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Research paper

Incorporation of camptothecin into *N*-phthaloyl chitosan-g-mPEG self-assembly micellar system

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Abstract

The capability of *N*-phthaloylchitosan-grafted poly (ethylene glycol) methyl ether (mPEG)(PLC-g-mPEG) to enhance the aqueous solubility and stability of the lactone form of camptothecin (CPT) was investigated. PLC-g-mPEG formed a core-shell micellar structure after dialysis of the polymer solutions in dimethyl sulfoxide (DMSO) or dimethylformamide (DMF) against water, with a critical micelle concentration (CMC) of 28 μg/ml. CPT was loaded into the inner core of the micelles by dialysis method. The results showed an increase in the CPT-loading amount with an increasing concentration of CPT. The stability of drug-loaded micelles was studied by gel-permeation chromatography (GPC), and their in vitro release behaviors were analyzed. Release of CPT from the micelles was sustained. When compared to the unprotected CPT, CPT-loaded PLC-g-mPEG micelles were able to prevent the hydrolysis of the lactone group of the drug. The kinetics of the CPT hydrolysis in human serum albumin (HSA) and fetal bovine serum (FBS) were pseudo-first order. The hydrolysis rate constants for CPT and CPT-loaded PLC-g-mPEG micelles in phosphate-buffered saline (PBS) pH 7.4, were 7.4×10^{-3} min^{-1} and 9.1×10^{-3} h^{-1}, parallel to an increase in half-life of CPT from 94 min to 76.15 h, respectively.

Keywords: Polymeric micelles; Anticancer agent; Camptothecin; *N*-Phthaloylchitosan

1. Introduction

Camptothecin (CPT) is a potent, anticancer agent acting through the inhibition of topoisomerase I during the S-phase of the cell cycle [1]. It exists in two forms depending on the pH value; an active lactone form at pH below 5 and an inactive carboxylate form at basic pH (Fig. 1) [2]. At physiological pH, most CPT molecules exist in an inactive carboxylate form. The stability of the lactone form of CPT is critical for its anticancer activity. In addition, the ring opening carboxylate form shows poorer diffusibility through the lipid bilayer than the lactone form. Therefore, factors influencing a lactone-carboxylate equilibrium within the CPT molecule are clearly important determinants of the agent's function. Human serum albumin (HSA) was shown to bind preferentially with the carboxylate form, resulting in the more rapid opening of the lactone ring [3]. The labile lactone ring and poor aqueous solubility pose many challenges for drug development and drug delivery system (DDS). Various types of DDS have been developed in order to reduce severe systemic toxicities and enhance antitumor effects by improving their pharmacokinetics. Previous studies have shown that the lactone ring of CPT was protected upon incorporation of the drug into a lipid bilayer structure like liposomes

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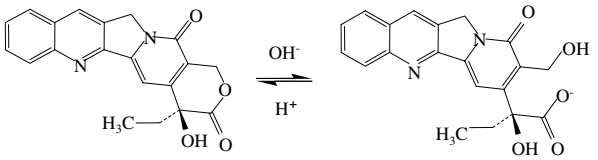


Fig. 1. Chemical structure of camptothecin (CPT) two forms depending on the pH value, namely, an active lactone form at pH below 5 and an inactive carboxylate form at basic pH.

[4–6], microspheres [7–9], upon conjugation to synthetic polymers [10–14], nanobiohybrids [15], and polymeric micelles [16–20].

Amphiphilic block or graft copolymers have recently emerged as a class of materials with a wide range of pharmaceutical applications. It can form nanoscopic core-shell structures above the critical micellar concentration (CMC) [21]. In the past 20 years, polymeric micelles have attracted great interest because of their potential utilized for a drug carrier system. The hydrophobic core serves as reservoirs for hydrophobic drugs while the hydrophilic part serves as interface between the bulk aqueous phase and the hydrophobic domain. Highly hydrated outer shells of the polymeric micelles can inhibit intermicellar aggregation of their hydrophobic inner cores. Consequently, the polymeric micelles maintain a satisfactory aqueous stability irrespective of high contents of hydrophobic drug incorporated into the inner core of micelles. Furthermore, a polymeric micelle in a size range <200 nm reduces non-selective reticuloendothelial system (RES) scavenge and shows enhanced permeability and retention effects (EPR effect) [22] at solid tumor sites for passive targeting. Reported elsewhere revealed that anticancer drugs such as adriamycin [23,24], KRN550 [25], camptothecin [18,19], all-*trans* retinoic acid [26], taxol [27] and paclitaxel [28] were successfully incorporated in polymeric micelles and showed highly selective delivery to solid tumors.

