Synthesis, characterization and drug delivery behaviors of new PCP polymeric micelles

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Abstract

A new PCP tri-block copolymer consisting of poly (ε-caprolactone)-b-chitooligosaccharide-b-poly(ethylene glycol) (PCL-b-COS-b-PEG, PCP) was synthesized and characterized. The potential for delivering doxorubicin (DOX), a model drug, with or without genipin crosslinking was evaluated. The PCP copolymers were analyzed by Fourier-transform infrared spectrometry (FT-IR) to confirm the amine and ester groups of the COS and the PCL of the copolymer, respectively. ^{1}H nuclear magnetic resonance (^{1}H NMR) was performed to determine the structure of the PCP copolymer for demonstrating both PCL and PEG blocks grafted onto the COS block. Moreover, gel permeation chromatography (GPC) was applied to determine the number average molecular weight of the tri-block copolymer (M_n), which was 11,340 Da/mole. The PCP form polymeric micelles at the critical micelle concentration (CMC) of 0.0107 wt% (or 1.0 μM) with the mean diameter 90 nm, as determined by a dynamic light-scattering (DLS) analyzer. Since PCP micelles contain COS, the zeta potentials of the micelles are changed from a neutral (e.g., -3.2 ± 1.3 mV at pH 7.4) to a cationic state (13.9 ± 4.4 mV at pH 3.0) when pH values of the suspension medium are varied. This change is a unique property of the micelles. In addition, drug delivery behavior of the PCP micelles is influenced by genipin crosslinking COS. The DOX release period of crosslinked micelles is significantly longer than that of the non-crosslinked ones (e.g., 8 days vs. 4 days, respectively) while the burst release of DOX of crosslinked ones is significantly reduced compared with that of non-crosslinked ones. In conclusion, a new tri-block COS-containing PCP copolymer/polymeric micelle has been synthesized and characterized. Moreover, the unique properties of COS-containing PCP micelles are demonstrated by varying zeta potentials via changing pH of medium and by influencing DOX delivering behaviors after genipin crosslinking.
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Keywords: Chitooligosaccharide (COS); Poly(ε-caprolactone); Polymeric micelles; Genipin; Drug delivery

1. Introduction

Polymeric micelles have attracted interest in recent years because they mimic the structural and functional aspects of biological transport systems (Jeong, Kibbey, Birnbaum, Won, & Gutowska, 2000; Park et al., 2002; Torchilin, 2001, 2002). Notably, micelles are usually self-assembled by amphiphilic block copolymers in aqueous solution, the structures of which contain a hydrophobic core and a hydrophilic shell. These cores are generally spherical

and nano-sized (10–100 nm) and can be used as nano-containers for the efficient loading of hydrophobic drugs or agents (Lavasanifar, Samuel, & Kwon, 2002; Torchilin, 2001, 2002). Thus, hydrophobic drugs that incorporate the micelles are effective drug delivery systems.

Poly(lactic-co-glycolic acid) (PLGA) and poly(ε-caprolactone) (PCL) are widely favored for the hydrophobic segments of micelles. Poly(ethylene-glycol) (PEG) polymers are usually adopted as the hydrophilic segments of micelles (Jeong et al., 2000; Lavasanifar et al., 2002; Park et al., 2002) because they provide a brush-like stability corona that increases the circulatory half-time of micelles in the blood stream (Savic, Luo, Eisenberg, & Maysinger, 2003; Yokoyama, Fukushima, Uehara, Okamoto, & Kataoka, 1998). For instance, PEO-b-PDLLA, PEG-PLGA, and PEG-PCL copolymers and micelles have been reported (Lavasanifar et al., 2002; Park et al., 2002; Savic et al., 2003; Torchilin, 2001; Yokoyama et al., 1998). This study prepares new polymeric micelles by inserting oligomers of chitosan, COS, between PCL and PEG polymers. These PCL-b-COS-b-PEG (PCP) tri-block copolymers/micelles provide their constituent copolymers with amine groups. The unique properties of the COS-containing PCP micelles are demonstrated by investigating the delivery behaviors of DOX, a model drug, with genipin crosslinking.

COS generally consists of fewer than ten D-glucosamine and N-acetyl-D-glucosamine units and is obtained by either the chemical or the enzymatic hydrolysis of chitosan (Jeon & Kim, 2000; Jeon, Park, & Kim, 2001), which consists of 2-amino-2-deoxy-(1-4β)-D-glucose-amine units and few or no N-acetyl-D-glucosamine units. COS biologically exhibits favorable biocompatibility and, chemically, includes finite numbers of hydroxyl and amine groups of the D-glucosamine units for chemical modifications such as those involving chitosan (Kurita, Tomita, Tada, Nishimura, & lshii, 1993; Nishiyama et al., 1999).

In synthesis of the PCP copolymer, the PCL and PEG polymers were designed to region-selectively react with the hydroxyl groups (Kurita, Ikeda, Yoshida, Shimojoh, & Harata, 2002), preserving amine groups of the D-glucosamine units of COS Therefore, the following steps were implemented (Scheme 1); (1) The amine groups of COS were first protected by the N-phthaloylation of COS; (2) PCL polymers were reacted with the hydroxyl groups of COS, and then PEG polymers were reacted with the residual hydroxyl groups of COS; (3) PCP copolymers were fabricated after the de-N-phthaloylation of the COS-yielded PCP copolymers.

To characterize the PCP copolymers, Fourier-transform infrared spectrometry (FT-IR), ^{1}H nuclear magnetic resonance (^{1}H NMR), and gel permeation chromatography (GPC) were performed. The characteristics of the PCP micelles such as the critical micelle concentration (CMC), the size by DLC, and morphology of micelles by TEM are reported. The unique properties of COS-containing PCP micelles are demonstrated by studying zeta potential of micelles in different pH media, and the delivery behavior of the PCP micelles of the model drug DOX, with or without genipin crosslinking.

