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Research paper

Development of novel polymeric micellar drug conjugates and nano-containers with hydrolyzable core structure for doxorubicin delivery

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Abstract

Novel micelle-forming poly(ethylene oxide)-*block*-poly(ε-caprolactone) (PEO-*b*-PCL) block copolymers bearing doxorubicin (DOX) side groups (PEO-*b*-P(CL-DOX)) on the PCL block were synthesized. Prepared block copolymers were characterized, assembled to polymeric micellar drug conjugates and assessed for the level of DOX release at pH 7.4 and pH 5.0 using a dialysis membrane to separate released and conjugated drug. The possibility for the degradation of PCL backbone for PEO-*b*-P(CL-DOX) micelles was investigated using gel permeation chromatography. Micelle-forming DOX conjugate did not show any signs of DOX release at 37 °C within 72 h of incubation at both pHs, but revealed signs of poly(ester) core degradation at pH 5.0. In further studies, PEO-*b*-PCL micelles bearing benzyl, carboxyl or DOX groups in the core were also used as micellar nano-containers for the physical encapsulation of DOX, where maximum level of drug-loading and control over the rate of DOX release was achieved by polymeric micelles containing benzyl groups in their core, i.e., PEO-*b*-poly(α-benzylcarboxylate-ε-caprolactone) (PEO-*b*-PBCL) micelles. The *in vitro* cytotoxicity of chemically conjugated DOX as part of PEO-*b*-P(CL-DOX) and physically encapsulated DOX in PEO-*b*-PBCL against B16F10 murine melanoma cells was assessed and compared to that of free DOX. Consistent with the results of *in vitro* release study, cytotoxicity of micellar PEO-*b*-P(CL-DOX) conjugate (IC_{50} of 3.65 μg/mL) was lower than that of free and physically encapsulated DOX in PEO-*b*-PBCL (IC_{50} of 0.09 and 3.07 μg/mL, respectively) after 24 h of incubation. After 48 h of incubation, the cytotoxicity of conjugated DOX (IC_{50} of 0.50 μg/mL) was still lower than the cytotoxicity of free DOX (IC_{50} of 0.03 μg/mL), but surpassed that of physically encapsulated DOX in PEO-*b*-PBCL (IC_{50} of 1.54 μg/mL). The results point to a potential for PEO-*b*-P(CL-DOX) and PEO-*b*-PBCL as novel polymeric micellar drug conjugates and nano-containers bearing hydrolyzable cores for DOX delivery.

Keywords: Doxorubicin; Cytotoxicity; Polymeric micelles; Polymer–drug conjugate; Poly(ethylene oxide)-*block*-poly(ε-caprolactone)

1. Introduction

Development of new dosage forms that can change the normal fate of drugs in a biological system and direct them toward their cellular or sub-cellular targets has been the focus of many pharmaceutical research efforts during the past few decades. Nano-delivery systems of appropriate stability, size, and surface properties have been designed that can avoid penetration through continuous capillary in normal tissue, escape glomerular filtration in kidneys, evade uptake by the reticuloendothelial system (RES), thus, circulate for longer periods in blood and eventually accumulate in solid tumors through enhanced permeability and retention (EPR) phenomenon. However, tumor accumulation of the carrier does not guarantee the preferential access of the incorporated drug to its targets. For efficient drug targeting, in addition to the above mentioned qualities, the carrier should be able to restrain the incorporated

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drug during blood circulation and preferentially release it in the extracellular space or appropriate intracellular organelle in tumor. Polymeric micelles have gained a lot of interest as promising delivery systems for drug targeting as they appear to have the potential for fulfilling several of these criteria [1–3]. Polymeric micelles have the right dimension and required surface properties for tumor accumulation as a result of EPR effect. More importantly, the structure of the core and shell in polymeric micelles can be chemically manipulated to achieve the required micellar stability, drug release and cellular interaction profile for the incorporated drug. In this context, the presence of free functional groups on micelle-forming poly(ethylene oxide)-*b*-poly(L-amino acid) (PEO-*b*-PLAA) block copolymers is considered an important advantage. It allows for the chemical conjugation or electrostatic complexation of different drugs, drug compatible groups and/or stimulus-sensitive structures in the micellar core [4–10].

Our research group has recently reported on the successful synthesis and assembly of novel PEO-*b*-poly(ester) copolymers bearing several functional side groups on the poly(ester) chain, i.e., PEO-*b*-poly(α-benzylcarboxylate-ε-caprolactone) (PEO-*b*-PBCL) and PEO-*b*-poly(α-carboxyl-ε-caprolactone) (PEO-*b*-PCCL) [11]. Functionalization of the poly(ester)s is mostly carried out through activation of their terminal hydroxyl group [12–15]. This approach, however, only provides opportunity for the conjugation of one drug molecule per polymer chain and may require utilization of a large polymer dose for biological administration. In the current manuscript, we report on the successful conjugation of DOX to the pendant carboxyl groups of PEO-*b*-PCCL by an amide bond. This has led to the formation of novel self-associating PEO-*b*-P(CL-DOX) conjugates that unlike PLAA bear a hydrolyzable polymeric backbone, i.e., poly(ε-caprolactone) (PCL), and at the same time can accommodate several DOX molecules per polymer chain. Chemical conjugation of DOX to the polymeric micellar core in PEO-*b*-P(CL-DOX) is expected to reduce the chance of premature drug release outside tumor tissue. On the other hand, since PCL backbone is prone to hydrolysis especially in acidic environment, core degradation followed by micellar dissociation and release of DOX-caprolactone (DOX-CL) derivatives may be facilitated in the acidic environment of the edosome/lysosomes after endocytosis of PEO-*b*-P(CL-DOX) micelles by tumor cells. The validity of this assumption was tested in this study assessing the possibility of polymeric chain cleavage for PEO-*b*-P(CL-DOX) in acidic medium by gel permeation chromatography (GPC).

In further studies, in an effort for the development of novel polymeric micellar nano-containers for DOX delivery, the effect of manipulations in the chemical structure of the micellar core in PEO-*b*-PCL micelles on physical encapsulation of DOX was assessed. As a result of increased hydrophobic or electrostatic interactions between DOX and PBCL or PCCL micellar core, respectively, an improvement in the solubilization and release profile of encapsulated DOX in these structures compared to PEO-*b*-PCL micelles was revealed. Finally, to define the superior polymeric micellar design and structure for targeted DOX delivery, the *in vitro* release and cytotoxicity of conjugated DOX as part of PEO-*b*-P(CL-DOX) micelles were assessed and compared to those of physically encapsulated DOX in PEO-*b*-PCL, PEO-*b*-PBCL, PEO-*b*-PCCL, and PEO-*b*-P(CL-DOX) nano-containers.