It can be expected that the DDS obtained from a biopolymer backbone has advantages of biodegradability, biocompatibility and non-toxicity. Chitosan is an aminopolysaccharide in a deacetylated form of chitin. Recently, the production of chitosan spheres for DDS was achieved by some specific processing techniques, such as suspension cross-linking, spray-drying coagulation, emulsification/solvent evaporation. Chitosan is soluble in aqueous acidic solutions, but cannot form micelles in water. However, some modified chitosans e.g. *N*-lauryl-carboxymethyl-chitosan [27] and *N*-octyl-*O*-sulfate chitosan [28] have been reported for the preparation of polymeric micelles for paclitaxel. In our previous study, we have concentrated on the modification of chitosan based on the balancing of polarity on the chain in order to obtain novel derivatives. We synthesized *N*-phthaloylchitosan-grafted poly (ethylene glycol) methyl ether (mPEG) (PLC-g-mPEG) which showed self-assembly nanolevel sphere-like particles (80–170 nm) depending on the chain length of mPEG as observed by SEM and TEM [29]. In this study, we aimed to develop the polymeric micellar system of PLC-g-mPEG for targeting of a water-insoluble anticancer agent, camptothecin (CPT). In order to achieve the stable CPT-loading micelles for tumor delivery, the factors affecting the incorporation efficiency and the stability of CPT-loading micelles are evaluated by changing the solvents and the initial drug loading content. In addition, its in vitro release behaviors were analyzed. We postulated that an optimal controlled release preparation would exclusively release the lactone form of CPT. Moreover, CPT-loaded PLC-g-mPEG micelles showed a longer kinetic half-life than CPT under the physiological conditions. The results revealed that CPT-loaded PLC-g-mPEG micelles are expected to have a long-circulating property in the bloodstream, which can contribute to targeting to solid tumor sites.

2. Materials and methods

2.1. Materials

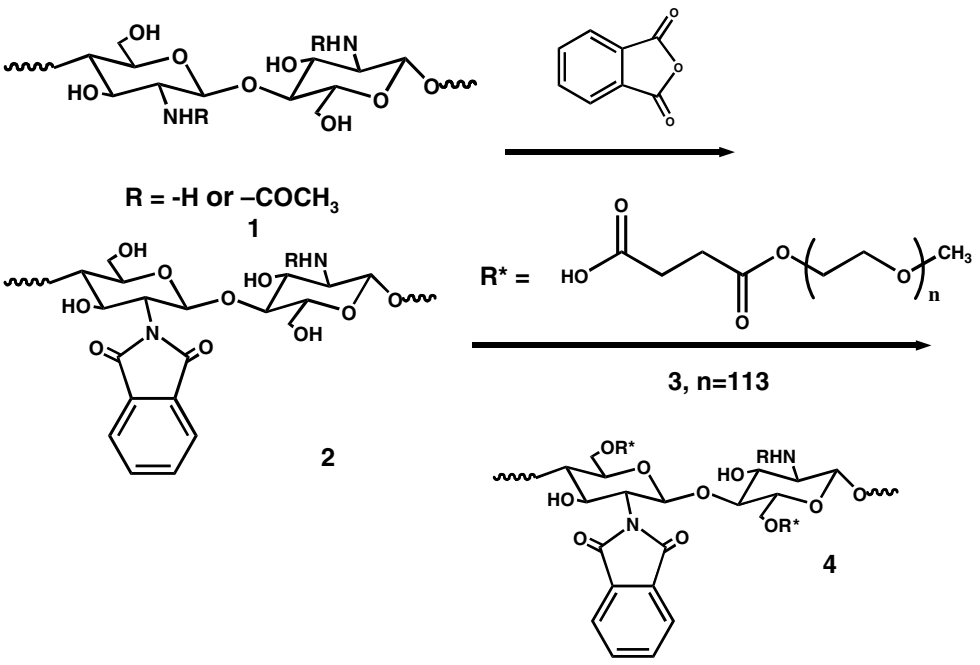
Chitosan with 95% deacetylation (Mv 5.78×10^5 Da) was provided by Seafreach Chitosan (Lab), Co., Thailand. Phthalic anhydride and succinic anhydride were purchased from Fluka Chemika, Switzerland. Poly(ethylene glycol) methyl ethers (mPEG, Mn 2000 Da) were obtained from Aldrich Chemical Company, USA. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was purchased from TCI, Japan. 1-Hydroxy-1H-benzotriazole, monohydrate (HOBt) was obtained from BDH Laboratory Supplies, UK. (*s*)-(+)-Camptothecin and pyrene were purchased from Aldrich Chem. Co., Milw., Wl, USA. All other chemicals were of analytical grade.

2.2. Synthesis of PLC-g-mPEG

N-Phthaloylchitosan **2** was prepared as previously reported [29]. Briefly, Chitosan **1** (DD = 0.95, Mv 5.78×10^5) was reacted with phthalic anhydride (5 mol equivalent to pyranose rings) in DMF at 100 °C under vacuum for 6 h. The temperature was reduced to 60 °C and left overnight. The product was reprecipitated in cold water. The precipitate was collected, washed with ethanol, and dried in vacuum to give pale yellow **2** (Scheme 1).

Anal. Calcd for **2** $(C_{14}H_{13}O_6N)_{0.67}$ $(C_6H_{11}O_4N)_{0.13}$ $(C_8H_{13}O_5N)_{0.05}$: (%): C, 55.54; H, 4.73; N, 6.14. Found (%): C, 56.54; H, 5.56; N, 3.43. FTIR (KBr, cm^{-1}): 3474 (OH), 1777 and 1713 (C=O anhydride), and 721 (aromatic ring).

Compound **2** (0.40 mol equivalent to **3**) was stirred with **3** in DMF solution. 1-Hydroxy-1H-benzotriazole, monohydrate (HOBt, 3 mol equivalent to **3**) was added as catalyst and stirred at room temperature until clear solution. 1-Ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide, hydrochloride (EDC, 3 mol equivalent to **3**) was reacted overnight. The mixture was dialyzed in water and washed with ethanol to obtain white particles, **4** (Scheme 1).



Scheme 1.