2. Materials and experimental methods

2.1. Materials

COS with at least a 95% de-acetylation and molecular weight about 2000 Da/mole was purchased from Dalwoo-chitosan (Dalwoo Corp., Korea). Monomer of ε-caprolactone (CL), methoxy-polyethylene glycol (m-PEG) with a number-average molecular weight of 2000, phthalic anhydride (PA), hexamethylene diisocyanate (HMDI), dibutyltin dilaurate (T12), stannous octoate (SnOct), 1,6-diphenyl-1,3,5-hexatriene (DPH), and dimethyl-sulfoxide (DMSO) were obtained from Aldrich (Aldrich Chem.Co., USA). All materials were used as received. Doxorubicin–HCl (DOX) was purchased from ICN (ICN Biomedical Inc., CA, USA). Triethylamine (TEA) was purchased from Aldrich (Sigma–Ardrich Laborchemikalien, GmbH, Germany). All solvents were of HPLC grade.
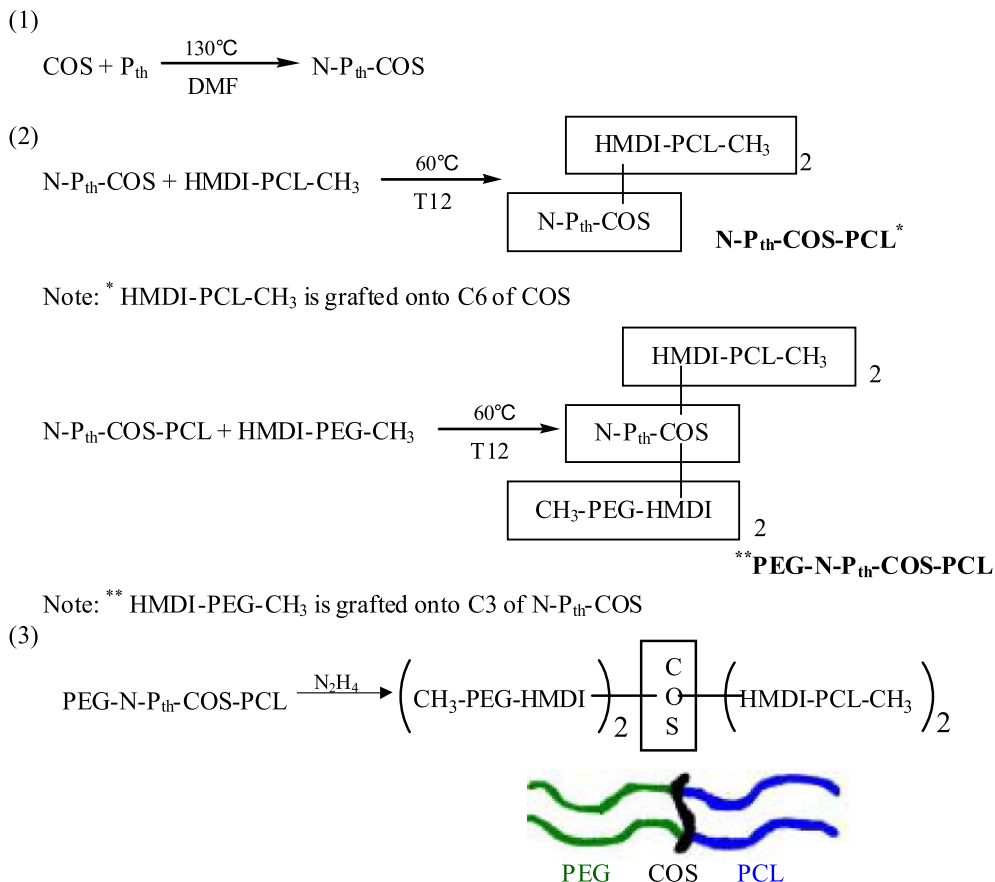
2.2. Phthaloylation of COS of blocked amine groups

The procedures for preparing the N-phthaloylation of COS (N-P_{th}-COS) were based on those of Kurita et al. (Kurita et al., 2002; Kurita et al., 1993) with some modifications (Scheme 1). First, 10 mmole of COS was suspended in 30 ml of solvent that contained DMF 95% (v/v) and water 5% (v/v). Then, 30 mmole of phthalic acid (PA) was added to the suspensions and heated at 130 °C in a nitrogen atmosphere with stirring. After 1 h of the reaction, the resulting pale tan mixture was cooled to room temperature and precipitated in ice water. After the precipitate had been filtered, washed in de-ionized water, and the un-reacted species of the precipitate had been removed with ethanol and ethyl ether in a Soxhlet extractor for 8 h, the precipitate was dried to yield a pale tan solid.
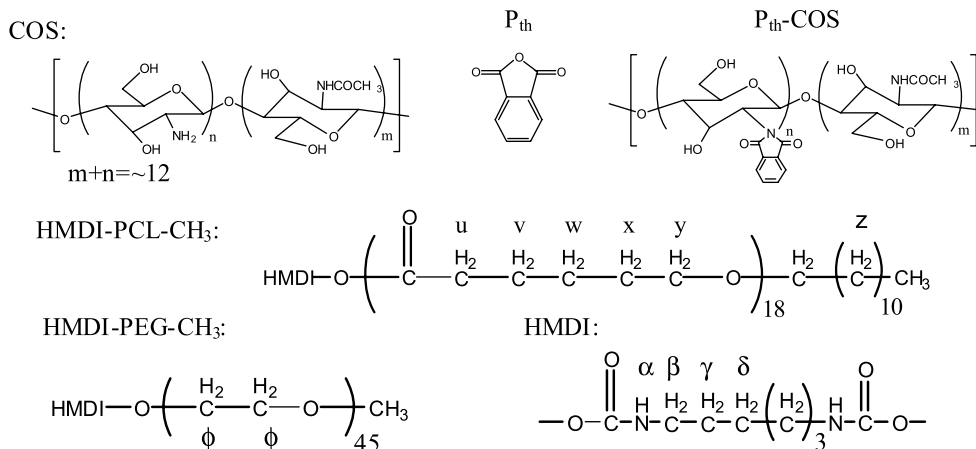
2.3. Synthesizing hydroxy-terminated poly(ε-caprolactone) (m-PCL)

The m-PCL was prepared as described by the work of Kim, Park, Lee, and Ihn (2001) with modifications. Briefly, 3 mol of CL monomer and 0.3 mol of DA in the presence of SnOct, as a catalyst, were reacted at 140 °C for 3 h for ring-opened polymerization. The reacted products were dissolved in chloroform, and then precipitated in dehydrated ethyl ether to obtain polymerized m-PCL, and the residual solvent was removed in a vacuum for over 48 h.

