the triblock copolymer. The average size of the empty micelles increased as the length of PCL segment increased. It could be attributed to the more hydrophobic the micellar core is, the more copolymer chains are aggregated into a micelle to minimize the interfacial energy. Additionally, the particle size of DOX-loaded micelles increased slightly compared to that of the DOX-free micelles. The particle size of the DOX-loaded micelles ranged from 50 to 92 nm (Table II). Similar results were also obtained in previous reports. The results showed that DLC and DLE were dependent on the composition of ABA triblock copolymers. The DOX was physically encapsulated into copolymeric nanoparticles due to the hydrophobic interaction of drug and PCL core. The DLC and DLE are affected by the interaction between DOX and PCL crystallinity and the interaction of hydrogen bonding. As results, the increase in the length of PCL, the high PCL crystallinity in the core of micelles increased DLC and DLE (Table II). It could be attributed to the increase of hydrophobic segment of block copolymers, the interaction between hydrophobic segment and hydrophobic drug was enhanced, leading to an increase in the loading content of drugs.

**In vitro release of DOX from polymeric micelles**

It was shown that the characteristics of ABA triblock copolymer micelles (size, CMC, DLE, and DLC) depend on the length of the hydrophobic PCL segment. Among the three triblock copolymers with different molecular weights of PCL blocks, the one with the longest PCL segment (EC220E: $M_w = 35,000$) was selected to study the release profile of drugs and cell assays. This polymer had the lowest CMC value ($5.1 \times 10^{-4} \text{ mg/mL}$), and its large hydrophobic block exhibited a good loading capacity of DOX. Figure 5 displays the *in vitro* release profiles of DOX from the polymeric micelles in PBS (0.1 M, pH 7.4) and acetate buffer solutions (0.1 M, pH 5.4) at 37°C. The results showed an initial burst release of DOX and followed by a sustained release for about 48 h. The initial burst release of DOX from micelles could be attributed to the diffusion of DOX located close to the surface of particles or within the hydrophilic shell. The total release of DOX in a period of 48 h with pH 7.4 and 5.4 was 25% and 37% of total DOX concentration, respectively. The relatively slow
release rate from nanoparticles could be attributed to strong interaction of drug molecules and hydrophobic core of the polymeric micelles, and short incubation time. The total release of DOX may be obtained by modulating the composition of triblock copolymer, incubation time, and pH of media. However, the release of DOX at a pH value of 5.4 was found faster than that at a pH value of 7.4. A similar pattern has been observed in the acidic condition by others. These results could be attributed to the re-protonation of the amino group of DOX and the faster degradation of the micelle core at lower pH values. This pH-dependent release profile is of particular interest. It is expected that the greater part of DOX-loaded micelles will remain in the micelles cores for a considerable time period in plasma after intravenous administration and have the potential for prolonged DOX retention time in the blood circulation. However, a faster release may occur at low local pH surrounding the tumor site or by the more acidic environment inside the endosome and lysosome of tumor cells after cellular uptake of micelles through endocytosis.

**In vitro cytotoxicity of DOX-loaded micelles**

The in vitro cytotoxicity of DOX-loaded micelles was conducted in two human breast cancer cell lines: wild-type MCF-7 and drug-resistant MCF-7/adr cells. Figure 6 shows the cell viability (%) treated with DOX-loaded micelles and free DOX, respectively, toward MCF-7 and MCF-7/adr cell lines. The cell viability was measured against various concentrations of DOX ranging from 0.01 to 10 μg/mL. The IC₅₀ value, the drug concentration at which 50% of cells were killed in a given period, was obtained from the experimental data (cell viability). The IC₅₀ values of MCF-7 cells that were treated with free

**Figure 5** The release profile of DOX-loaded EC₂₂₀E micelles.

**Figure 6** In vitro cytotoxicity of free DOX and DOX-loaded EC₂₂₀E micelles in human MCF-7 and MCF-7/adr breast cancer cells.
can avoid being effluxed out by the tumor cells that express P-gp. We reported previously that the polymeric solution (placebo) exhibited no toxic effects to cells at a concentration up to 0.25 mg/mL.30