^{1}H NMR exhibited the peaks at 3.1–4.1 ppm belonging to methylene protons of mPEG, whereas the peaks at 3.1–4.2, and 7.6–7.9 refer to pyranose ring, and aromatic protons, respectively. Substitution degree of mPEG onto phthalimido was 16.50% as calculated by ^{1}H NMR.

2.3. Suitable solvents investigation

The suitable solvents for dissolving PLC-g-mPEG were studied by dissolved in various solvents such as water, methanol, ethanol, 2-propanol, chloroform, dimethyl sulfoxide (DMSO), *N*-dimethylformamide (DMF), toluene, and *n*-hexane.

2.4. Incorporation of CPT into polymeric micelles

The incorporation of CPT into polymeric micelles was carried out by a dialysis method [23]. Briefly, 5 mg of PLC-g-mPEG polymer and CPT (5–40% of polymer) was dissolved in 2 ml of DMSO or DMF in a glass tube. The mixture was stirred at room temperature until completely dissolved. The mixture was then placed in a dialysis bag (membrane: Spectra/Por® 12,000–14,000 MWCO, Spectrum Laboratories, USA), dialyzed against distilled water overnight. The mean particle diameters were determined by dynamic laser light scattering (DLS) using Zetasizer®3000 (Malvern Instruments, Southborough, MA, USA). CPT-loaded polymeric micelles were dissolved in a mixture of DMSO:H_2O (9:1). The amount of CPT incorporated into polymeric micelles was determined by UV–vis absorption at 365 nm. The incorporation efficiency was calculated as the percentage share of the initial drug used in the preparation for incorporation into the micelles.

2.5. Gel-permeation chromatography (GPC)

The stability of drug-loaded micelles was determined by GPC as described previously [24]. High performance liquid chromatography (HPLC) was carried out using an Agilent HPLC system (Agilent 1100 series, USA) equipped with a Tosoh TSKgel G3000PW$_{XL}$ column at 40 °C. Samples (50 μl) were injected into the column and eluted with distilled water at a flow rate of 1.0 ml/min. The detection was performed by absorption at 351 nm using a UV detector and a refractive index (RI) detector.

2.6. Critical micelle concentration (CMC) determination

The CMC of PLC-g-mPEG polymer was determined using a spectrofluorophotometer (RF-1501, Shimadzu, Japan) with pyrene as a fluorescence probe. Experiments were set up with excitation and emission wavelengths of 352 and 383 nm, respectively. The concentrations of pyrene were 6.0×10^{-7} M. The emission and excitation spectra of pyrene fluorescence were recorded with a micelle concentration that ranged from 0.125 to 256 μg/ml. In each experiment, a 5 μl pyrene in acetone solution was added to a 4 ml polymeric micelle solution and stirred for 24 h until the acetone was completely evaporated prior to measurement. For pyrene excitation spectra, the ratio of fluorescence intensity at 334 and 337 nm (I_{334}/I_{337}) was calculated and plotted against the logarithm concentration of micelles.

2.7. In vitro release

Release of CPT from CPT-loaded micelles was measured using a dialysis bag (membrane: Spectra/Por® 12,000–14,000

MWCO, Spectrum Laboratories, USA) as described previously [19]. One hundred milliliters of phosphate-buffered saline (PBS) at pH 7.4, was used as a medium at 37 ± 0.1 °C under constant stirring. One milliliter CPT-loaded micelles were placed in a dialysis bag and immersed in the medium. At certain time intervals, 1 ml aliquots of the medium were withdrawn and the same volume of fresh medium was added. The sample solution was analyzed by reverse-phase HPLC. All experiments were performed in triplicate.

2.8. Reverse-phase HPLC analysis of CPT

Concentrations of CPT were determined using a reverse-phase HPLC system [19]. A lactone form and the open carboxylated form of CPT were separated within a single chromatographic run. The reverse-phase HPLC system for this determination consisted of an Agilent HPLC system (Agilent 1100 series, USA) at a flow rate of 1.0 ml/min at 40 °C. For separation a Waters μBondasphere C_{18} reverse-phase column (3.9 × 150 mm, Nihon Waters, Tokyo, Japan) was used. The mobile phase was composed of 23% acetonitrile and 77% aqueous buffer (0.1 M KH_2PO_4, 0.5 mM tetrabutylammonium dihydrogen phosphate and 0.4 mM triethylamine at pH 6).The detection was performed using a fluorescence detector with an excitation wavelength of 360 nm and emission wavelength of 430 nm.

2.9. Effect of micelles on lactone ring protection

To elucidate the effects of polymeric micelles on the lactone-carboxylate hydrolysis over time at physiological pH (7.4), CPT-loaded polymeric micelles were incubated in the PBS buffer at pH 7.4, in 50% fetal bovine serum (FBS) and in 4% human serum albumin (HSA) at a CPT concentration of 55 μg/ml. Ten microliters of aliquots was withdrawn at time intervals (1, 2, 4, 6, 8 and 24 h), followed by immediate reverse-phase HPLC analysis of the lactone and carboxylate forms of CPT. For comparison, a CPT solution of 10 μg/ml in a PBS buffer at pH 7.4, FBS and HSA was investigated by the same method as that for the micelles.

2.10. Statistical analysis

All measurements were collected in triplicate experiments. Values are expressed as means ± standard deviation (SD). Statistical significance of differences in amount of CPT lactone form was examined using one-way analysis of variance (ANOVA) followed by LSD post hoc test. The significance level was set at $p < 0.05$.