The molecular weight of m-PCL was determined using gel permeation chromatography (GPC) at 80 °C in NMP at a flow rate of 0.5 ml min^{-1} on a Waters 515 HPLC pump with a series of columns (Phenogel 5μ with column Number:

(1)

$$COS + P_{th} \xrightarrow[DMF]{130℃} N\text{-}P_{th}\text{-}COS$$

(2)

$$N\text{-}P_{th}\text{-}COS + HMDI\text{-}PCL\text{-}CH_3 \xrightarrow[T12]{60℃}$$

```
          ┌─────────────────┐
          │  HMDI-PCL-CH₃   │₂
          └────────┬────────┘
          ┌────────┴────────┐
          │   N-Pₜₕ-COS     │
          └─────────────────┘
```

N-P$_{th}$-COS-PCL*

Note: * HMDI-PCL-CH$_3$ is grafted onto C6 of COS

$$N\text{-}P_{th}\text{-}COS\text{-}PCL + HMDI\text{-}PEG\text{-}CH_3 \xrightarrow[T12]{60℃}$$

```
          ┌─────────────────┐
          │  HMDI-PCL-CH₃   │₂
          └────────┬────────┘
          ┌────────┴────────┐
          │   N-Pₜₕ-COS     │
          └────────┬────────┘
          ┌────────┴────────┐
          │  CH₃-PEG-HMDI   │₂
          └─────────────────┘
```

****PEG-N-P$_{th}$-COS-PCL**

Note: ** HMDI-PEG-CH$_3$ is grafted onto C3 of N-P$_{th}$-COS

(3)

$$PEG\text{-}N\text{-}P_{th}\text{-}COS\text{-}PCL \xrightarrow{N_2H_4} \left(CH_3\text{-}PEG\text{-}HMDI\right)_2 - \boxed{\begin{array}{c}C\\O\\S\end{array}} - \left(HMDI\text{-}PCL\text{-}CH_3\right)_2$$

PEG   COS   PCL

Chemical structure:

COS:

m+n=~12

P$_{th}$

P$_{th}$-COS

HMDI-PCL-CH$_3$:

HMDI-PEG-CH$_3$:   HMDI:

Scheme 1. Shows synthetic diagrams of the PCP copolymers, chemical structures, and symbols for each structure. The steps are (1). The amine groups of COS are protected by the *N*-phthaloylation of COS; (2). PCL polymers are region-selectively reacted with the hydroxyl groups of C$_6$ of *N*-P$_{th}$-COS, and then PEG polymers are reacted with the hydroxyl groups of C$_3$ of *N*-P$_{th}$-COS-PCL; (3). PCP copolymers are yielded after the de-*N*- phthaloyation of the PEG-*N*-P$_{th}$-COS-PCL.

00H-0445-K0, 00H-0444-K0 and 00H-0441-K0 with length of 300 × 7.8 mm of ID], Phenomenex, USA), using a Waters 2414 RI-detector (Waters Instrument, USA). The injection volume was 100 μl, and the solution contained 0.5 wt% m-PCL in NMP. The determinations of the numerical average molecular weight (M_n) and the molecular weight (M_w) of the tested samples were based on a linear calibration curve of six molecular weights of polystyrene standards with M_n value of 1260, 3370, 13,900, 30,300, 52,400, and 189,000. The molecular weight (M_w) of the synthesized m-PCL was recorded as 2300. In addition, the molecular weight of the synthesized reaction intermediate and final products, such as the PCL-COS and PCP copolymers, were determined on the same equipment.

2.4. Preparing PCL-NCO and PEG-NCO polymer

A mixture of 10 mmol (milli-mole) of HMDI and 10 mmol of synthesized m-PCL was dissolved in 45.0 g of DMF, reacted at 50 °C in the presence of T12, as a catalyst, in a nitrogen atmosphere for 8 h to prepare PCL-*NCO*. The solution product, PCL-*NCO*, was isolated by precipitation in dehydrated ethyl ether, vacuumed, and stored in a dehydrated nitrogen atmosphere.

PEG-*NCO* was prepared by dissolving a mixture of 10 mmol HMDI and 10 mmol m-PEG (M_w, 2000) in 40.3 g of DMF, and all other reaction and purification conditions were the same as those for preparing PCL-*NCO*.

2.5. Synthesizing PCL-P_{th}-COS-PEG copolymers (P-P_{th}-C-P)

Ten millimole of the PCL-*NCO* solution was slowly added to 12 g of DMSO with 10 mmol of N-P_{th}-COS in the presence of T12 in a three-neck flask in a nitrogen atmosphere. The flask was then heated at 60 °C to induce the chemical reactions between the hydroxyl groups of N-P_{th}-COS and PCL-*NCO* (Scheme 1). After this reaction had proceeded for 8 h, 10 mmol of PEG-*NCO* solution was slowly added to the flask to react with the residual hydroxyl groups of N-P_{th}-COS at 60 °C for 8 more hours (Scheme 1). The products of the reaction were dissolved in 1 L of THF, and then precipitated in dehydrated ethyl ether in an ice bath. The precipitated products were filtered and further dried in vacuo to remove the residual solvent at room temperature for more than 48 h.

2.6. Preparing PCP copolymers by de-phthaloylation (N-P_{th})

The N-P_{th} groups of the P-P_{th}-C-P copolymers were selectively removed by using hydrazine hydrate as described by (Nishiyama et al. (1999)). Briefly, 20 g of P-P_{th}-C-P copolymer was suspended in 50 ml of hydrazine hydrate and heated in a nitrogen atmosphere at 100 °C for 1 h (Scheme 1). After the reactions, the mixture was cooled to room temperature, filtered, and washed with ethyl ether. The remaining solid products, which contained PCP copolymer, were further extracted using dehydrated ethyl ether in a Soxhlet extractor for 8 h and drying in vacuum at room temperature for at least 48 h.

2.7. Purifying PCP copolymers

The PCP copolymer was dissolved in DMSO at 50 °C for 30 min to a final concentration of 5 wt%. It was first dialyzed for several days at 50 °C against DMSO using a membrane with a cut-off molecular weight of 6–8 kDa/mole (Spectrum Medical Industries Inc., USA) to remove un-reacted hydrophobic PCL, PA, and other unwanted polymers. Then, the DMSO medium was replaced with distilled water without changing the membrane, dialyzing was performed a few more days at 4 °C to remove un-reacted

hydrophilic COS, PEG, and DMSO solvent. The purified PCP copolymer solution was filtered through a membrane with 0.22 μm pores and then freeze-dried for characterization and application.

2.8. Characterizing PCP copolymers by FT-IR, and ^{1}H NMR

FT-IR absorption spectra of synthesized m-PCL, PCL-COS, and PCP co-polymers were analyzed by using an FT-IR spectrum analyzer (Digilab. FTS-3000MX, Digilab Corp., MA, USA), collected at a resolution of 2 cm^{-1}, and analyzed using a built-in standard software package to characterize the functional groups, including amine and carbonyl.

The shifts in the ^{1}H NMR signals associated with the molecular structure of PCP copolymers were determined using a Varian Unity Inova (Varian Inc., CA, USA) at 600 MHz as done in other groups (Liu, Li, & Fang, 2004; Sugimoto, Morimoto, Sashiwa, Saimoto, & Shigemasa, 1998). The ^{1}H NMR measurement of COS involved dissolving it in D$_2$O at 80 °C while reaction intermediates and PCP were dissolved in DMSO at the same temperature.