2. Materials

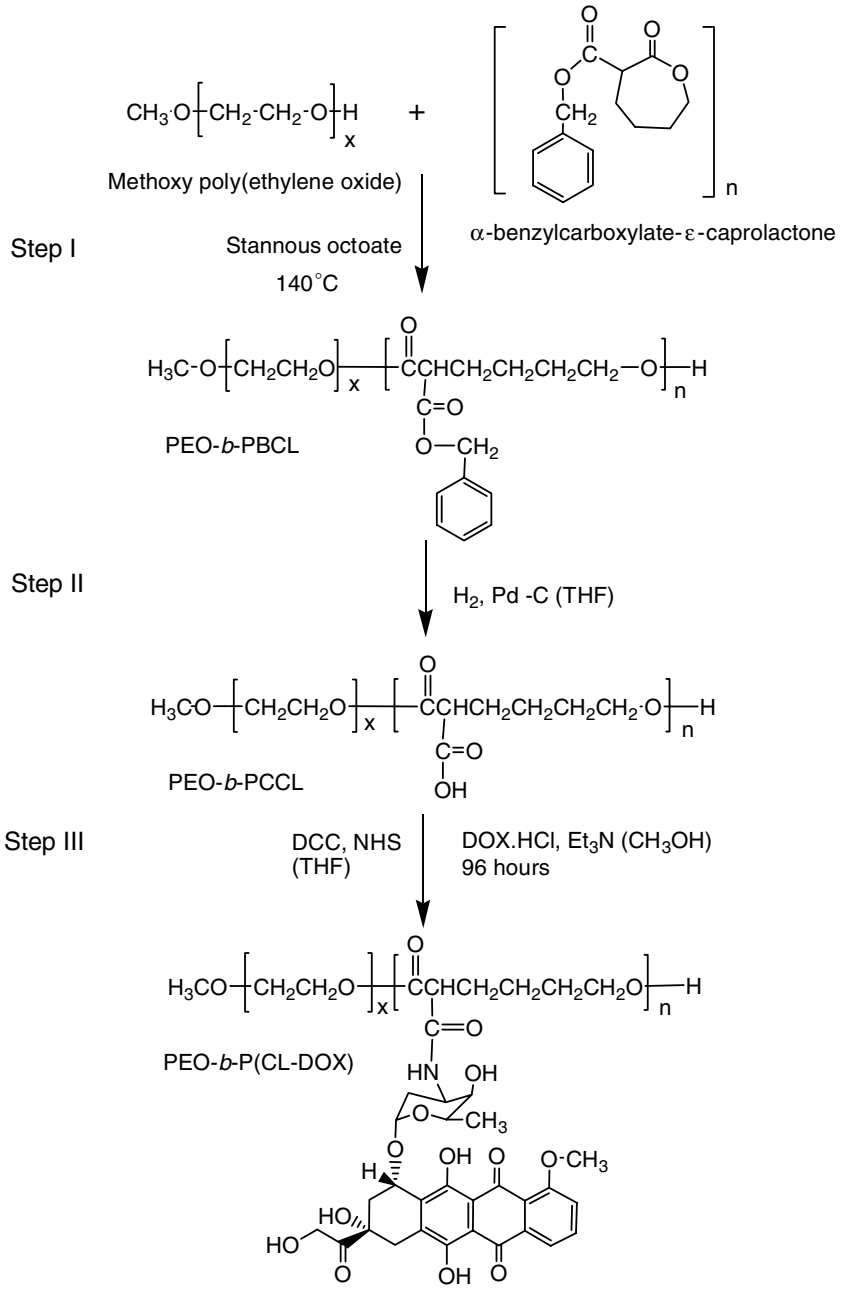
Methoxy poly(ethylene oxide) (average molecular weight of 5000 g mol^{-1}), diisopropyl amine (99%), benzyl chloroformate (tech. 95%), sodium (in Kerosin), butyl lithium (Bu-Li) in hexane (2.5 M solution), palladium-coated charcoal, *N*, *N* dicylcohexyl carbodiimide (DCC), *N*-hydroxy succinimide (NHS), triethylamine, and pyrene were purchased from Sigma (St. Louis, MO, USA). ε-Caprolactone was purchased from Lancaster Synthesis, UK. DOX.HCl was purchased from Hisun Pharmaceutical Co. (Zhejiang, China). Stannous octoate was purchased from MP Biomedicals Inc, Germany. Fluorescent probe 1,3-(1,1′-dipyrenyl)propane was purchased from Molecular Probes, USA. Sephadex LH20 was purchased from Amersham Biosciences (Sweden). Cell culture media RPMI 1640, penicillin–streptomycin, fetal bovine serum, L-glutamine and Hepes buffer solution (1 M) were purchased from GIBCO, Invitrogen Corp. All other chemicals were of reagent grade.

3. Methods

3.1. Synthesis of PEO-b-P(CL-DOX) and its characterization

Synthesis of PEO-*b*-P(CL-DOX) was accomplished in three steps: (I) ring-opening polymerization of functionalized caprolactone with methoxy PEO to prepare PEO-*b*-PBCL; (II) reduction of PEO-*b*-PBCL to PEO-*b*-PCCL; and (III) conjugation of DOX to free carboxyl groups of PEO-*b*-PCCL (Scheme 1).

Synthesis of functionalized monomer, i.e., α-benzylcarboxylate-ε-caprolactone, was reported by our group in a previous publication [11]. Briefly, to a solution of dry diisopropylamine in dry tetrahydrofuran (THF) in a 3-neck round bottom flask, BuLi in hexane was slowly added at −30 °C under vigorous stirring with continuous argon supply. The solution was cooled to −78 °C and kept stirring for additional 20 min. Freshly distilled ε-caprolactone was dissolved in dry THF and added to the above mentioned mixture slowly, followed by the addition of benzyl chloroformate. The temperature was allowed to rise to 0 °C after 1.5 h and the reaction was quenched with saturated ammonium chloride solution. The reaction mixture was diluted with water and extracted with ethyl acetate. The combined extracts were dried over Na_2SO_4 and purified by chromatography using an eluent of 33% EtOAc in hexane. The PEO-*b*-PBCL block copolymer was then



Scheme 1. Synthetic scheme for the preparation of PEO-*b*-P(CL-DOX) block copolymer.

synthesized (Scheme 1 – step I) by reacting methoxy PEO (M_w: 5000 g mol^{-1}) (3.5 g), α-benzylcarboxylate-ε-caprolactone (3.5 g), and stannous octoate (0.002 eq of monomer) in a 10 mL previously flamed ampoule, nitrogen purged and sealed under vacuum. The polymerization reaction was allowed to proceed for 4 h at 140 °C in oven. The reaction was terminated by cooling the product to room temperature. Carboxyl group bearing block copolymer, i.e, PEO-*b*-PCCL was obtained (Scheme 1 – step II) by the catalytic debenzylation of PEO-*b*-PBCL in the presence of hydrogen gas as reducing agent and palladium-coated charcoal as catalyst according to the procedure described in our previous publication [11].

For the conjugation of DOX to PEO-*b*-PCCL, NHS (17.3 mg, 0.15 mM) and DCC (31 mg, 0.15 mM) were added to a stirred solution of PEO-*b*-PCCL (M_w: 7530 g mol^{-1}) (200 mg, 0.03 mM) in anhydrous THF (15 mL) under nitrogen. The reaction mixture was stirred for 2 h at room temperature. A solution of DOX.HCl (34.8 mg, 0.06 mM) and triethylamine (Et_3N) (16.8 μL, 0.12 mM) in anhydrous methanol (2 mL) was then added and the reaction continued for additional 96 h. Thin-layer chromatography in the presence of butan-1-ol:acetic acid:water (4:1:4) as the mobile phase was used to monitor the reaction progress. Evaporation of the reaction mixture gave a residue that was dissolved in HPLC grade methanol (10 mL).

DOX conjugated PEO-*b*-PCCL block copolymer, i.e., PEO-*b*-P(CL-DOX), was purified twice using Sephadex LH 20 column and methanol as eluent to remove the unreacted DOX and any other by-product. PEO-*b*-P(CL-DOX) was lyophilized to a deep orange powder for further use.

Prepared block copolymers were characterized for their average molecular weights and polydispersity by ^{1}H NMR and GPC. ^{1}H NMR was carried out by a Bruker Unity-300 spectrometer at room temperature, using deuterated dimethyl sulfoxide (DMSO-d_6) or deuterated chloroform ($CDCl_3$) as solvent and tetramethylsilane as internal reference. Gel permeation chromatography was carried out at 25 °C with an HP instrument equipped with Waters Styragel HT4 column (Waters Inc., Milford, MA). The elution pattern was detected at 35 °C by refractive index (PD2000, Percision Detectors, Inc.)/light scattering (Model 410, Waters Inc) detectors. THF was used as eluent at a flow rate of 1.0 mL/min. The column was calibrated with a series of standard methoxy poly(ethylene glycol)s with varying molecular weights.