Cellular uptake of DOX-loaded micelles

Because DOX is fluorescent, it can be used directly to measure its cell uptake without introducing additional fluorescent probes. Figure 7 shows the histogram of fluorescence measured on both MCF-7 and MCF-7/adr cells that had been incubated with either free DOX or DOX-loaded micelles. DOX-free medium was used as a control. As expected, only autofluorescence from cells was observed for the control. The MCF-7 cells incubated with DOX-loaded micelles showed a lower fluorescence intensity in comparison with that incubated with free DOX. The results suggested that free DOX was taken up with a much greater efficiency than DOX-loaded micelles by the cells [Fig. 7(a)]. These data, which are in agreement with the characterization of cytotoxicity discussed in the previous section, indicated once again that the free DOX has higher toxicity than DOX-loaded micelles toward drug-sensitive MCF-7 cells. On the other hand, when the free DOX was added to drug-resistant MCF-7/adr cells, the fluorescence intensity was much lower in comparison with the situation when the DOX-loaded micelles were introduced [Fig. 7(b)]. This difference could be attributed to the high expression of P-glycoprotein in the MCF-7/adr cell.31 The results obtained from flow cytometry also suggested that the DOX-loaded micelles can potentially be used to treat multidrug resistant tumors.

Confocal microscopy was employed to visualize the cellular uptake of DOX and the internalization of DOX-loaded micelles and free DOX into the drug-sensitive MCF-7 and drug-resistant MCF-7/adr cells. As shown in Figure 8, the distribution of free DOX in cells was drastically different from that of DOX-loaded micelles. The cellular uptake of free DOX was faster than that of DOX-loaded micelles in the drug-sensitive breast cancer cells, MCF-7 [Fig. 8(a–d)]. More interesting, faster uptake and greater amount of DOX accumulation into drug-resistant cells was observed if DOX was pre-loaded in

Figure 7 Flow cytometry histogram profile of MCF-7 (a) and MCF-7/adr (b) cells that were incubated with free DOX and DOX-loaded EC220E micelles (DOX concentration 10 μg/mL).

Figure 8 Confocal images of MCF-7 and MCF-7/adr cells after incubation at the equivalent 10 μg/mL DOX concentration. MCF-7: free DOX 2 h (a) and 24 h (c); DOX-loaded EC220E micelles 2 h (b) and 24 h (d); MCF-7/adr: free DOX 24 h (e), and DOX-loaded EC220E micelles 24 h (f). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
micelles [Fig. 8(e,f)]. After 2 h, the drug-sensitive cells that had been incubated with free DOX or DOX-loaded micelles exhibited strong fluorescence of DOX, and the intensity of fluorescence continued to increase after 24 h of incubation time. Conversely, only weak fluorescence was observed in drug-resistant cells after 2 h of exposure (data not shown), although minor increase in the fluorescence intensity was also observed after 24 h treatment with free DOX. Furthermore, when the MCF-7 cells were incubated with free DOX, fluorescence signals were observed only near the nuclei of cells but not in the cytoplasm [Fig. 8(a,c)]. This is reasonable since DOX is a small molecule and could transport freely through both the plasma membrane and nuclear membrane via a passive pathway of diffusion. In the case of relatively larger DOX-loaded micelles, strong DOX fluorescence was observed only in the cytoplasm, whereas the fluorescence in the nuclei was very dim [Fig. 8(b,d)]. The signal observed in the nuclei was attributed to the release of DOX molecules from the micelles. These images displayed the enhanced cellular internalization of DOX-loaded micelles and reversal drug-resistant cells. The observation of fluorescence in cytoplasm indicated that the DOX-loaded micelles were internalized by the cells through endocytosis and DOX was distributed in the cytoplasm after escaping from the endosome and/or the lysosome.26,27 In addition, the lower fluorescence intensity observed in cells treated with DOX-loaded micelles suggested that either some micelles were not internalized or the fluorescence of DOX was quenched by the micelles.32

CONCLUSIONS

In this work, three biodegradable triblock copolymers with various PCL blocks were synthesized for the delivery of anticancer drugs. The structures of copolymers were characterized by 1H NMR, FT-IR, GPC, DSC, and XRD, respectively. The copolymers that formed nanosized micellar structures in aqueous solution were studied by DLS and AFM, showing particle sizes ranging from 40 to 92 nm. These hydrophobic PCL segments contained copolymers could encapsulate DOX into the micelle cores. The in vitro release profiles of drugs from the micelles exhibited a pH dependence. Furthermore, in comparison with free DOX, DOX-loaded micelles showed a similar cytotoxicity to MCF-7/adr cells. However, the cytotoxicity of DOX-loaded micelles was higher to that of free DOX in MCF-7/adr cells when the concentration was lower than 1.0 μg/mL. Additionally, the micelles displayed higher cellular internalization and cytoplasm residence in drug-resistant cells. These observations suggest that DOX-loaded micelle is a promising means of treating multidrug resistant tumors.

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