2.9. Preparing PCP micelles and determining CMC

Micelles of PCP copolymers were prepared by a direct dissolution method (Ge et al., 2002). Generally, 1 mg of PCP copolymer was suspended in distilled water at 40 °C for 1 h to induce self-assembly of PCP copolymers in micelle form. They were then filtered through a membrane with a pore size of 0.22 μm. The filtrated suspension was further equilibrated for 5 h at room temperature. The CMC value of the PCP micelles was determined as reported (Jeong et al., 2000; Park et al., 2002). Briefly, 20 μl of 0.4 mM DPH dissolved in methanol was injected into 2.0 ml of PCP copolymer suspensions with concentrations ranging from 2×10^{-3} to 1.0 wt%, and then equilibrated for 5 h to form DPH-loaded PCP micelles. Un-encapsulated DPH was removed using a dialysis column (Spectrum Laboratory Inc. CA, USA) with a cut-off molecular weight of 6–8 kDa within half-an-hour.

The absorbance spectra of PCP micelle suspensions were obtained using a UV–Vis spectrometer (Jasco V-530, Jasco Inc., Japan) in the range 350–430 nm at 25 °C. The CMC value of PCP micelles was determined by plotting the difference between the absorbance at 386 nm (the peak of the absorption spectra) and that at 401 nm against the logarithmic concentration of the PCP reported by other groups (Jeong et al., 2000; Park et al., 2002; Torchilin, 2001).

2.10. Size and morphology of PCP micelles

The size of the PCP micelles in aqueous solution was determined at 25 °C using a DLS analyzer (Malvern Zeta

Sizer 1000, Malvern Instruments, Worcestershire, England) with a 5 mW He–Ne laser ($\lambda = 633$ nm). The morphology of the micelles was observed by TEM, by placing a drop of PCP micelle solution on a 300 mesh copper grid coated with carbon. Then, 2.0 wt% of phosphotungstic acid was used to stain the micelles on the copper grid (Ge et al., 2002). After the micelle samples were air-dried at room temperature, the morphology of the stained micelles was observed and imaged by TEM (JEOL 1200EX, JEOL Ltd., Tokyo, Japan).

2.11. Zeta potential measurements

Surface charges or zeta potentials of the micelles were determined using a Brookhaven Zetasizer (ZetaPlus 90, Brookhaven Instruments Corporation, USA) at 1.0 wt% of PCP copolymers/micelles in de-ionized water at 25 °C. The pH values of suspended medium varied from 3.0 to 7.4 and were adjusted by using 0.1 N of HCl or NaOH solutions.

2.12. Preparing and characterizing delivery behaviors of DOX of PCP micelles

Five milligrams of PCP copolymers was dissolved in 2 ml of PBS buffer, and 1 mg of DOX–HCl was dissolved in 0.5 ml of pure ethanol in the presence of TEA (molar ratio to DOX, 1.4:1) solution (Ouchi et al., 2002). The suspensions were further filtered through of membrane with a pore size of 0.22 μm to remove un-dissolved copolymers. Those solutions were then mixed at 4 °C for 5 h to yield stable DOX-loaded PCP micelles. The hydrochloride salt of TEA, free DOX, and unwanted polymer aggregates were removed by dialyzing the micelle suspensions three times using a membrane with a cut-off molecular weight of 6–8 kDa/mole (Spectrum Medical Industries Inc., USA) in 30 ml of PBS solution at 4 °C. The concentration of DOX in the dialysate obtained from the collections in the aforementioned purification process was further analyzed to calculate the quantity of DOX encapsulated in PCP micelles (Wong, Bendayan, Rauth, & Wu, 2004). The concentration of DOX in the medium was determined by measuring the absorbent intensity of the medium at 487 nm using a UV–Vis spectrometer (Jasco V-530, Jasco Inc., Japan) (Shuai, Ai, Nasongkla, Kim, & Gao, 2004). The encapsulation efficiency of DOX in micelles is defined as the ratio of the weight of DOX in micelles to the initial DOX-loaded weight, expressed as a percentage. The loading content of the micelles is defined as the percentage of the weight of DOX in micelles divided by the weight of micelles.

The micelle suspension was further concentrated to 1.0 ml using a column filtered through a cross-flow syringe filter with a cut-off molecular weight of 10 kDa/mole to study the release of DOX from the micelles (Column Number: X11S-200-04 N, Spectrum Medical Industries Inc., USA). Then, the concentrated DOX-loaded PCP micelles

were placed in a dialysis membrane (Spectrum Laboratories, USA) with a cut-off molecular weight of 6–8 kDa, and the membrane was placed in 10 ml PBS dissolution medium at 37 °C in a shaker bath (Tungtec Instruments Co., Taiwan) at 70 rpm. At the selected time, the dissolution medium was removed for DOX analysis and replaced with the same volume of fresh medium. The concentration of DOX in the medium was determined by measuring the absorbance intensity of the medium at 487 nm with a UV–Vis spectrometer (Jasco V-530, Jasco Inc., Japan).

2.13. Post-crosslinking the DOX-loaded PCP micelles by genipin

Genipin, a natural crosslinking agent, has been widely applied to the crosslinking of the amine groups of proteins or chitosan (Mi, Sung, Shyu, Su, & Peng, 2003; Mi, Tan, Liang, & Sung, 2002). The mild conditions for the crosslinking of micelles by genipin were applied as described elsewhere with some modifications. 0.7 ml of 1.0 wt% genipin solution (Mi et al., 2003, 2002) was added to 7 ml of a suspension of DOX-loaded PCP micelles at 37 °C to crosslink the amine groups of PCP micelles. After the crosslinking reactions, the suspensions were dialyzed using a column filter (Column #: X11S-200-04N, Spectrum Medical Industries Inc., USA) to concentrate them and remove residual genipin. The analysis protocols for determining encapsulation efficiency, loading percentage, and the release study of DOX for post-crosslinked PCP micelles were the same as those for non-crosslinked PCP micelles.

The statistical analysis was performed using Sigmastat statistical software (Jandel Science Corp., San Rafael, CA, USA). Statistical significance corresponded to a confidence level of 95% or better. Data measured at least thrice are presented as mean ± SD.