3.2. Measurement of DOX-conjugated levels

Reversed-phase chromatography was carried out with a 10 μm C18-125 Å column (3.9 × 300 mm, Waters) heated at 40 °C using a Waters 625 LC system. Samples of 20 μL were injected in a gradient elution using 0.05% trifluoroacetic acid aqueous solution and acetonitrile at a flow rate of 1.0 mL/min. The percentage of acetonitrile in the mobile phase was 15% at time 0, which was increased to 85% within 15 min at a constant rate. The detection was performed at 485 nm using a Waters 486 tunable UV/Vis absorbance detector. The level of conjugated DOX was estimated from the integration of PEO-*b*-P(CL-DOX)-related peak in HPLC and also UV/Vis spectroscopy for PEO-*b*-P(CL-DOX) at 485 nm based on a calibration curve of free DOX under the same HPLC condition assuming an identical molar absorptivity for free DOX and polymer-conjugated DOX.

3.3. Self-assembly of block copolymers and physical encapsulation of DOX in the assembled structures

Self-assembly of block copolymers was accomplished through a co-solvent evaporation method where block copolymer (10 mg) was dissolved in THF (2 mL) and added to doubly distilled water (10 mL) in a drop-wise manner under moderate stirring. After 4 h of stirring at room temperature, vacuum was applied to ensure the complete removal of organic solvent. Encapsulation of DOX in PEO-*b*-PCL, PEO-*b*-PBCL, PEO-*b*-PCCL, and PEO-*b*-P(CL-DOX) micelles was carried out by an identical procedure with 1 mg of DOX and 20 μL of triethylamine added to the polymeric THF solution at the initial step. During encapsulation, the glycosidic amino group in DOX was deprotonated in the presence of triethylamine to increase the hydrophobicity of DOX. All the resulting micellar solutions were dialyzed (Spectra Por, M_w cutoff 3500 Da) against distilled water for 8 h exchanging the medium at 2 h intervals to remove un-encapsulated DOX.

3.4. Characterization of polymeric micelles

A change in the fluorescence excitation spectra of pyrene in the presence of varied concentrations of PEO-*b*-P(CL-DOX) block copolymer was used to measure its CMC according to the method described previously [16]. Briefly, pyrene was dissolved in acetone and added to 5 mL volumetric flasks to provide a concentration of 6×10^{-7} M in the final solutions. Acetone was then evaporated and replaced with aqueous polymeric micellar solutions with concentrations ranging from 0.05 to 5000 μg/mL. Samples were heated at 65 °C for an hour, cooled to room temperature overnight, and deoxygenated with nitrogen gas prior to fluorescence measurements. The excitation spectrum of pyrene for each sample was obtained at room temperature using a Varian Cary Eclipse fluorescence spectrophotometer (Victoria, Australia). The scan was performed at medium speed (600 nm/min) and at PMT detector voltage 575 V. Emission wavelength and excitation/emission slit were set at 390 and 5 nm, respectively. The intensity ratio of peak at 339 nm to that at 334 nm was plotted against the logarithm of copolymer concentration. CMC was measured from a sharp raise in intensity ratios (I_{339}/I_{334}) at the onset of micellization. The viscosity of prepared micellar core was estimated by measuring excimer to monomer intensity ratio (I_e/I_m) from the emission spectra of 1,3-(1,1′-dipyrenyl)propane at 373 and 480 nm, respectively, according to the method described previously [17]. Average diameters and size distribution of prepared micelles were estimated by dynamic light scattering (DLS) using Malvern Zetasizer 3000 at a polymer concentration of 10 mg/mL.

The level of entrapped DOX in polymeric micelles was determined in an aliquot of the micellar solution in water (200 μL) diluted five times with DMSO using DOX absorbance at 485 nm by a UV/Vis spectrophotometer (Beckman DU 640, USA). A calibration curve was constructed using different concentrations of free DOX (1–50 μg/mL) in an identical solvent mixture. The level of DOX-loading and encapsulation efficiency were calculated from the following equations:

$$\text{DOX-loading } [M(\text{DOX})/M(\text{monomer})]\ (\%) = \frac{\text{Moles of loaded DOX}}{\text{Moles of monomer}} \times 100$$

$$\text{DOX-loading [weight/weight] } (\%) = \frac{\text{amount of loaded DOX in mg}}{\text{amount of copolymer in mg}} \times 100$$

$$\text{DOX-loading } [M(\text{DOX})/M(\text{monomer})]\ (\%) = \frac{\text{Moles of loaded DOX}}{\text{Moles of copolymer}} \times 100$$

$$\text{Encapsulation efficiency (\%)} = \frac{\text{amount of loaded DOX in mg}}{\text{amount of DOX added in mg}} \times 100$$

The level of physically loaded DOX in PEO-*b*-P(CL-DOX) was calculated by deducting the level of chemically conjugated DOX (obtained by HPLC measurement as described above) from the total DOX content.

3.5. Release of DOX from polymeric micelles

DOX-loaded micellar solutions (15 mL) were prepared at 1 mg/mL polymer concentration from PEO-*b*-PCL, PEO-*b*-PBCL, PEO-*b*-PCCL, and PEO-*b*-P(CL-DOX) block copolymers according to the above-mentioned method. The micellar solutions were transferred into a dialysis bag (MW cutoff: 3500 Da, supplied by Spectrum Laboratories, USA). The dialysis bags were placed into 500 mL of phosphate buffer (pH 7.4) or acetate buffer (pH 5.0) solutions. Release study was performed at 37 °C in a Julabo SW 22 shaking water bath (Germany). At selected time intervals, 200 μL of micellar solution was withdrawn from inside the dialysis bag for UV/Vis analysis. DOX concentration was calculated based on the absorbance intensity at 485 nm as described in the previous section. An identical procedure was performed to investigate the level of DOX release from PEO-*b*-P(CL-DOX) conjugates. For this test group, at the end of the study, a sample of the remained polymer in the dialysis bag was dialyzed against distilled water for 8 h to remove the dissolved salt in the micellar solution. The sample was freeze-dried, dissolved in THF, centrifuged, and injected to the GPC system to assess the possibility of chain cleavage by hydrolysis for PEO-*b*-P(CL-DOX) block copolymers. The condition used in GPC was identical to that described for polymer characterization.

3.6. Assessing the hydrolysis of poly(ester) backbone in PEO-b-P(CL-DOX) micelles

PEO-*b*-P(CL-DOX) micellar solutions in acetate buffer (pH 5.0) were prepared at a polymer concentration of 1 mg/mL. Prepared micellar solution (3 mL) was incubated in a closed vial at 37 °C in a Julabo SW 22 shaking water bath (Germany). After 72 h, micellar solution was withdrawn from incubation and freeze-dried. The freeze-dried PEO-*b*-P(CL-DOX) micelle was dissolved in THF and centrifuged at 12,000×*g* to remove the salt and other insoluble ingredients. A sample (20 μL) of this solution was injected to the GPC system. The condition used in GPC was identical to that described for polymer characterization. The eluent from the GPC system was analyzed by mass spectroscopy (Waters, Micromass ZQ^{TM} 4000 Quadrupole Mass Analyzer, USA).

3.7. In vitro cytotoxicity of polymeric micellar DOX nanocontainers and drug conjugate against mouse melanoma cells

The cytotoxicity of free DOX, PEO-*b*-P(CL-DOX) conjugates and DOX-loaded PEO-*b*-PCL, PEO-*b*-PBCL, and PEO-*b*-P(CL-DOX) block copolymer micelles against B16F10 murine melanoma cells was investigated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were grown in RPMI 1640 complete growth media supplemented with 10% fetal bovine serum, 1% L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin and maintained at 37 °C with 5% CO_2 in a tissue culture incubator. In the logarithmic growth phase the cells were harvested and seeded into 96-well plates at a density of 5×10^3 cells/well in 100 μL of RPMI 1640 media. After 24 h when the cells had adhered, micellar solutions and free DOX at different concentrations were incubated separately with the cells for 24 and 48 h. After this time, MTT solution (20 μL; 5 mg/ml in sterile-filtered PBS) was added to each well and the plates were re-incubated for further 3 h. The yellow MTT is reduced to purple formazan in the mitochondria of living cells. The formazan crystals were dissolved in DMSO, and the absorbance was read by a Power Wave X 340 microplate reader (Bio-Tek Instruments, Inc. USA) at 550 nm. Cell viability (%) was plotted against logarithm of DOX concentration and used to calculate the DOX concentration required to reach 50% inhibition of cell growth compared to PBS treated cells (IC_{50}).

