Block Copolymer Design for Camptothecin Incorporation into Polymeric Micelles for Passive Tumor Targeting

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Purpose. Polymeric micelles were designed for targeting of a water-insoluble anticancer agent, camptothecin (CPT). Chemical structures of inner core segment were optimized to achieve high incorporation efficiency and stable CPT-loaded micelles.

Methods. Poly(ethylene glycol)-poly(β-benzyl L-aspartate) block copolymer (PEG-PBLA) was synthesized. The PBLA chain was modified by alkaline hydrolysis of its benzyl group followed by esterification with benzyl, *n*-butyl, and lauryl groups. Incorporation of CPT into micelles was carried out by an evaporation method. The stability of drug-loaded micelles was studied by gel-permeation chromatography (GPC), and their *in vitro* release behaviors were analyzed.

Results. CPT was incorporated into polymeric micelles constructed by various block copolymers. Among the esterified groups, block copolymers with high benzyl ester contents showed high CPT loading efficiency and stable CPT-loaded micelles. In chain lengths, 5-27 Bz-69 showed the highest incorporation efficiency. In contrast, 5-52 Bz-67, which had a longer hydrophobic chain, showed low incorporation efficiency. Release of CPT from the micelles was dependent on the benzyl contents and chain lengths. Sustained release was obtained when the benzyl content was high.

Conclusions. CPT was successfully incorporated into polymeric micelles with high efficiency and stability by optimizing chemical structures of the inner core segment.

KEY WORDS: anticancer agent; camptothecin; polymeric micelles; stability.

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, namely, an active lactone form at pH below 5 and an inactive carboxylate form at basic pH (Fig. 1) (2). At physiologic pH, most CPT molecules exist in the inactive carboxylate form. The stability of the lactone form of CPT is crucial for its anticancer activity. 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 showed that the lactone ring of CPT was protected upon incorporation of the drug into a lipid bilayer structure like liposomes (3–5) and microspheres (6–8) and upon conjugation to synthetic polymers (9–11) and nanobiohybrids (12).

Recently, polymeric micelles prepared from block copolymers have been proposed to attain effective novel drug delivery for antitumor drugs (13–17), diagnostic reagents (18), DNA (19–20), and enzymes (23). Furthermore, temperature-modulated drug delivery of block copolymer micelles have been studied (21–22). AB-type block copolymers possessing both hydrophilic and hydrophobic segments are known to form micellar structures in aqueous media due to their amphiphilic character (24). 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 nonselective reticuloendothelial system (RES) scavenge and shows enhanced permeability and retention effects (EPR effect) (25) at solid tumor sites for passive targeting.

In our previous reports, an anticancer drug, adriamycin (doxorubicin), was used as our first example for the polymeric micelle drug delivery system, and these adriamycin-containing polymeric micelles showed dramatically higher *in vivo* antitumor activity than free adriamycin and highly selective delivery to a solid tumor by the EPR effect (15).The second example was of KRN 5500 (6-[4-deoxy-4-(2E,4E)-tetradecadienoylglycyl]amino-L-glycero-β-L-mannoheptopyranosylamino-9H-purine). Its successful incorporation into polymeric micelles was achieved by optimizing the block copolymer structure and the incorporation condition (26).

Our previous study for CPT showed that poly(ethylene glycol)-poly(β-benzyl L-aspartate) block copolymer (PEG-PBLA) incorporated CPT into its micelles. However, the stability of these micelles was very poor. These micelles could be used for solubilization of CPT, but not as a stable drug carrier system. We have also successfully synthesized various AB-type block copolymers by varying chemical structures of hydrophobic segment of the poly(ethylene glycol)-poly(aspartate) block copolymers. We found that the CPT incorporation efficiency and stability were improved with the modified hydrophobic segments of block copolymers and the incorporation method (27). In the current work, in order to achieve the more stable CPT-loading micelles for tumor delivery, the factors affecting the incorporation efficiency and the stability of CPT-loading micelles are systematically evaluated by changing the esterified moieties of the hydrophobic segment, the

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ABBREVIATIONS: CMC, critical micelle concentration; CPT, camptothecin; DBU, 1, 8-diazabicyclo[5,4,0]7-undecene; DDS, drug delivery system; DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide; EPR effect, enhanced permeability and retention effect; FBS, fetal bovine serum; GPC, gel-permeation chromatography; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; PEG-P(Asp), poly(ethylene glycol)-poly(aspartic acid) block copolymer; PEG-PBLA, poly(ethylene glycol)-poly(β-benzyl L-aspartate) block copolymer; RES, reticuloendothelial system.

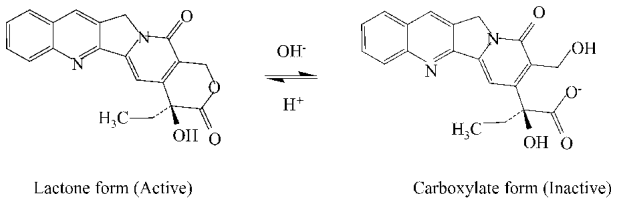

Fig. 1. Chemical structure of camptothecin.

initial drug loading content, and the block copolymer chain lengths. In addition, their *in vitro* release behaviors are also analyzed. We postulated that an optimal controlled release preparation would exclusively release the lactone form of CPT, therefore, the stability of CPT-loaded micelles is examined under the physiologic conditions in a phosphate buffer saline at pH 7.4 or serum. These stable CPT-loaded micelles are expected to have a long-circulating property in the blood stream, which can contribute to targeting to solid tumor sites.