3. Results and discussion

3.1. Characterization of CPT-incorporating polymeric micelles

Initially, suitable solvents for dissolving PLC-g-mPEG were investigated by observing the physical appearance of PLC-g-mPEG when various solvents were used. It was found that the degree of turbidity largely depended on the types of solvents. This effect was mainly due to the hydrogen bonding and polar–polar interaction. While PLC-g-mPEG was completely dissolved in DMSO and DMF, it formed colloid (as seen from turbidity) in water, methanol, ethanol, 2-propanol and chloroform, and was insoluble in toluene and *n*-hexane. The ability of DMSO and DMF to dissolve PLC-g-mPEG was probably due to a high dielectric constant and a high dipole moment of the solvents.

As shown in previous studies, several strategies i.e. the evaporation, dialysis and emulsion method were used for physical incorporation of CPT into the polymeric micelles [18]. In this study, CPT was incorporated by the dialysis method since PLC-g-mPEG and CPT were dissolved in DMSO and DMF. Both solvents could not be evaporated, therefore evaporation and emulsion method may not be employed. Fig. 2 shows the effect of solvents on the CPT incorporation efficiency in polymeric micelles. The *X*-axis represents the initial drug used in preparation (percentage of CPT), ranging from 5% to 40%, and *Y*-axis represents the percentage of CPT incorporated into the micelles (% CPT-loaded). It was found that the incorporation efficiency of CPT loaded in micelles when using DMSO as the solvent was slightly higher than DMF in all percentage of CPT. The incorporation efficiency increased ranging from 30% to 90% with an increase in the initial CPT loading from 5% to 40%. Such a high content indicates successful incorporation of water-insoluble drug to polymeric micelles with good water solubility. Micelle formation and drug incorporation into micelle were expected to occur simultaneously. Interactions, mainly hydrophobic interactions, among the hydrophobic *N*-phthaloylchitosan chain, CPT, and solvent may be an important key to control this incorporation process. If CPT interacts more favorably with the hydrophobic polymer chain than with solvent, incorporation efficiency should be high. If CPT molecules

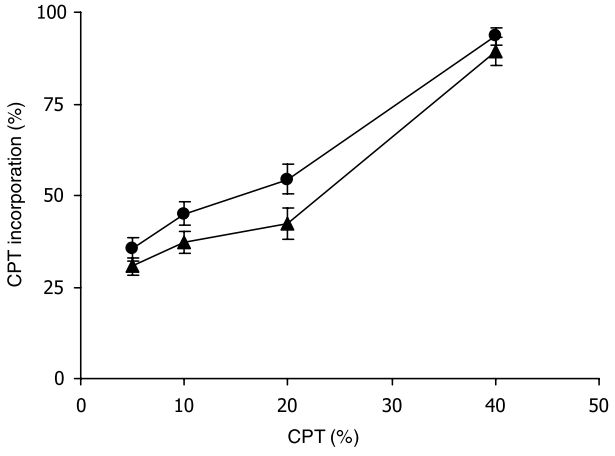


Fig. 2. Effect of solvent on the CPT incorporation efficiency in polymeric micelles: (●) dimethyl sulfoxide (DMSO); (▲) dimethylformamide (DMF). Data are plotted means ± SD of three measurements.

interact with each other more strongly than with the hydrophobic polymer chain, CPT will precipitate rather than being incorporated into micelles. Chitosan is hydrophilic and cannot interact with CPT molecules via hydrophobic interactions. As a result, chitosan graft mPEG cannot incorporate CPT and form micelles (data not shown). However, *N*-phthaloylchitosan (phthalimido group substitution of 80%) showed high micelle stability upon the CPT incorporation. This suggests a large contribution of π–π interaction between aromatic groups of CPT molecules and the hydrophobic inner core (phthalimido group) of the PLC-g-mPEG polymers. These results showed advantages of this novel polymer since it is preferable to design drug carrier systems without using drug molecules as a chemically conjugated species. These results are likely to be consistent with previous results in which benzyl and methylnaphthyl PEG-poly(aspartate) block copolymer (phenyl/naphthyl group) at high esterification degree provided high CPT incorporation stability. In contrast, *n*-butyl and lauryl PEG-poly(aspartate) block copolymer showed lower stability than benzyl and methylnaphthyl PEG-poly(aspartate) block copolymer [18,19]. The diameter and distribution of the polymeric micelles were measured by dynamic laser light scattering (DLS). The mean particle sizes of the CPT-loaded PLC-g-mPEG micelles ranged from 200 to 267 nm in diameter and had a larger size than the bare micelles (170 nm). The narrow size distribution was observed in both bare and CPT-loaded PLC-g-mPEG micelles (data not shown). The diameter increased with an increase in the weight ratio of CPT to polymer (5, 10, 20 and 40% CPT to polymer). Fig. 3 shows the TEM micrographs of CPT-loaded polymeric micelles (40% CPT to polymer). In all case the micelles were spherical in shape and nanosize.