3.8. Statistical analysis

Data are reported as means ± standard deviation (SD). Differences among the mean of formulation characteristics for polymeric micelles were compared by either one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls post hoc test for multiple comparisons using Sigma stat software or Student's unpaired *t*-test assuming unequal variance. Differences between means of IC_{50} were assessed using one-way ANOVA followed by post hoc analysis using Dunnett T3 test (SSPS for Windows v.13, Cary, NC). The level of significance was set at $\alpha = 0.05$.

4. Results

4.1. Synthesis of PEO-b-P(CL-DOX) and its characterization

DOX-conjugated PEO_{114}-*b*-P(CL-DOX)$_{16}$ was synthesized by forming an amide bond between carboxyl groups of PEO-*b*-PCCL and a primary amine group of DOX using DCC as coupling agent and NHS as catalyst (Scheme 1). The conjugation of the DOX molecule with block copolymer was confirmed by thin-layer chromatography (TLC), where free DOX eluted with the solvent and showed a spot at R_f value of 0.68, but the polymer-conjugated DOX did not elute and stayed at the baseline (data not shown). Fur-

ther evidence for the conjugation of DOX to PEO-*b*-PCCL polymer was provided by the ^{1}H NMR spectrum of PEO-*b*-P(CL-DOX) (Fig. 1A), free DOX (Fig. 1B) and PEO-*b*-PCCL (Fig. 1C). ^{1}H NMR spectrum of PEO-*b*-P(CL-DOX) in $CDCl_3$ (Fig. 1A) showed characteristic DOX peaks at δ 7.4–7.8 ppm as well as ^{1}H NMR spectrum in DMSO-d_6 revealed the characteristic DOX peaks (Fig. 1A) at δ (ppm): 5.6; 5.3; 3.6; 3.3; 2.25; 2.0; and 1.2. Similar peaks were also present in the ^{1}H NMR spectrum of free DOX (Fig. 1B). The characteristic peaks of PEO-*b*-PCCL block copolymer observed in its ^{1}H NMR spectrum at δ (ppm): 4.05; 3.5, 3.30; 1.72; 1.55; and 1.28 are also present in the ^{1}H NMR spectrum of PEO-*b*-P(CL-DOX) at identical positions (Fig. 1A and C). The dependence of ^{1}H NMR spectrum of DOX–polymer conjugates on the organic solvent used for ^{1}H NMR spectroscopy is due to a difference in the conformation of DOX–polymer conjugate in various organic solvents. In the HPLC chromatogram of PEO-*b*-P(CL-DOX) block copolymer, peak related to free DOX was absent (Fig. 2). The ^{1}H NMR spectra of PEO-*b*-PCL(DOX) along with HPLC data and TLC results provided evidence for the conjugation of DOX to PEO-*b*-PCL backbone and the efficient removal of free DOX from the PEO-*b*-P(CL-DOX) polymer–drug conjugate after the purification process.

The substitution level of DOX on the polymer backbone was 14% (mole DOX/mole monomer) as measured by UV analysis at 485 nm, which was consistent with the DOX conjugation level, estimated from the HPLC analysis (Fig. 2). This corresponds to 2.25 DOX molecules per

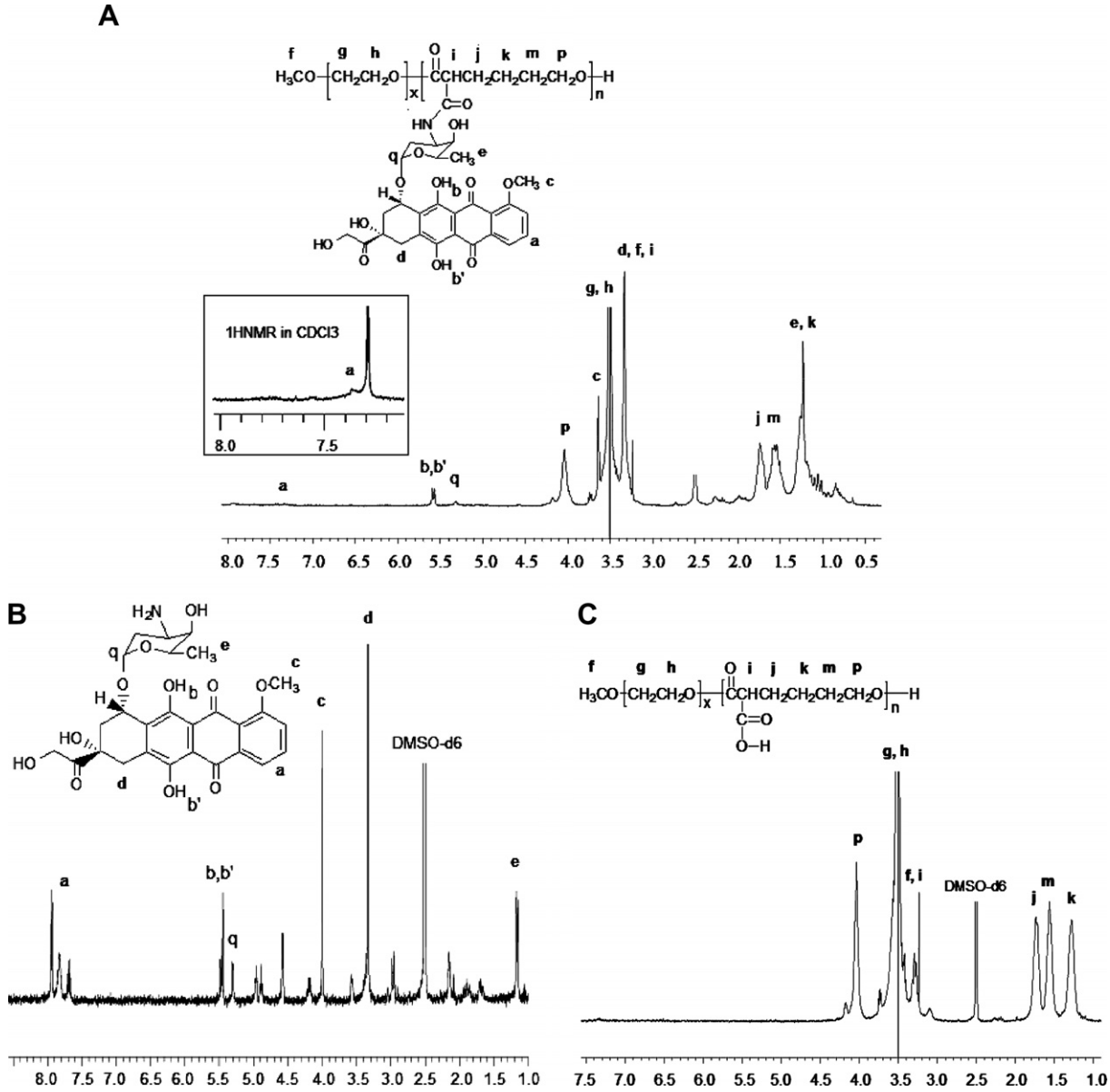


Fig. 1. ^{1}H NMR spectrum and peak assignments of (A) PEO-*b*-P(CL-DOX) block copolymer in DMSO-d_6 and in $CDCl_3$ (A, window); (B) free DOX in DMSO-d_6; (C) PEO-*b*-PCCL block copolymer in DMSO-d_6 (Reproduced with permission from Ref. [11]).