MATERIALS AND METHODS

Materials

Poly(ethylene glycol)-poly(benzyl L-aspartate) block copolymer (PEG-PBLA) was synthesized as described previously(28). (*s*)-(+)-Camptothecin was purchased from Aldrich Chem. Co. (Milwaukee, Wl, USA). 1, 8-Diazabicyclo[5,4,0]7-undecene (DBU) was purchased from Wako Pure Chemicals (Tokyo, Japan). *N,N*-Dimethylformamide was dried over molecular sieve 4A, followed by distillation under reduced pressure. All other chemicals were of analytical grade.

Synthesis of Esterified Block Copolymer

Poly(ethylene glycol)-poly(aspartic acid) block copolymer [PEG-P(Asp)] was obtained by alkaline hydrolysis of PEG-PBLA as reported previously (28). The molecular weight of the PEG chain was 5000 or 12,000. The average number of benzyl aspartate units was 27 or 52 for block copolymers composed of a PEG chain with a molecular weight of 5000, and 26 or 50 for block copolymers composed of a PEG chain with a molecular weight of 12,000. PEG-P(Asp) block copolymer was dissolved in *N,N*-dimethylformamide (DMF) and added by a halogen compound (benzyl bromide, n-butyl bromide, or lauryl bromide), and DBU. The reaction mixture was stirred at 50°C for 15.5 h. Polymers were obtained by precipitation into an excess amount of diethyl ether and collected by centrifugation at 3000 rpm for 10 min. The dried polymer was dissolved in dimethyl sulfoxide (DMSO) and added by an excess amount of 6 N HCl. These solutions were placed in dialysis bags (Spectrapor 6 MWCO = 1000) and dialyzed against distilled water for 24 h, and followed by freeze-drying.

In order to determine the esterified contents, ^{1}H-NMR spectra were measured with 1% solutions in 6D-DMSO added by 3% trifluoroacetic acid using a Varian UNITY INOVA NMR spectrometer at 400 MHz.

Incorporation of CPT into Polymeric Micelles

The incorporation of CPT into polymeric micelles was carried out by an evaporation method (29). Briefly, a block copolymer and CPT were dissolved in chloroform in a glass tube. The mixture was stirred at room temperature under nitrogen gas flow until the solvent completely evaporated. Distilled water was added, and the solution was sonicated using a probe-type sonicator (model VC 100, Sonics & Materials Inc., Newton, CT, USA) in a cycle with a sonication time of 0.5 s and a standby time of 0.5 s at 80°C for 2 min. The solution was centrifuged at 3900 rpm for 10 min. Subsequently, the supernatant was collected and filtrated through a nylon membrane filter with a 1-μm pore (Puradisc 25NYL, 6751-2510, Whatman, Clifton, NJ, USA). The mean particle diameters were determined using a dynamic light scattering particle size analyzer (DLS-7000, Otsuka Electronics, Osaka, Japan).

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.

Gel Permeation Chromatography

The stability of drug-loaded micelles was determined by gel permeation chromatography (GPC) as described previously (30). High-performance liquid chromatography (HPLC) was carried out using a Tosoh HPLC system SC-8010 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 Tosoh UV-8010 detector and a refractive index (RI) detector.

In Vitro Release

Release of CPT from CPT-loaded micelles was measured using a dialysis bag (membrane: Spectra/Por-4 12,000–14,000 MWCO, Spectrum Laboratories, Rancho Dominguez, CA, USA). 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 duplicate.

Reverse-Phase HPLC Analysis of CPT

Concentrations of CPT were determined using a reverse -phase HPLC system (31). 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 a JASCO HyPer LC-800 system (Tokyo, Japan) 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 triethyl amine at pH 6). The detection was performed using a fluorescence detector with an excitation wavelength of 360 nm and emission wavelength of 430 nm.

Critical Micelle Concentration Determination

The critical micelle concentration (CMC) of esterified block copolymers was determined using a fluorescence spectrophotometer (FP-6500, Jasco, Tokyo, 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, 1.5×10^{-7} M, and 0.375×10^{-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. The micelle solutions were measured on days 1, 3, 5, and 7. 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 emission spectra, the intensity (peak height) ratio (I_1/I_3) of the first band (374 nm) to the third band (385 nm) was calculated and plotted against the logarithm of the concentration of micelles. 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 the concentration of micelles.

Effect of Micelles on Lactone Ring Protection

To elucidate the effects of polymeric micelles on the lactone-carboxylate hydrolysis over time at physiologic pH (7.4), CPT-loaded polymeric micelles (5-27 Bz-69) were incubated in the PBS buffer at pH 7.4 or in fetal bovine serum (FBS) at a CPT concentration of 75 μg/ml. Ten microliters of aliquots were 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 or FBS was investigated by the same method as that for the micelles.