The stability of CPT-loaded micelles was characterized by GPC. It was observed that all samples formed polymeric micelle structures with micelle peaks near the gel-exclusion volume. Micelle peaks detected by the RI detector (for polymers) showed the same retention time (4.2 min) as detected by UV absorption at 351 nm (for CPT). GPC with UV detection allowed us to evaluate the nature of the polymeric micelles obtained and the degree of drug incorporation. Therefore, the stability of CPT-loaded micelles was characterized by the peak area detected by UV absorption at 351 nm. This peak area represents the amount of CPT loaded into the micelles. The ratio of this peak area/CPT concentration, [CPT], was larger, thus CPT was more stably incorporated into the micelles. The small values of the peak area/[CPT] mean that most of the CPT was adsorbed to the GPC column by hydrophobic interactions due to unstable packaging of CPT in the micelles. The values of peak area/[CPT] of this CPT-loaded micelles were decreased with an increase in the weight ratio of CPT to polymer (5–40% CPT to polymer), as shown in Fig. 4. These results implied that this novel PLC-g-mPEG polymer acts not only as a solubilizer for water-insoluble drug but also as a drug carrier.

3.2. Critical micelle concentration (CMC)

The critical micelle concentration (CMC) of the self-assembly micellar system of PLC-g-mPEG was determined by measuring the fluorescence intensity of the pyrene as a fluorescent probe [30]. Pyrene was used as a fluorescence probe because its fluorescence spectrum is sensitive to the polarity of the environment. With an increase in PLC-g-mPEG polymer concentrations, the total fluorescent intensity (I) increased, and fluorescence spectrum shifted. The ratio I_{337}/I_{334} of the pyrene excitation spectra was used to determine CMC of block copolymers in water. The plot of the intensity ratio I_{337}/I_{334} of the pyrene excitation spectra against the logarithm of the polymer concentration is shown in Fig. 5. The CMC value can be determined at a polymer concentration of onset of the I_{337}/I_{334} ratio increase. The CMC value of PLC-g-mPEG is 28 μg/ml.

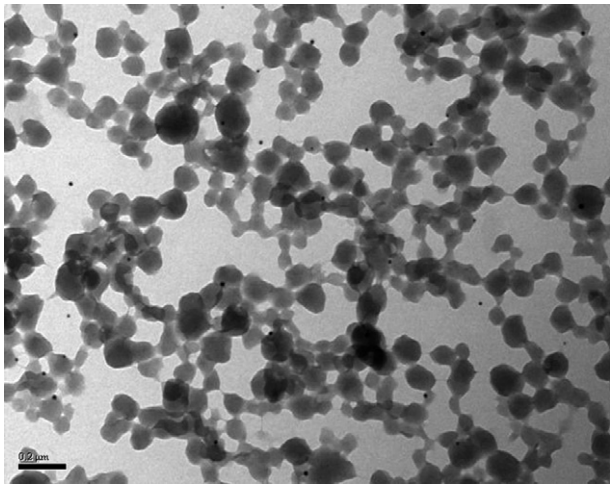


Fig. 3. TEM micrographs of camptothecin (CPT)-loaded polymeric micelles forming from *N*-phthaloylchitosan-grafted mPEG polymer (magnification 6000×).

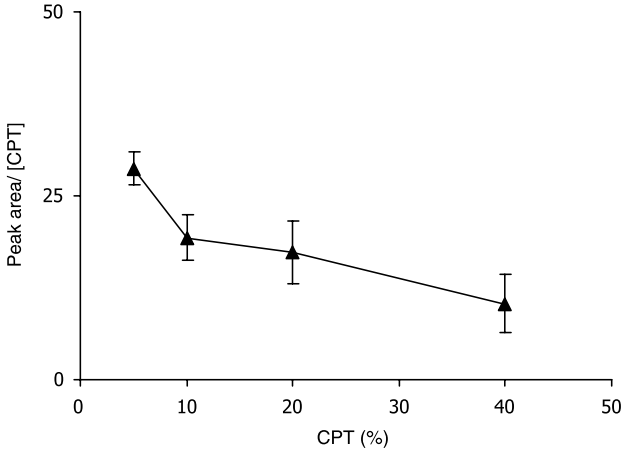


Fig. 4. Effect of CPT concentration on the CPT-loaded micelles stability by using the ratio of peak area/CPT concentration. Data are plotted as means ± SD of two measurements.

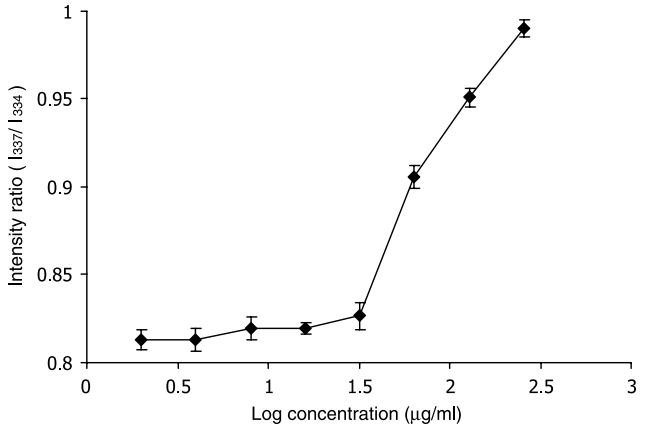


Fig. 5. I_{337}/I_{334} band intensity ratio of pyrene as a function of logarithm concentration of micells forming from *N*-phthaloylchitosan-grafted mPEG. Data are plotted as means ± SD of three measurements.