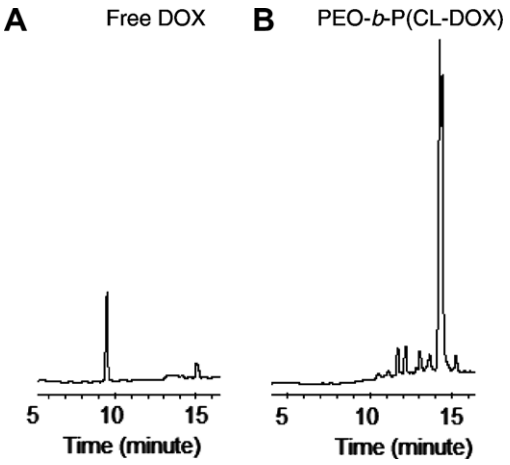


Fig. 2. Typical HPCL chromatogram of (A) free DOX; and (B) PEO-*b*-P(CL-DOX) polymer. Reversed-phase chromatography was carried out with a Waters 10 μm C18-125 Å column (3.9 × 300 mm) in a gradient elution using 0.05% trifluoroacetic acid aqueous solution and acetonitrile.

PEO_{114}-*b*-P(CL-DOX)$_{16}$ chain on average. Conjugation by DCC is known to produce low substitution of DOX on the polymeric backbone [18]. The number average molecular weight of PEO-*b*-P(CL-DOX) obtained from ^{1}H NMR by comparing the peak related to methylene protons (CH_2) of PCL at δ 4.0 ppm to that for the methylene protons (CH_2–CH_2) of PEO at δ 3.5 ppm and considering the number of DOX attached to PCL chain (~2.25 for each PCL chain on average) was 8800 g mol^{-1}. The number average molecular weight of PEO-*b*-P(CL-DOX) block copolymer determined by GPC showed a small increase ($M_w = 9600$ g mol^{-1}) compared to the molecular weight determined by ^{1}H NMR and a relatively broad molecular weight distribution ($M_w/M_n = 1.47$).

4.2. Self-assembly of PEO-b-P(CL-DOX) and characterization of micellar structures

Characteristics of prepared polymeric micelles are summarized in Table 1. The average diameter of the unloaded PEO-*b*-PCL, PEO-*b*-PBCL, PEO-*b*-PCCL, and PEO-*b*-P(CL-DOX) block copolymer micelles was 40.0, 65.5, 55.3, and 81.6 nm, respectively. The broad polydispersity index (0.58) for PEO-*b*-P(CL-DOX) indicated existence of secondary aggregation. Despite the presence of secondary association, the micellar solutions were still transparent.

Low CMC values were revealed for PEO-*b*-PCL and PEO-*b*-PBCL (Table 1), whereas PEO-*b*-PCCL block copolymers showed lower tendency for self-association reflected by higher CMC values (1.22×10^{-2} mM). DOX attachment reduced the CMC of PEO-*b*-PCCL by 3.5 times (370×10^{-2} μM) although the polymer chain contained a considerable number of free COOH groups. A reverse relationship between the hydrophobicity of the core-forming block and CMC has been shown in many studies [17,19,20].

Evidence for the rigidity in the core of PEO-*b*-PCL-based micelles prepared in this study was obtained from the fluorescence emission spectra of 1,3 (1,1′ dipyrenyl)propane in the presence of block copolymers at concentration above CMC (~1000 μg/mL) [17,21]. 1, 3 (1,1′ dipyrenyl) propane forms intermolecular excimers that emit light at 480 nm when excited at 390 nm. In a highly viscous environment, such as in the core of PEO-*b*-PCL based micelles, excimer formation is restricted and exhibits a very low value of I_e/I_m ratio. Attachment of DOX to the PEO-*b*-PCCL decreased the microviscosity of micellar core exhibiting a significantly higher I_e/I_m ratio (0.045) than PEO-*b*-PCCL micelles (0.025) ($P < 0.05$, unpaired Student's *t*-test).

4.3. Preparation of DOX-loaded micelles and their characterization

The level of loaded DOX to polymer in molar ratio was found to be significantly higher for PEO-*b*-PBCL compared to PEO-*b*-PCL (Table 2). This level was significantly lower for micelles formed from PEO-*b*-PCCL and PEO-*b*-P(CL-DOX) block copolymers. To account for differences in the polymerization degree of hydrophobic block between block copolymers under study and demonstrate the contribution of each monomer to the drug-loading efficiency in the micellar core, the mole% of loaded DOX to monomer

Table 1
Characteristics of empty block copolymer micelles ($n = 3$)

Block copolymer	Average micellar size ± SD (nm)[a]	Average size of secondary peaks (nm)	PDI[c]	CMC[d] ± SD (μM)	I_e/I_m ± SD[e]
PEO_{114}-*b*-PCL_{42}	40.0 ± 2.0	–	0.19	$18.2 \times 10^{-2} \pm 0.01$[f]	0.055 ± .007[f]
PEO_{114}-*b*-$PBCL_{19}$	65.5 ± 3.6	–	0.31	$9.8 \times 10^{-2} \pm 0.01$[f]	0.028 ± .002[f]
PEO_{114}-*b*-$PCCL_{16}$	55.3 ± 4.0	–	0.15	$1220 \times 10^{-2} \pm 0.42$[f]	0.025 ± .002[f]
PEO_{114}-*b*-P(CL-DOX)$_{16}$	81.6 ± 3.6	347 (60%)[b]	0.58	$370 \times 10^{-2} \pm 0.36$	0.045 ± .030

[a] Intensity mean estimated by dynamic light scattering technique.
[b] Numbers in the parenthesis indicate the frequency of secondary peak in micellar population in percentage.
[c] Polydispersity index (PDI).
[d] Measured from the onset of a rise in the intensity ratio of peaks at 339 nm to peaks at 334 nm in the fluorescence excitation spectra of pyrene plotted versus logarithm of polymer concentration.
[e] Intensity ratio (excimer/monomer) from emission spectrum of 1,3-(1,1′ dipyrenyl) propane in the presence of polymeric micelle.
[f] The data are reproduced from Refs. [11] for comparison.

Table 2
Characteristics of DOX-loaded block copolymer micelles ($n = 3$)

Block copolymer micelle	DOX-loading content (%) ± SD			Encapsulation efficiency (%) ± SD	Average diameter ± SD	PDI[d]	DOX release at 24 h (%)[e]	
	DOX/monomer (molar)[a]	DOX/polymer (molar)[b]	DOX/polymer (wt)[c]				pH 5.0	pH 7.4
PEO_{114}-*b*-PCL_{42}	2.0 ± 0.1	75.1 ± 4.9	4.3 ± 0.3	48.3 ± 3.1	35.9 ± 4.0	0.08	35.9 ± 3.0	24.7 ± 1.4
PEO_{114}-*b*-$PBCL_{19}$	5.0 ± 0.2[g]	83.5 ± 1.0[g]	5.0 ± 0.1[g]	54.9 ± 1.0[g]	63.9 ± 2.8[g]	0.29	20.0 ± 1.9[g]	13.2 ± 1.0[g]
PEO_{114}-*b*-$PCCL_{16}$	2.6 ± 0.3[g]	35.7 ± 3.4[g]	2.8 ± 0.3[g]	31.8 ± 2.9[g]	120 ± 9.0[g]	0.23	47.2 ± 1.0[g]	32.0 ± 1.7[g]
PEO_{114}-*b*-P(CL-DOX)$_{16}$	3.6 ± 0.2[f,g]	63.5 ± 4.2[f,g]	4.0 ± 0.5[f]	43.3 ± 2.8[g]	125.5 ± 15	0.38	40.2 ± 2.3	22.5 ± 1.0

[a] DOX-loading content, calculated as moles of DOX/moles of monomer of core-forming block.
[b] DOX-loading content, calculated as moles of DOX/moles of copolymer.
[c] DOX-loading content, calculated as weight of DOX/weight of copolymer used to form micelles.
[d] Polydispersity index (PDI) for micellar size distribution.
[e] Release study was performed in acetate buffer (pH 5.0) and in phosphate buffer (pH 7.4).
[f] The level is estimated for physically encapsulated DOX only, by subtracting the concentration of conjugated DOX from its total concentration.
[g] Significantly different from PEO-*b*-PCL ($P < 0.05$).

was calculated. The loading content (mole DOX/mole monomer in core-forming block) of DOX in PEO-*b*-PCL micelles was 2.0, which agrees well with the previously reported results [22]. Compared to PEO-*b*-PCL, DOX-loading content (mole DOX/mole monomer in core-forming block) was increased by 2.5- and 1.8-fold in PEO-*b*-PBCL and PEO-*b*-P(CL-DOX) micelles, respectively. The loading content of DOX (mole DOX/mole monomer in core-forming block) in PEO-*b*-PCCL was 2.6, slightly higher than that of PEO-*b*-PCL ($P < 0.05$, one-way ANOVA).