3. Results and discussion

3.1. Characterizing PCP copolymers

Fig. 1 shows the FT-IR transmittance spectra of COS, COS-PCL, and PCP copolymers. The transmittance spectra of COS show the characteristic peaks of amide (I) at 1640 cm^{-1} and the amide (II) band at 1560 cm^{-1} (Sugimoto et al., 1998). Moreover, the spectra for COS-PCL (Fig. 1) show a strong absorption band at 2850–3000 cm^{-1} which is due to the C–H stretching band of PCL (e.g., 2945 cm^{-1}). They also show a new absorption band at 1700 cm^{-1} attributable to the ester groups of PCL. The amide (I) and (II) bands, on the other hand, have shifted slightly. The spectra and the peaks grafted onto the hydroxyl groups of chitosan were consistent with observations of other reporters of PCL (Liu, Li, Liu, & Fang, 2004). The spectra of PCP copolymer exhibit characteristic peaks like those of the ester and amide groups of PCL, PEG, and COS. These are the peaks of the ester groups of PCL at 1720 cm^{-1} as presented in (b), a C–H stretching band at
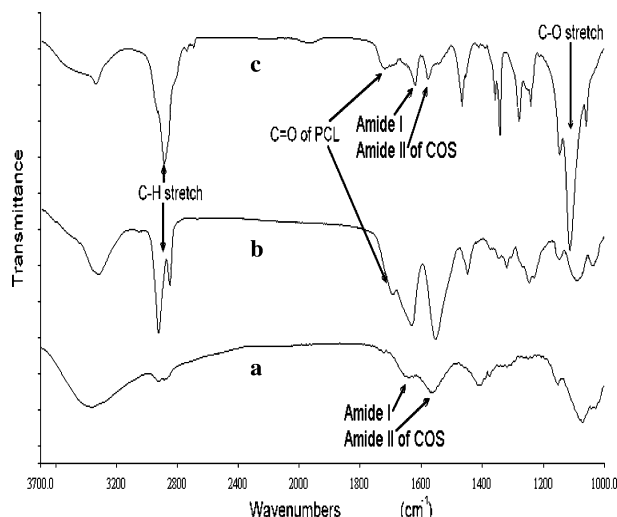
Fig. 1. FT-IR transmittance spectra of (a) COS, (b) COS-PCL copolymer and (c) PCP copolymer. The characteristic peaks/functional groups of each copolymer are assigned. The spectra of PCP copolymers exhibit characteristic peaks of ester, amide groups and C–H stretch of PCL polymers, COS and PEG polymers, respectively.

2850–$3000 \, \text{cm}^{-1}$ much stronger than that in (a) associated with the aliphatic C–H stretching of PEG and PCL, and the slightly shifted amide (I) and amide (II) bands of COS. The FT-IR study of Chitosan-PEG and PCL-PEG-PCL copolymers revealed the C–H stretching of PCP copolymers (Ge et al., 2002; Sugimoto et al., 1998). The results of FT-IR transmittance spectra of the COS-PCL and PCP copolymers in Fig. 1 are evidence of PCP copolymers that consist of PCL, COS, and PEG polymers.

The molecular weight and the de-acetylation of COS are about 2 kDa/mole and 95%, respectively, and the mean ratio of n to m (COS structure of the Scheme 1) of COS is 5–10. To gain insight into the chemical structure of the intermediates and PCP copolymers, the ^1H NMR spectra of COS, intermediates, and PCP copolymers were studied. The peaks corresponding to the chemical shifts of the H_1 proton in the D-glucosamine unit of COS in D_2O are about the same as in other reports (Ge et al., 2002; Kurita et al., 2002; Sugimoto et al., 1998). For instance, the peaks at 1.8, 3.45, 4.0–4.6 and 5.2 ppm correspond to the backbone hydrogen of HAc, H_2–H_6 and H_1 of COS (Kurita et al., 2002; Liu et al., 2004; Sugimoto et al., 1998).

Fig. 2(a) shows the ^1H NMR spectra of N-P_{th}-COS-PCL dissolved in DMSO solvent. The ^1H NMR peaks of PCL are at 1.22 and 1.55 (methylene 6 H), 2.27 (methylene 2H adjacent to carbonyl), and 4.01 ppm (methylene 2H adjacent to oxygen) with an area ratio of 3:1:1, respectively. The peaks of DMSO, backbone hydrogen of the COS, and the N-P_{th} groups are at 2.50, 1.35 (with two splits), and 2.9–5.2, and 7.22–7.95 ppm, respectively, while the peak at 2.75 ppm may correspond to residual DMF (Liu et al., 2004) although the area of the peak is relatively small. Hence, the peaks of the ^1H NMR spectra suggest that PCL-NCO regio-selectively binds to the hydroxyl groups

of C6 of N-P_{th}-COS because of the steric effect of the phthalimido groups (Kurita et al., 1993).

Fig. 2(b) presents the ^1H NMR spectra of PCP copolymers in DMSO; the characteristic peaks for PCL and DMSO are the same as in Fig. 1(a), but a little shifted (e.g., 1.51, 2.23, and 3.96 ppm for the methylene H of PCL, respectively) while the other peaks of PCP are assigned to COS and PEG. The peaks at 1.20, 1.31, 2.91, and 4.02 ppm correspond to the hydrogen backbone of COS while multiple peaks at 3.0–4.0 ppm overlap and correspond to COS and PEG of the PCP copolymers. The peak at 3.22 ppm is the terminal methoxy, and those at 3.4–3.6 ppm correspond to the methylene 4 H that is adjacent to the oxygen of ether of the m-PEGs of the PCP copolymer (Liu et al., 2004; Sugimoto et al., 1998). However, the ^1H NMR spectra of PCP copolymers may nevertheless contain impure H_2O (Liu et al., 2004). In addition, the weak peak at 7.15 ppm from the PCP copolymers is attributable to residual phthalimido groups (Fig. 2(a)), suggesting a very weak and incomplete de-protection of the amine of COS occurred. Since PCL polymers regio-selectively bind to the hydroxyl groups of C6 of COS (Kurita et al., 1993), PEG polymers would regio-selectively bind to the hydroxyl groups of C3 of COS while the amine groups of the COS remained mostly intact (Scheme 1).

The molecular weights of the products were determined by GPC to characterize further the intermediated products and PCP copolymer. The measured M_n of N-P_{th}-COS is 3760, and that indicates most of the amine groups of the D-glucosamine of COS are protected by the phthalimido groups (M_w 132 g/mole) after the N-P_{th} procedure because the reported molecular weight of COS is about 2 kDa/mole with a de-acetylation of 95%. Additionally, the calculated M_n of PCL-COS copolymer is 6870 Da/mole, and that of the PCP copolymer is 11.3 kDa/mole. Furthermore, molecular weight measurements show that the M_w and PDI (polydispersity index) for N-P_{th}-COS, PCL-COS, and PCP copolymers are 3940, 7210 and 12,508 Da/mole and 1.05, 1.07 and 1.16, respectively. Since the molecular weight of m-PCL synthesized by this laboratory is 2450, the molecular weight of PCL-COS indicates that only two PCL-NCO polymers bind to the hydroxyl groups of N-P_{th}-COS. Moreover, the molecular weight of PCP copolymer indicates that only two PEG-NCO polymers bind to the residual hydroxyl groups of N-P_{th}-COS- PCL copolymers.