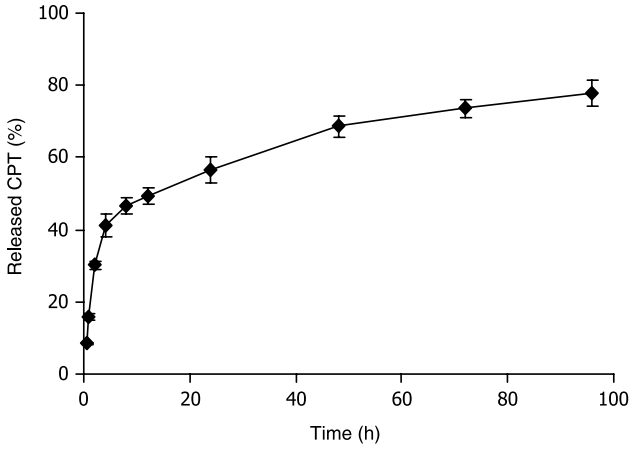


Fig. 6. Release profile of CPT from the micelles forming from *N*-phthaloylchitosan-grafted mPEG. Data are plotted as means ± SD of three measurements.

3.3. Micelle drug release

Based on the fact that the delivery of the lactone form of CPT is necessary for antitumor activity, we postulated that an optimal controlled release preparation would exclusively release the lactone form of CPT. Yokoyama et al. have reported that the hydrophobic inner core functioned as a good reservoir of the drugs by protecting them from inactivation reactions [31]. Polymeric micelle drug delivery systems are advantageous for their wide applicability in delivering hydrophobic drugs. Micelle stability, long-circulation properties, and sustained drug release are critical factors for achieving highly selective delivery to tumor target sites. As previous reported, the CPT incorporation behavior was analyzed by systematically varying both the chemical structure and the content of hydrophobic ester groups of PEG-poly(aspartate) block copolymers. The GPC stability results were correlated with the release rates and the CPT release rate was slowest when it showed the highest GPC stability [19]. The weak interaction between drug and inner core of micelles partially contributes to the fastest release of drug [32]. In this studies, we therefore investigated the release behavior of CPT-loaded PLC-g-mPEG micelles. The CPT release from PLC-g-mPEG micelles initially loaded with 10% CPT is shown in Fig. 6. The results showed that CPT release from PLC-g-mPEG micelles was sustained over 96 h. This implied that PLC-g-mPEG micelles might be a good carrier for CPT incorporation and sustained release for passive tumor targeting.

3.4. Effect of micelles on lactone ring protection

From several preclinical trials being conducted worldwide, unexpected severe systematic toxicity and poor tumor response were reported mainly due to rapid formation of the open ring from of CPT, which is 10-fold less potent than the CPT lactone form. The carboxylate form of CPT preferentially binds to human serum albumin (HSA), which further reduces the equilibrium amount of active lactone and greatly lowers antitumor efficacy [3]. As determined by reverse-phase HPLC, the lactone form of CPT was preserved in the inner core of micelles to an extent of 95% and more (data not shown). The micelle structure was found to greatly contribute toward keeping CPT in its biologically active lactone form. Fig. 7 compares CPT hydrolysis profiles (panel a) and CPT-loaded micelles (panel b) at 25 ± 0.5 °C of 10 μg/ml CPT in PBS buffer, pH 7.4, for the control, in the presence of purified human serum albumins (HSA), and in serum sample (FBS). The inverse linear relationship between the concentration of CPT lactone form and time indicates that the hydrolysis of CPT follows pseudo-first-order kinetics. The amount of lactone versus time was fitted to the equation $y = a\exp(-kt)$, where a is equivalent to the total concentration of lactone at $t = 0$. The hydrolysis rate constants (k) and the corresponding half-life values are summarized in Table 1. The half-life of hydrolysis was calculated from $t_{1/2} = 0.693/K$, where K represents the pseudo-first-order hydrolysis rate constant [20]. Both HSA and FBS showed rapid CPT lactone ring opening more than in PBS buffer. Micelles protected the lactone ring after 24 h with 80% in PBS, 57% in serum, and 43% in HSA, respectively (Fig. 7b). On the other hand, free CPT dissolved in PBS, in serum, and in HSA significantly exhibited ring opening. Only 32%, 20% and 5% of the lactone ring remained in PBS, in serum, and in HSA, respectively, after 3 h (Fig. 7a). This indicated that the incorporation of CPT into the hydrophobic inner core of micelles was advantageous for preservation of the active lactone form at a high concentration for a long period of time. As can be seen, CPT unprotected by the polymers was hydrolyzed very rapidly with the lactone being fully converted into carboxylate, while the CPT-loaded micelles proved to be an effective barrier against the drug decomposition. It is expected that after the CPT-loaded polymeric micelles were incubated in the physiological environment, only the released CPT was