DOX loading did not lead to any significant change in the average size of PEO-*b*-PCL and PEO-*b*-PBCL micelles ($P > 0.05$, unpaired student's *t*-test). DOX-loaded PEO-*b*-PCCL and PEO-*b*-P(CL-DOX) block copolymer micelle had an average size of 120 and 125.5 nm, respectively, which was significantly higher than the size of their unloaded counterparts ($P < 0.01$, unpaired student's *t*-test) with no sign of secondary aggregation (Table 2).

4.4. Release of DOX from polymeric micelles

The release profile of DOX from micellar nano-containers and DOX–polymer conjugate was studied within 72 h, using a dialysis membrane immersed either in phosphate buffer (pH 7.4, 0.1 M) or acetate buffer (pH 5.0, 0.1 M) at 37 °C temperature. The concentration of copolymers was fixed at 1 mg/mL, which is much higher than their corresponding CMCs. Transfer of released DOX through dialysis membrane to buffer solution was assumed to take place rapidly, and the release of DOX from its vehicle to medium was assumed to be the rate-limiting step in this process. In fact, 73% of free DOX was transferred to the release medium from the dialysis bag within 2 h at pH 5.0 (Fig. 3). The transfer of free DOX at pH 7.4 was slower (63% was transferred to the medium at 2 h-time point). The release of DOX from polymeric micelles at both pHs was strongly affected by the micellar core composition, with micelles bearing the benzyl core, i.e., PEO-*b*-PBCL, showing the minimum rate of drug release. At pH 5, PEO-*b*-PCL, PEO-*b*-PBCL, and PEO-*b*-PCCL micelles released 35%, 20%, and 47% of encapsulated DOX after 24 h, respectively (Table 2). Similar to what has been observed for free DOX, DOX release from polymeric micelles was found to be slower at pH 7.4 compared to pH 5.0, but followed the same trend between different core structures (Fig. 3). At pH 7.4, 25%, 13%, and 32% of encapsulated DOX were released from PEO-*b*-PCL, PEO-*b*-PBCL, and PEO-*b*-PCCL micelles after 24 h, respectively. PEO-*b*-P(CL-DOX) micelles did not show any superiority in sustaining

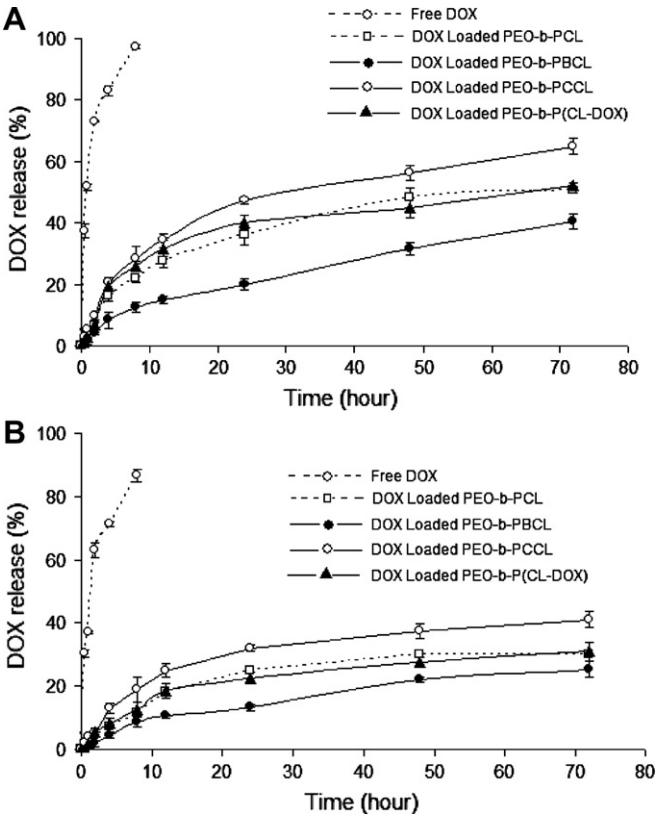


Fig. 3. *In vitro* DOX release profile from polymeric micelles at (A) pH 5.0, and (B) pH 7.4 at 37 °C. Each point represents mean ± SD ($n = 3$).

the release of physically loaded DOX over PEO-*b*-PCL micelles at both pHs (Fig. 3).

No significant change in the level of DOX remaining in the dialysis bag for PEO-*b*-P(CL-DOX) conjugate was observed within the time frame of the study, reflecting the stability of the amide linkage between DOX and the polymeric backbone (data not shown). To assess the possibility of poly(ester) cleavage, a sample from the remaining PEO-*b*-P(CL-DOX) in the dialysis bag at the end of the release study was freeze-dried, dissolved in THF and injected to the GPC system. No significant change in the retention time of the polymer-related peak was observed, but the polydispersity of PEO-*b*-P(CL-DOX) at pH 5.0 was reduced from 1.47 to 1.20 pointing to a possibility of chain cleavage in the poly(ester) backbone.

4.5. Hydrolysis of poly(ester) backbone of PEO-b-P(CL-DOX) micelles

GPC analysis revealed a dramatic change in the retention time of PEO-*b*-P(CL-DOX) block copolymer from 9.6 to 10.3 min after its incubation in a closed vial at pH 5.0 for 72 h at 37 °C (Fig. 4). Also, the generation of a new peak at 11.3 min which was absent in the GPC chromatogram of PEO-*b*-P(CL-DOX) before incubation at pH 5.0 (Fig. 4) strongly implicated the possibility for the hydrolysis of polyester core at acidic pH after 72 h. Analysis of the eluent at 11.3 min by mass spectroscopy revealed the presence of a peak at *m/z* of 701 (data not shown) corresponding to DOX-(6-hydroxy caproic acid), i.e., the possible product of PCL chain cleavage in PEO-*b*-P(CL-DOX) block copolymers at pH 5.0.

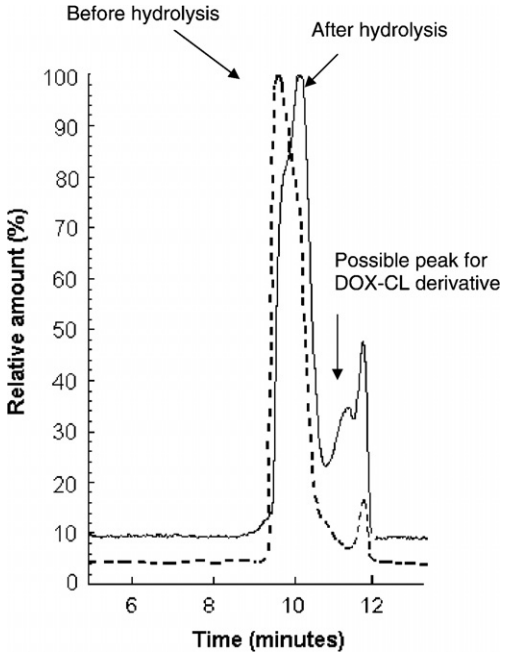


Fig. 4. Gel permeation chromatogram of PEO-*b*-P(CL-DOX) before and after incubation at pH 5.0 for 72 h.