Since the maximum chain length of a PCL polymer is about 17 nm and the length of 12 D-glucosamine subunits are about 8 nm, the segment of the first PCL polymer regio-selectively grafted into C6 would hinder another polymer from grafting to similar positions. Therefore, only two PCL polymers are likely grafted onto the C6 of the 12 D-glucosamine subunits of COS. Moreover, since the chain length of PEG polymer is also about 17 nm, the same argument can be applied to support the assertion that two PEG polymers graft onto the C3 of COS. Therefore,
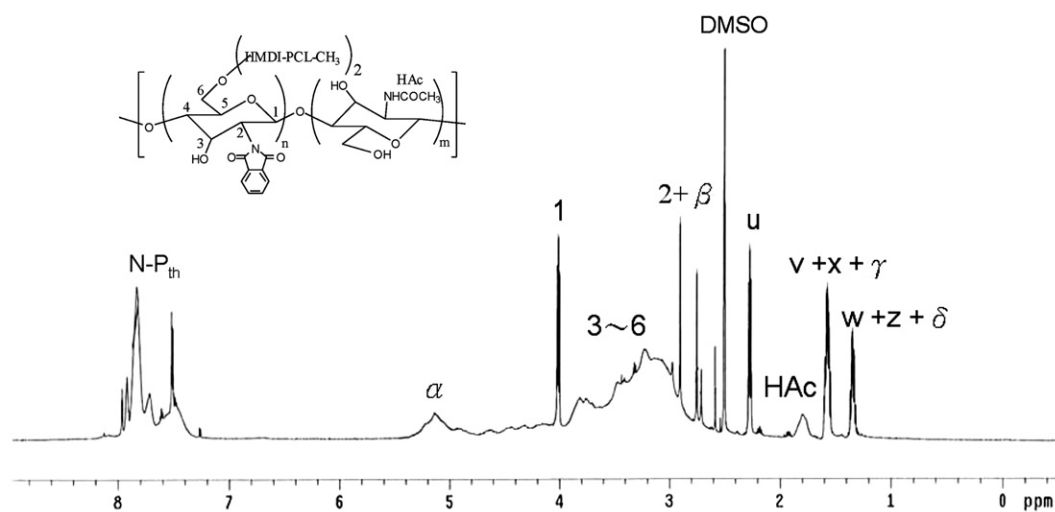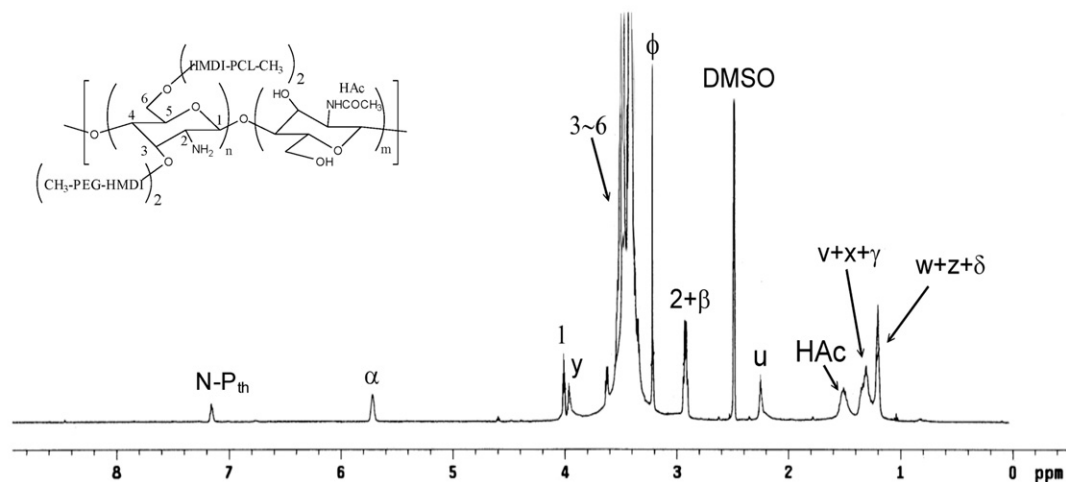
Fig. 2. (a) ^{1}H NMR spectra of $N\text{-}P_{th}$-COS-PCL copolymers. The numbers, Greek and other symbols such as α and w, respectively, are correspondent to the backbone carbon of COS and other chemical components such as PCL polymers stated in Scheme 1. (b) ^{1}H NMR spectra of PCP copolymers. The numbers are correspondent to the backbone carbon of COS. The Greek symbols such as α and β are the ^{1}H of different components of PCP copolymers such as PCL and PEG polymers stated in Scheme 1.

the structure of PCP copolymers contains two PCL and PEG polymers.

3.2. PCP micelles and CMC value

Since the structure of the PCP copolymers comprises hydrophobic and hydrophilic segments, the possibility of forming PCP micelles as reservoirs for drug delivery is of interest. The fluorescent hydrophobic dye, DPH, a strong chromophore showing UV–Vis absorption spectra between 350 nm and 415 nm, was loaded and applied to evaluate the CMC value of PCP copolymers (Chang, Prange, Allcock, Lee, & Kim, 2002; Jeong et al., 2000; Savic et al., 2003). Notably, the absorbance of DPH in a hydrophobic environment greatly exceeds that under aqueous conditions, suggesting that DPH is entrapped into the

PCL core of PCP micelles. Fig. 3 shows that the CMC value of the PCP micelles was 1.07×10^{-2} wt% PCP copolymers, as determined by extrapolating the difference between the absorbance of the suspension at 384 and 401 nm, which was plotted against the logarithmic of the concentration of the PCP copolymers. Moreover, the CMC value of the PCP micelles is of about one order of magnitude higher than values for PLGA-PEG and PCL-PEG-based micelles, as reported by other groups (Chang et al., 2002; Jeong et al., 2000; Savic et al., 2003). That may be due to the ring structure of the COS of PCP copolymers instead of to linear-chain copolymers.