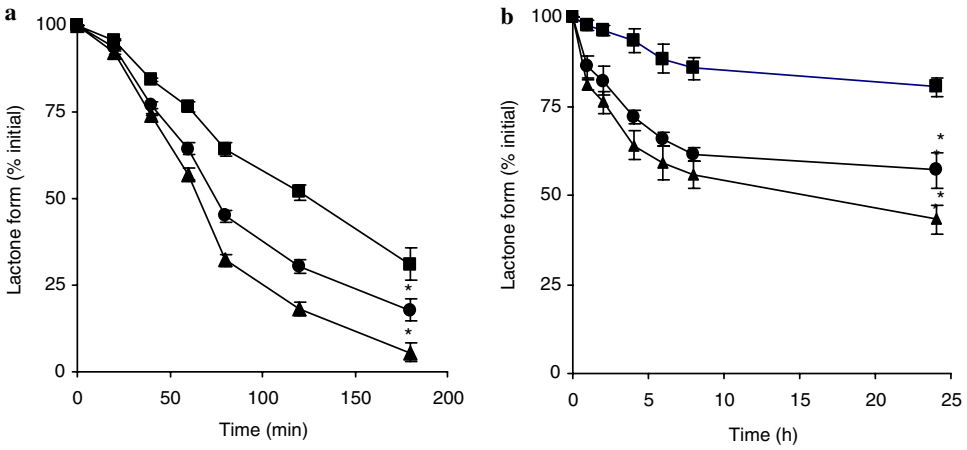


Fig. 7. Kinetic evaluation of the rate of lactone ring opening for (a) camptothecin (CPT) and (b) camptothecin (CPT)-loaded polymeric micelles forming from *N*-phthaloylchitosan-grafted mPEG polymer evaluated by reversed-phase HPLC in the presence of (■) PBS (control); (●) 50% FBS; (▲) 4% HSA ($n = 3$); $*p < 0.05$ compared with PBS (control).

Table 1
Hydrolysis rate and half-life ($t_{1/2}$) of CPT and CPT-loaded polymeric micelles at 25 ± 0.5 °C in the presence of serum and albumins ($n = 3$)

Compounds	CPT		CPT-loaded polymeric micelles	
	Hydrolysis rate ($\times 10^{-3}$ min^{-1})	Half-life (min)	Hydrolysis rate ($\times 10^{-3}$ h^{-1})	Half-life (h)
PBS buffer (control)	7.4	94	9.1	76.15
Fetal bovine serum (FBS)	10.8	64	22.4	30.93
Human serum albumin (HSA)	17.7	39	30.7	22.57

hydrolyzed to the carboxylate form, resulting in a decrease in the lactone form of CPT. It is well known that at a physiological pH more than 80% of CPT exists as the carboxylate form at equilibrium [33].

4. Conclusion

The novel chitosan derivative PLC-g-mPEG can form polymeric micellar system. A water-insoluble anticancer agent, CPT, was successfully incorporated into these polymeric micelles using a dialysis method. The drug was incorporated into polymeric micelles in considerably high yields, with high stability and with a sustained release against PBS buffer, pH 7.4. Furthermore, the internalization of the drug in the micelles notably hindered the hydrolytic opening of the lactone ring in physiological environment (PBS buffer, pH 7.4), in serum and in HSA. Therefore, this novel PLC-g-mPEG polymer presents considerable potential interest in the development of CPT carrier for long circulation of passive tumor targeting.

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References

[1] R.P. Hertzberg, M.J. Caranfa, S.M. Hecht, On the mechanism of topoisomerase I inhibition by camptothecin: evidence for binding to an enzyme–DNA complex, Biochemistry 28 (1989) 4629–4638.
[2] J. Fassberg, V.J. Stella, A kinetic and mechanistic study of the hydrolysis of camptothecin and some analogues, J. Pharm. Sci. 81 (1992) 676–684.
[3] Z. Mi, T.G. Burke, Differential interactions of camptothecin lactone and carboxylate forms with human blood components, Biochemistry 33 (1994) 10325–10336.
[4] X. Liu, B.C. Lynn, J. Zhang, L. Song, D. Bom, W. Du, D.P. Curran, T.G. Burke, A versatile prodrug approach for liposomal core-loading of water-insoluble camptothecin anticancer drugs, J. Am. Chem. Soc. 124 (2002) 7650–7661.
[5] B.B. Lundberg, Biologically active camptothecin derivatives for incorporation into liposome bilayers and lipid emulsions, Anticancer Drug Des. 13 (1998) 453–461.
[6] D.S. Chow, L. Gong, M.D. Wolfe, B.C. Giovanella, Modified lactone/carboxylate salt equilibria in vivo by liposomal delivery of 9-nitro-camptothecin, Ann. NY Acad. Sci. 922 (2000) 164–174.
[7] W. Tong, L. Wang, M.J. D'Souza, Evaluation of PLGA microspheres as delivery system for antitumor agent-camptothecin, Drug Dev. Ind. Pharm. 29 (2003) 745–756.
[8] V. Kumar, J. Kang, R.J. Hohl, Improved dissolution and cytotoxicity of camptothecin incorporated into oxidized-cellulose microspheres prepared by spray drying, Pharm. Dev. Technol. 6 (2001) 459–467.