4.6. In vitro cytotoxicity study

The results of cytotoxicity study on free DOX, PEO-*b*-P(CL-DOX), as well as DOX-loaded PEO-*b*-PCL, PEO-*b*-PBCL, and PEO-*b*-P(CL-DOX) micelles against murine melanoma B16F10 cells after 24 and 48 h of incubation are shown in Fig. 5. Overall, free DOX was shown to be more effective than the polymeric micellar formulations at both incubation times. The IC_{50} of DOX as part of polymeric micelles was between 1.54 and 3.65 μg/mL after 24 h of incubation with conjugated DOX showing the least cytotoxicity among different formulations. In comparison, the IC_{50} of free DOX was 0.09 μg/mL after 24 h of incubation (Fig. 5c and d). Interestingly, the IC_{50} of conjugated DOX in PEO-*b*-P(CL-DOX) after 48 h of incubation (0.50 μg/mL) was lower than the IC_{50} of physically encapsulated DOX in PEO-*b*-PCL and PEO-*b*-PBCL micelles (1.05 and 1.54 μg/mL, respectively) ($P < 0.01$, one-way ANOVA). Free DOX showed an IC_{50} of 0.03 μg/mL after 48 h of incubation with this cell line. DOX loaded in PEO-*b*-P(CL-DOX) micelles (chemically conjugated + physically loaded) showed higher cytotoxicity (threefold lower IC_{50}) than conjugated DOX in PEO-*b*-P(CL-DOX) micelles at 24 h, but did not show superiority over conjugated DOX after 48 h of incubation. PEO-*b*-PBCL and PEO-*b*-PCCL alone, did not show any sign of cytotoxicity up to 500 μg/mL concentrations in this cell line.

5. Discussion

Conjugation of DOX to the block copolymer and further self-association of the copolymers to micellar structure is expected to minimize the chance of DOX leakage from carrier during blood circulation and restrict the distribution of conjugated drug only to tissues accessible for the carrier. As a result, the conjugated DOX will follow the fate of the polymeric micellar delivery system, circulate for prolonged period in blood and preferentially accumulate in solid tumor by EPR effect [23,24]. The major concern is the excessive stability of the polymer–drug conjugate at target site which may lead to substantial reduction in the efficacy of the conjugated drug. In contrast to conjugated DOX, physically encapsulated DOX in polymeric micelles is expected to show a better efficacy as a result of more rapid DOX release. However, by the same token, the carrier may lose most of its drug content before reaching the target site in the biological system. In this paper, we report on the preparation of novel self-associating PEO-*b*-P(CL-DOX) conjugates with four distinct characteristics: (a) a possibility for the incorporation of several DOX molecules per polymer chain, which can lower the required polymer dose of administration; (b) thermodynamic stability induced by a great tendency for micellization due to the presence of hydrophobic PCL backbone as the core-forming block; (c) stabilization of DOX within the carrier through covalent conjugation to the polymer and further self-association of the polymer, which will

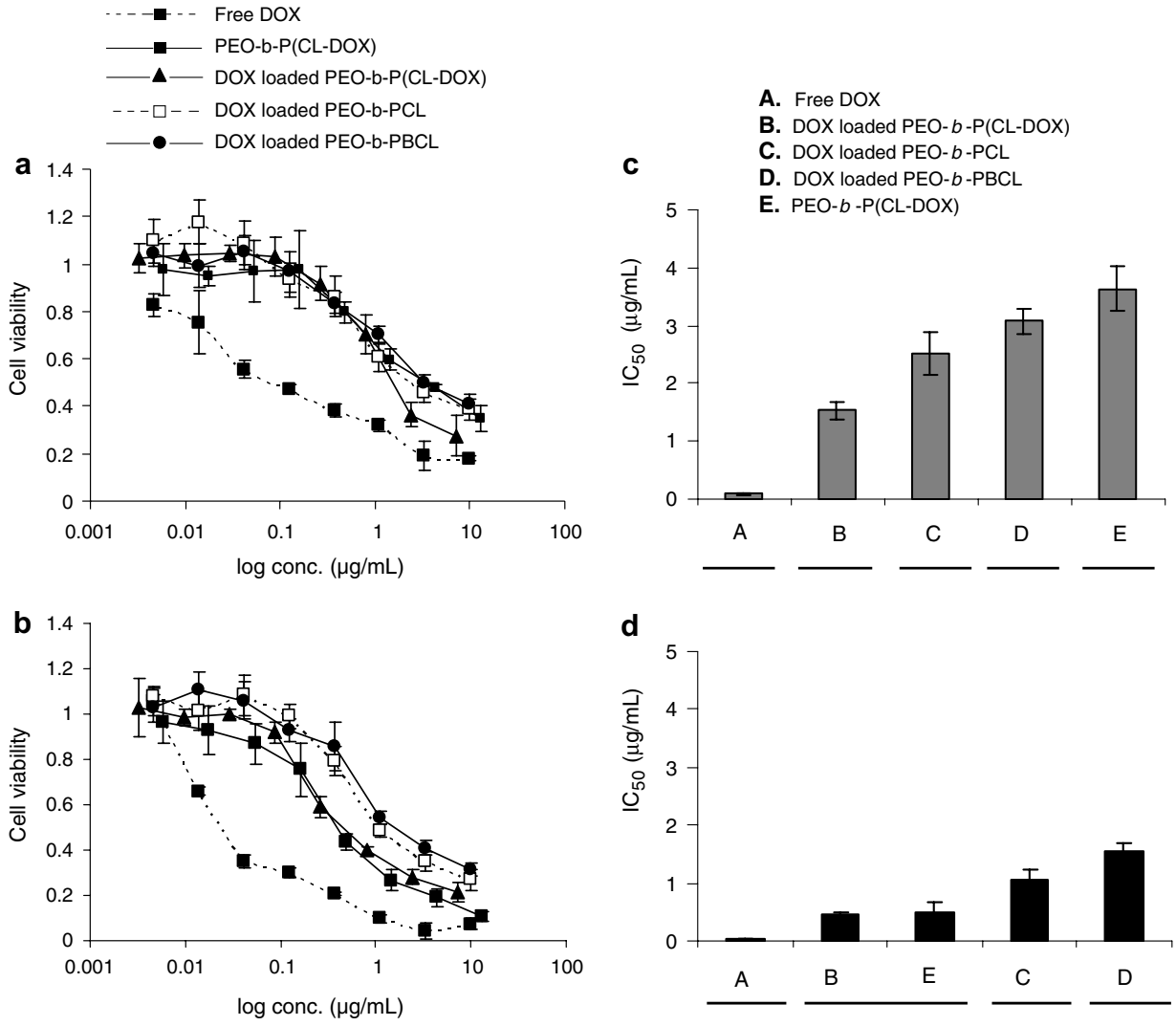


Fig. 5. *In vitro* cytotoxicity of free and polymeric micellar DOX formulations against B16F10 mouse melanoma-carcinoma cells after (a) and (c) 24 h, and (b) and (d) 48 h of incubation. (a) and (b) The cell viabilities are expressed as a function of the logarithm of the DOX concentration. Each point represents mean ± SD ($n = 3$). For DOX-loaded in PEO-*b*-P(CL-DOX) micelles, the concentration of total DOX in the micelle (physically encapsulated + chemically conjugated DOX) is calculated and used in the graph. (c) and (d) IC_{50} values of free DOX and DOX loaded polymeric micellar DOX formulations against B16F10 cells were calculated form the plots of *in vitro* cytotoxicity (plot A and B). Discontinuation of the line under the bars indicates the significant difference of the IC_{50} values among different formulations ($P < 0.05$). Differences between means ($n = 3$) of IC_{50} were assessed using one-way ANOVA followed by post hoc analysis using Dunnett T3 test (SSPS for Windows v.13, Cary, NC). The level of significance was set at $\alpha = 0.05$.

lower the chance of premature DOX release in blood circulation; (d) degradability of the hydrophobic backbone, to which DOX is covalently attached, by hydrolysis that is catalyzed in acidic condition. The latter property may trigger micellar dissociation and DOX release in acidic extra cellular microenvironment or intracellular organelles of solid tumors leading to a better release and cytotoxicity profile for the conjugated DOX.