The size and the morphology of the micelles were determined and examined using a DLS analyzer and TEM, respectively, to further characterize the PCP micelles (Fig. 4). The mean diameter of the PCP micelles measured
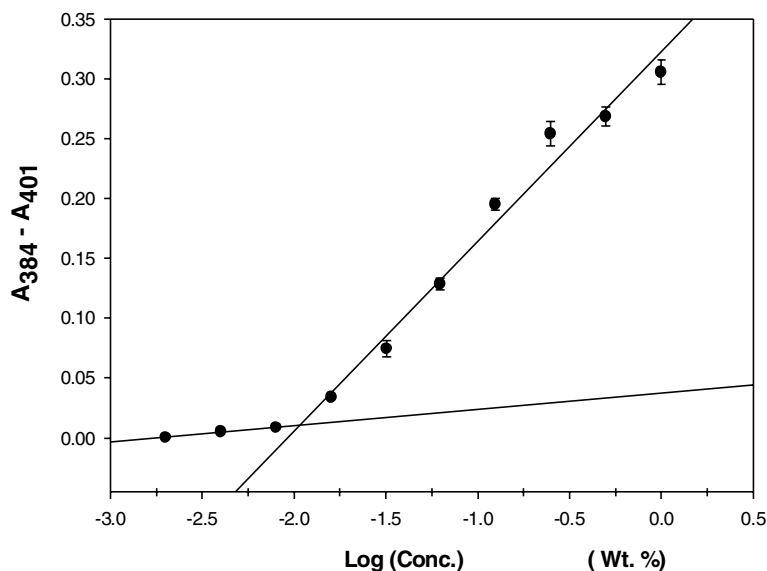
Fig. 3. The plot differences of absorbance between 384 and 401 nm of DPH absorption spectra versus Log (Conc.) (wt%), the concentrations of PCP copolymers, for determining the CMC value of the micelles. The CMC value is the intersection of two linear regression lines.

using the analyzer was 90 nm, and the polydispersity index (PDI) was 0.35, suggesting that the sizes of the micelles are narrowly distributed. Furthermore, the morphologies of the micelles without or with genipin crosslinking were imaged by TEM to show the spherical particles with nanometer dimensions with sizes ranging from 40 to 120 nm (Figs. 4a and b), which was consistent with the measurements made by the DLS analyzer. The size of micelles was similar to P(d,l)LA-based copolymers (Kohori, Yokoyama, Sakai, & Okano, 2002) or DOX-PLGA-mPEG copolymers (e.g., 112 nm) (Yoo & Park, 2004). It has been suggested that a low milli-molar region of CMC values and a 10–100 nm size of the polymeric micelles, make the micelles suitable for drug delivery applications (Torchilin, 2001). The CMC value results and particle size of the PCP micelles are eminently suited to the requirements of drug delivery.

3.3. The role of COS on zeta potentials of PCP micelles

To demonstrate one of the unique properties of COS-containing PCP micelles, zeta potentials of micelles suspended in media of different pH values were investigated (Table 1). The results show that the zeta potential of PCP micelles varies with changing pH in the medium. For instance, the values are -3.2 ± 1.3 mV at pH 7.4 but 13.9 ± 4.4 mV at pH 3.0. This can mainly be attributed to the COS of PCP copolymers since the amine groups of COS are protonized in acidic media. The results indicate that the surface properties of PCP micelles can be manipulated from neutral to a cationic state that may facilitate the delivery by PCP micelles of drug into cancer cells or tissues. The cytoplasm of cancer cells is reportedly more acidic than that of normal cells (Gerweck & Seetharaman, 1996). This may enhance the migration of micelles within

the cancer cell cytoplasm during delivery of anti-cancer drugs. The property of varying surface potential of PCP micelles cannot be achieved for PLGA-b-PEG or other polyester-based micelle systems without further modifications.

3.4. The delivery behaviors of PCP micelles of DOX with or without genipin post-crosslinking

Although many polyester-based polymeric micelles have been fabricated, only few of them that consist of functional groups (e.g., carboxyl group) in the PLA block can be chemically modified (Lee, Cho, & Cho, 2004). To demonstrate one of the advantages of COS-containing PCP micelles in drug delivery, the delivery behaviors of DOX-loaded micelles with or without genipin crosslinking the amine groups of COS were studied and compared. Interestingly, the results showed that the encapsulation efficiencies of DOX-laden PCP micelles with or without genipin crosslinking were 54.0% and 56.5%, respectively ($n = 3$). In addition, the genipin crosslinking process of PCP micelles did not affect the loading capacities of DOX in PCP micelles (e.g., 11.3 and 10.8 wt% ($n = 3$) for the micelles with or without crosslinking, respectively). No difference in the particle sizes of crosslinking and non-crosslinking PCP micelles was observed (data not shown). In general, the macroscopic characteristics of micelles were not affected by genipin crosslinking.

For comparison, the encapsulation efficiencies of DOX into PCP micelles of this study markedly exceed those of micelles assembled by PLGA-PEG or PCL-PEG copolymers (Nasongkla et al., 2004; Ouchi et al., 2002; Shuai et al., 2004) or thermo-sensitive molecule conjugated P(d,l)-LA copolymers (Kohori et al., 2002). Additionally, the loading capacity of the PCP micelles exceeds the
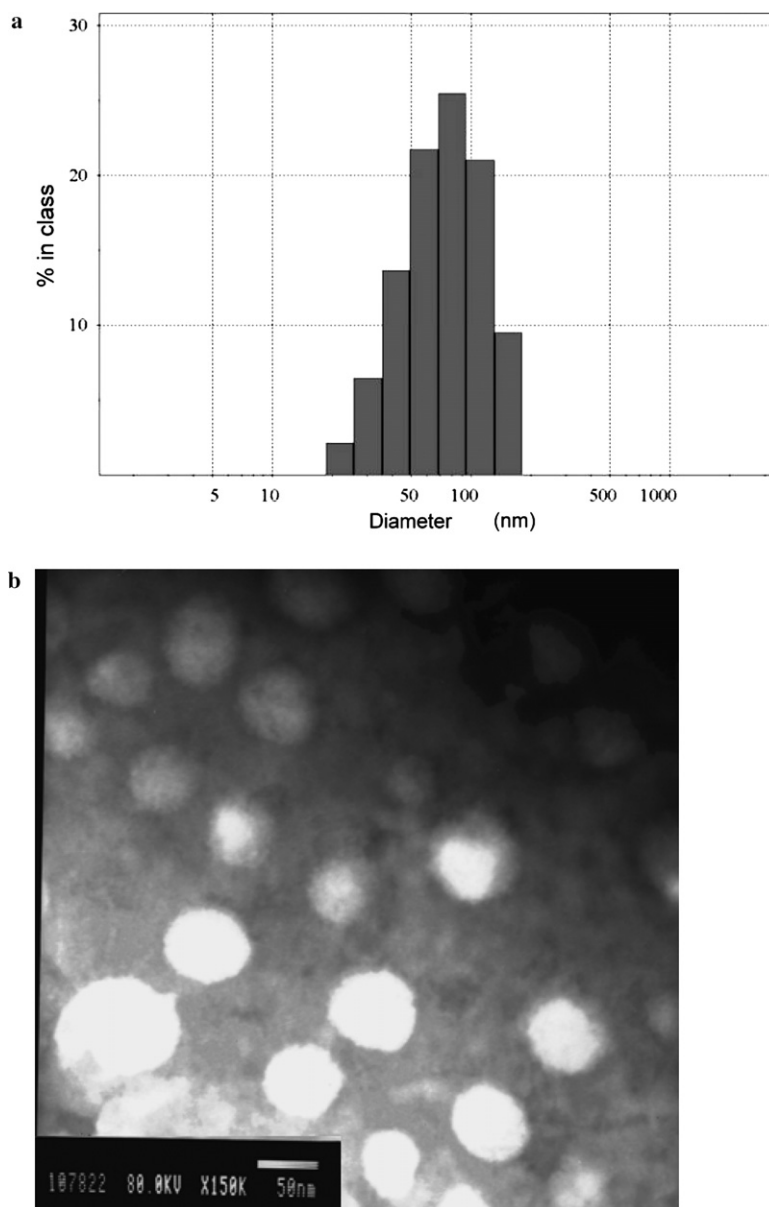
Fig. 4. (a) The particle size distribution of PCP micelles measured by DLS. The mean diameter of the PCP micelles is 90 nm with the polydispersity index (PDI) of 0.35, suggesting that the sizes of the micelles are narrowly distributed. (b) TEM micrographs of the PCP micelles stained with phosphotungstic acid (×50 K, bar represents 100 nm). The imaged micelle sizes range from 40 to 120 nm.