[9] B. Ertl, P. Platzer, M. Wirth, F. Gabor, Poly(D,L-lactic-co-glycolic acid) microspheres for sustained delivery and stabilization of camptothecin, J. Control Release 61 (1999) 305–317.
[10] J.W. Singer, P. De Vries, R. Bhatt, J. Tulinsky, P. Klein, C. Li, L. Milas, R.A. Lewis, S. Wallace, Conjugation of camptothecins to poly-(L-glutamic acid), Ann. NY Acad. Sci. 922 (2000) 136–150.
[11] C.D. Conover, R.B. Greenwald, A. Pendri, K.L. Shum, Camptothecin delivery systems: the utility of amino acid spacers for the conjugation of camptothecin with polyethylene glycol to create prodrugs, Anticancer Drug Des. 14 (1999) 499–506.
[12] B. Qiu, G.C. Williams, P. Sinko, S. Stein, T. Minko, Molecular targeting of drug delivery systems to ovarian cancer by BH3 and LHRH peptides, J. Control Release 91 (2003) 61–73.
[13] S.S. Dharap, Y. Wang, P. Chandna, J.J. Khandare, B. Qiu, S. Gunaseelan, P.J. Sinko, S. Stein, A. Farmanfarmaian, T. Minko, Tumor-specific targeting of an anticancer drug delivery system by LHRH peptide, Proc. Natl. Acad. Sci. USA 102 (2005) 12962–12967.
[14] P.V. Paranjpe, S. Stein, P.J. Sinko, Tumor-targeted and activated bioconjugates for improved camptothecin delivery, Anticancer Drugs 16 (2005) 763–775.
[15] K.M. Tyner, S.R. Schiffman, E.P. Giannelis, Nanobiohybrids as delivery vehicles for camptothecin, J. Control Release 95 (2004) 501–514.
[16] L. Mu, T.A. Elbayoumi, V.P. Torchilin, Mixed micelles made of poly(ethylene glycol)-phosphatidylethanolamine conjugate and *d*-alpha-tocopheryl polyethylene glycol 1000 succinate as pharmaceutical nanocarriers for camptothecin, Int. J. Pharm. 306 (2005) 142–149.
[17] M. Watanabe, K. Kawano, M. Yokoyama, P. Opanasopit, T. Okano, Y. Maitani, Preparation of camptothecin-loaded polymeric micelles and evaluation of their incorporation and circulation stability, Int. J. Pharm. 308 (2006) 183–189.
[18] M. Yokoyama, P. Opanasopit, T. Okano, K. Kawano, Y. Maitani, Polymer design and incorporation methods for polymeric micelle carrier system containing water-insoluble anti-cancer agent camptothecin, J. Drug Target 12 (2004) 373–384.
[19] P. Opanasopit, M. Yokoyama, M. Watanabe, K. Kawano, Y. Maitani, T. Okano, Block copolymer design for camptothecin incorporation into polymeric micelles for passive tumor targeting, Pharm. Res. 21 (2004) 2001–2008.
[20] P. Opanasopit, M. Yokoyama, M. Watanabe, K. Kawano, Y. Maitani, T. Okano, Influence of serum and albumins from different species on stability of camptothecin-loaded micelles, J. Control Release 104 (2005) 313–321.
[21] Z. Tuzar, P. Kratochvil, Block and graft copolymer micelles in solution, Adv. Colloid Interface Sci. 6 (1976) 201–232.
[22] K. Greish, J. Fang, T. Inutsuka, A. Nagamitsu, H. Maeda, Macromolecular therapeutics: advantages and prospects with special emphasis on solid tumour targeting, Clin. Pharmacokinet. 42 (2003) 1089–1105.
[23] M. Yokoyama, T. Okano, Y. Sakurai, S. Fukushima, K. Okamoto, K. Kataoka, Selective delivery of adriamycin to a solid tumor using a polymeric micelle carrier system, J. Drug Target 7 (1999) 171–186.
[24] M. Yokoyama, G.S. Kwon, T. Okano, Y. Sakurai, K. Kataoka, Influencing factors on in vitro micelle stability of adriamycin-block copolymer conjugates, J. Control Release 28 (1994) 59–65.
[25] Y. Matsumura, M. Yokoyama, K. Kataoka, T. Okano, Y. Sakurai, T. Kawaguchi, T. Kakizoe, Reduction of the side effects of an antitumor agent, KRN5500, by incorporation of the drug into polymeric micelles, Jpn. J. Cancer Res. 90 (1999) 122–128.
[26] S. Kawakami, P. Opanasopit, M. Yokoyama, N. Chansri, T. Yamamoto, T. Okano, F. Yamashita, M. Hashida, Biodistribution characteristics of all-*trans* retinoic acid incorporated in liposomes and polymeric micelles following intravenous administration, J. Pharm. Sci. 94 (2005) 2606–2615.
[27] A. Miwa, A. Ishibe, M. Nakano, T. Yamahira, S. Itai, S. Jinno, H. Kawahara, Development of novel chitosan derivatives as micellar carriers of taxol, Pharm. Res. 15 (1998) 1844–1850.
[28] C. Zhang, P. Qineng, H. Zhang, Self-assembly and characterization of paclitaxel-loaded *N*-octyl-*O*-sulfate chitosan micellar system, Colloids Surf. Biointerfaces 39 (2004) 69–75.
[29] R. Yoksan, M. Matsusaki, M. Akashi, S. Chirachanchai, Controlled hydrophobic/hydro- philic chitosan: colloidal phenomena and nanosphere formation, Colloid Polym. Sci. 282 (2004) 337–342.
[30] C. Zhao, Y. Wang, M.A. Winnik, G. Riess, M.D. Croucher, Fluorescence probe technique used to study micelle formation in water-soluble block co-polymer, Langmuir 6 (1990) 514–516.
[31] M. Yokoyama, M. Miyauchi, N. Yamada, T. Okano, Y. Sakurai, K. Kataoka, S. Inoue, Characterization and anticancer activity of the micelle-forming polymeric anticancer drug adriamycin-conjugated poly(ethylene glycol)-poly(aspartic acid) block copolymer, Cancer Res. 50 (1990) 1693–1700.
[32] W.J. Lin, L.W. Juang, C.C. Lin, Stability and release performance of a series of pegylated copolymeric micelles, Pharm. Res. 20 (2003) 668–673.
[33] J. Fassberg, V.J. Stella, A kinetic and mechanistic study of the hydrolysis of camptothecin and some analogues, J. Pharm. Sci. 81 (1992) 676–684.