Characterization studies on the prepared polymer revealed successful conjugation of DOX to PEO-*b*-PCCL (Fig. 1) and absence of free DOX after polymer purification (Fig. 2). Although the substitution level of DOX to the PEO-*b*-PCCL backbone was only 14% the system has the potential for increasing the conjugation level of drug to the polymeric backbone through use of excess DOX or catalysts in the reaction and/or elongation of PCL backbone.

A second particle population at higher size for PEO-*b*-P(CL-DOX) micelles, possibly formed through aggregation of polymeric micelles, was observed in DLS measurements. Secondary association of polymeric micelles has been described by several authors previously [25–28]. Nevertheless, it may be prevented by optimizing the hydrophilic lipophilic balance (HLB) of the block copolymer, increasing the level of DOX conjugation, modification of the micellization process [28] and/or addition of lyoprotectants [29]. DOX conjugation to PEO-*b*-PCCL reduced the CMC of this polymer, but the CMC of PEO-*b*-P(CL-DOX) was higher than that of PEO-*b*-PCL (Table 1). DOX conjugation to PEO-*b*-PCCL had a negative impact

on the viscosity of micellar core, probably as a result of interruption in intramicellar hydrogen bonds between carboxyl groups of PEO-*b*-PCCL within the micellar core by substituted DOX. Polymeric micellar PEO-*b*-P(CL-DOX) that contained conjugated DOX did not show any significant release of free DOX at physiological (pH 7.4) or acidic condition (pH 5.0) using the dialysis method (data not shown). The observation was attributed to the stability of the amide bond between caprolactone and DOX in the micellar core and was consistent with the findings of Kataoka et al. on DOX release from PEO-*b*-P(Asp-DOX) micellar drug conjugates [30]. A decrease in the polydispersity of the PEO-*b*-P(CL-DOX) remained in the dialysis bag after the release experiment at pH 5.0, pointed to a possibility for chain cleavage in poly(ester) backbone of P(CL-DOX) at pH 5.0. This assumption was further confirmed in a study where PEO-*b*-P(CL-DOX) micelles were incubated at acidic pH in a closed system for three days. Change in the retention time of polymer-related peak and generation of an extra peak at 11.3 min in the GPC chromatogram of the incubated polymer strongly supports the possibility for the hydrolysis of P(CL-DOX) backbone and release of active DOX-CL derivative (Fig. 4). Mass spectrum analysis of the fraction corresponding to the peak at 11.3 min lends further support to this assumption due to the presence of ion at *m*/*z* 701 (data not shown) showing the presence of DOX-CL derivative, i.e., DOX-(6-hydroxy caproic acid), produced after hydrolysis in the sample incubated at pH 5.0. Recently, Geng et al. has shown PCL-end hydrolysis and production of 6-hydroxy caproic acid as hydrolyzed product from PEO-*b*-PCL block copolymer worm-like micelle at pH 5.0 [31]. Degradation of aliphatic poly(ester)s occurs via random hydrolytic scission of ester bonds [32]. The degradation rate of PCL is considerably slower compared to other aliphatic poly(ester)s due to its hydrophobicity and high crystallinity [33,34]. However, Gimenez et al. demonstrated a dramatic increase in the hydrolytic degradation rate of PCL polymer bearing pendant COOH group compared to the parent PCL due to the increase in hydrophilicity of the polymer backbone [35]. Degradation is also accelerated by the surface area of the polymeric scaffold and acidic pH of the media [34]. Moreover, poly(ester)s may degrade faster *in vivo* than *in vitro* due to the presence of lysosomal enzyme (e.g., lysosomal esterase, cathepsin B, etc.) at cellular level [34,36,37].

In further studies, physical encapsulation of DOX in PEO-*b*-PCL block copolymers and its PCL-modified derivatives was carried out. Elongation of the chain length, introduction of hydrophobic groups or changing the net charge of the polymeric core has also been utilized to increase the loading efficiency and limit the rate of release for hydrophobic drugs from polymeric micelles [38,39,5]. Increasing the molecular weight of PCL has shown limited benefit in terms of DOX-loading efficiency [22]. Alternatively, modification of the PCL with benzyl or DOX groups was expected to increase DOX loading as a result of increased interaction between the micellar core and DOX. Introduction of carboxyl groups in the micellar core was also hypothesized to have a positive impact on DOX encapsulation because of the possibility for hydrogen-bonding and/or electrostatic complexation between DOX and COOH in the micellar cores [11]. In effect, the mole% of loaded DOX to monomer was ranked as PBCL > P(CL-DOX) > PCCL > PCL (Table 2). The π–π interaction between the aromatic rings of PBCL or conjugated DOX and physically encapsulated DOX may account for higher DOX solubilization in PBCL and P(CL-DOX) cores [40]. Whereas formation of hydrogen bonds between the carboxyl groups of DOX and PCCL may explain the better solubilization of DOX in PCCL, an alternative possibility is the formation of electrostatic complex between the amine group of DOX and carboxyl group of PCCL as shown in previous studies for poly(acrylic acid)/DOX pairs [41,42].

Among different micellar core structures a core of PBCL provided the most control over the rate of DOX release in both pHs. A more rapid rate of DOX release from all micellar nano-containers under study was seen at pH 5.0 compared to pH 7.4. The faster release of encapsulated DOX from PEO-*b*-poly(β-benzyl-L-aspartic acid) (PEO-*b*-PBLA) and PEO-*b*-PCL in acidic pH has been observed in previous studies[22,40]. Protonation of the 3′-NH_2 group in the DOX–sugar moiety at acidic pH, which increases the partition of DOX from the micellar core to the aqueous environment is assumed to be the reason behind this observation [40]. Accelerated release of DOX at acidic pH is an added advantage for its tumor targeted delivery, because it allows preferential drug release at the extracellular space of solid tumors or cellular endosomes where pH is acidic (5.0–5.5).

The cytotoxicity of physically encapsulated DOX in different polymeric micelles followed a similar trend to what has been observed in the release study where polymeric micelles with higher level of DOX release showed higher cytotoxicity (lower IC_{50}) values at both incubation times (Fig. 5). Accordingly, the IC_{50} of conjugated DOX was higher than physically encapsulated DOX after 24 h of incubation. However, after 48 h of incubation, The IC_{50} of conjugated DOX was found to be lower than physically encapsulated DOX in PEO-*b*-PCL and PEO-*b*-PBCL and similar to that of DOX-loaded PEO-*b*-P(CL-DOX) micelles. The micellar DOX conjugate was also less cytotoxic than free drug, but the difference in the cytotoxicity of conjugated and free DOX decreased as the incubation time was raised. Given that the results of *in vitro* release study did not show any significant release of free DOX from the micellar polymer–DOX conjugate; the cytotoxicity of PEO-*b*-P(CL-DOX) conjugate may be an indication for the release of active DOX derivatives from the polymer–drug conjugate due to PCL chain cleavage within 48 h of incubation and/or an anti-growth activity for the PEO-*b*-P(CL-DOX) conjugate in B16F10 cells. Several mechanisms have been proposed to explain the antitumor activity of DOX [43,44] including intercalation into DNA, interaction with plasma membranes and the forma-

tion of free radicals through bioreductive activation. Although release of free DOX from conjugate is essential for the first mechanism, it may not be necessary if alternative mechanisms play a crucial role in the cytotoxicity. Cytotoxic effects for conjugated DOX as part of polyacetal-DOX conjugates [18] and PEO-*b* poly(Glutamic acid-DOX) micelles [45] against different cancer cells have also been reported.

6. Conclusion

Manipulation of the micellar core in PEO-*b*-PCL micelles through introduction of benzyl and carboxyl groups endows polymeric micelles superior DOX-solubilizing capacity as well as a better control over DOX rate of release from the colloidal carrier. Modification of the core in PEO-*b*-PCL micelles through conjugation of DOX, on the other hand, provides viable means for the development of polymeric micellar drug conjugates that can afford efficient control over the rate of DOX release in physiological pH, core hydrolysis in acidic pH and maintained cytotoxicity in cancer cells.

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