Table 1
Zeta potentials of PCP micelles change from neutral to cationic state when pH values of the suspension medium are varied from 7.4 to 3.0

pH of suspension medium	Zeta potential (mV) of PCP micelles ($n = 3$)
7.4	-3.23 ± 1.27
6.0	-0.20 ± 0.98
5.0	1.23 ± 0.81
4.0	7.48 ± 3.08
3.0	13.87 ± 4.41

corresponding values obtained in the above-mentioned studies. For instance, DOX loading capacity in crosslinked PCP micelles is 11.3 wt% while that for PCL-PEG ones is 3.10 wt% (Nasongkla et al., 2004) although particle size of the latter is smaller than that of the former. However, the efficiency and loading capacity of PCP micelles of this study are somewhat lower than what has been reported for DOX-PLGA-PEG copolymers (Yoo & Park, 2004).

Fig. 5 presents the release behaviors of DOX-laden PCP micelles with or without genipin crosslinking (Fig. 5). The burst release and release periods of DOX for crosslinked micelles are $22.0 \pm 2.3\%$ (at 24 h) and 192 h (with a cumulative releases of $69.4 \pm 3.9\%$, $n = 3$), respectively, significantly reduced from $37.6 \pm 3.3\%$ (at 24 h) and longer than 96 h (with a cumulative releases of $73.4 \pm 3.6\%$, $n = 3$), respectively, for non-crosslinked ones. The reducing initial burst while increasing the release period of DOX
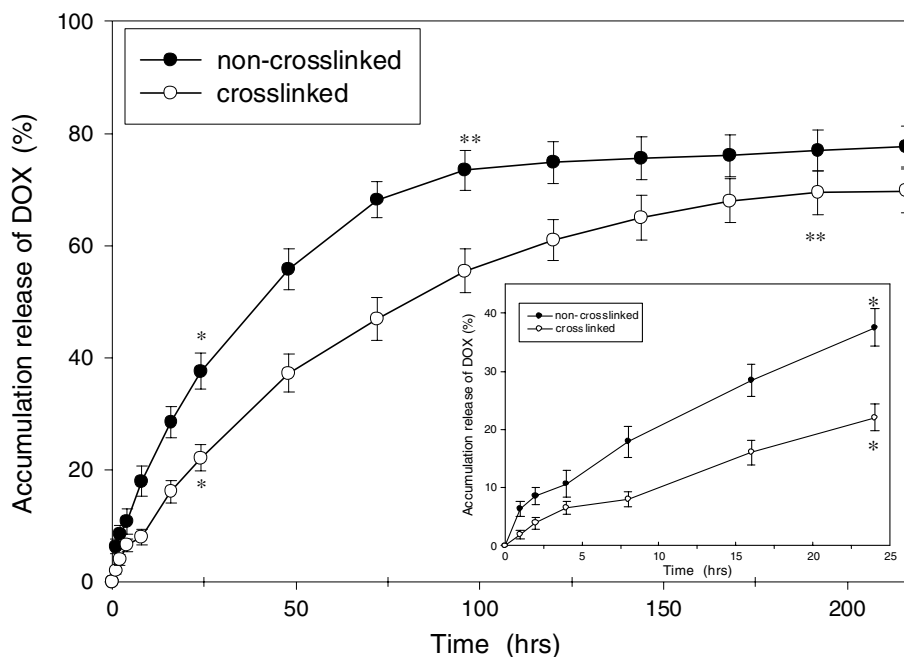
Fig. 5. The cumulative release profiles of DOX loaded PCP micelles with or without genipin crosslinking. The burst release and release period of DOX from micelles with genipin crosslinking are significantly reduced and sustained (e.g., $22.0 \pm 2.34\%$ and 8 days, respectively) (both * and $^{**}P < 0.01$, $n = 3$) compared to non-crosslinked micelles (e.g., $37.6 \pm 3.26\%$ and 4 days, respectively).

from PCP micelles with genipin crosslinking are mainly contributed by decreasing the mobility of the COS segment of PCP copolymers and enhancing the stability of the micelles (data not shown) due to crosslinking the amine groups of COS. However, the total percentage of DOX releases of crosslinked micelles being less than that of non-crosslinked ones that may be due to the possibly crosslinking reactions between both amine groups of DOX and PCP micelles.

The sustained release of DOX from crosslinked PCP micelles is longer than that of DOX encapsulated within PLGA-PEG micelles (Kataoka et al., 2000) and is similar to that of micelles formed by PLA polymers grafted with amino acids such as lysine (Ouchi et al., 2002). However, the release period and quantity of DOX of this study are less than those from PCL-PEG micelles in acidic media (Shuai et al., 2004). Nevertheless, the genipin crosslinking PCP micelles support the advantages of COS-containing PCP copolymers and of micelles in drug delivery. Although this paper only presents the characterizations and drug delivery behaviors of PCP micelles, the biocompatibility of PCP micelles to fibroblasts, determined by MTT assay, has been investigated, and no toxicity is observed when the cells are incubated with 2500 µg/ml of PCP micelles (data not shown).

4. Conclusion

New COS-containing tri-block PCP copolymers were synthesized that can self-assemble to form polymeric micelles with 0.0107 wt% CMC and a mean size of 90 nm. Varying zeta potentials of PCP micelles from a neutral to cationic state and the advantage of genipin crosslinking COS of the micelles in delivering DOX demonstrate the unique property of the COS of PCP micelles.

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