RESULTS

Esterified Block Copolymer

Poly(ethylene glycol)-poly(aspartate ester) block copolymers were successfully synthesized from PEG-P(Asp) with various chain lengths of the PEG and P(Asp) as shown in Fig. 2. Ester formation in the side chain of the P(Asp) block was confirmed by ^{1}H-NMR spectrum measurements. The block copolymers obtained are coded by chain lengths of the PEG and P(Asp), the name of the hydrophobic group, and degree of esterification as summarized in Table I. For example, 5-27 Bz-75 represents a block copolymer composed of the PEG block of molecular weight of 5,000, the P(Asp) block possessing 27 units of aspartic acid, and 75% of the aspartic acid residue that was esterified to the benzyl aspartate residue.

Characterization of Polymeric Micelles Incorporating CPT

As shown in previous studies, the evaporation method was found to be suitable in comparison with other methods (dialysis and emulsion) for the incorporation of CPT into the polymeric micelles (27). In this study, CPT was incorporated only by the evaporation method, and all the block copolymer systems yielded clear solutions after sonication. The mean particle sizes of the micelles ranged from 60 to 110 nm in diameter. The diameter increased with an increase in the

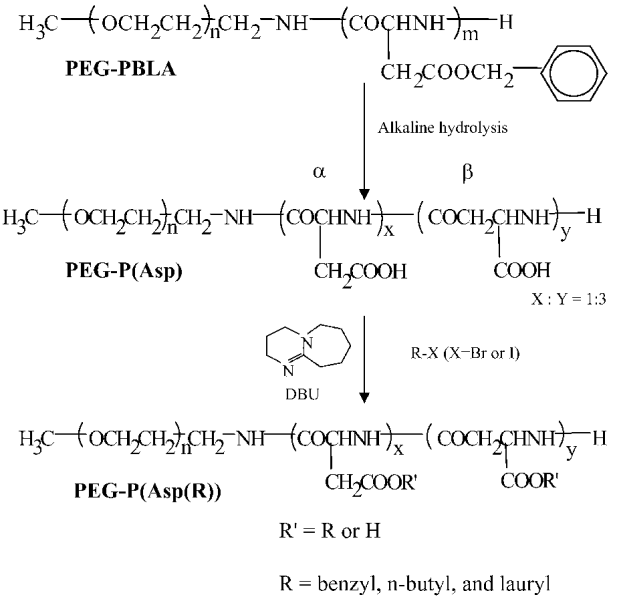

Fig. 2. Synthesis of esterified block copolymer PEG-P(Asp(R)) consisting of hydrophilic PEG and hydrophobic P(Asp(R)) from poly(ethylene glycol)-poly(aspartic acid) block copolymer (PEG-P(Asp)).

weight ratio of CPT to polymer. The stability of CPT-loaded micelles was characterized by GPC. It was observed that all the 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 of the peak 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

Table I. Esterification of PEG-P(Asp) Block Copolymers

Polymer[a]	MW of PEG	No. of Asp units	Esterified groups	Esterification (%)
5-27 Bz-75	5000	27	Benzyl	75
5-27 Bz-69	5000	27	Benzyl	69
5-27 Bz-57	5000	27	Benzyl	57
5-27 Bz-44	5000	27	Benzyl	44
5-27 Bz-25	5000	27	Benzyl	25
5-27 n-Bu-47	5000	27	*n*-Butyl	47
5-27 Lau-43	5000	27	Lauryl	43
12-26 Bz-64	12,000	26	Benzyl	64
5-52 Bz-67	5000	52	Benzyl	67
12-50 Bz-63	12,000	50	Benzyl	63

[a] The block copolymers are coded by chain lengths of the PEG and P(Asp), the name of the hydrophobic group, and degree of esterification. For example, 5-27 Bz-75 represents a block copolymer composed of the PEG block of molecular weight of 5000, the P(Asp) block possessing 27 units of aspartic acid, and 75% of the aspartic acid residue that was esterified to the benzyl aspartate residue.

values of the peak area/[CPT] means that most of the CPT was adsorbed to the GPC column by hydrophobic interactions due to unstable packaging of CPT in the micelles. The results of CPT-loaded micelles formed from benzyl, n-butyl, and lauryl ester block copolymers with esterification degree ca. 45% are shown in Fig. 3. Figure 3a shows incorporation efficiency. The x-axis is the initial drug used in preparation (percentage of CPT), ranging from 5% to 40%, and the percentage of CPT incorporated into the micelles (% CPT-loaded) is represented on the y-axis. Polymeric micelles formed from 5-27 Bz-44 exhibited lower incorporation efficiency than those formed from 5-27 n-Bu-47 and 5-27 Lau-43, as shown in Fig. 3a. At 5% of the initial CPT, the CPT incorporation yield of 5-27 Bz-44 was significantly lower than that of 5-27 n-Bu-47, judged by Student's *t* test ($p < 0.05$). The CPT incorporation stability results exhibited behaviors which were different from the CPT incorporation efficiency results. Polymeric micelles formed from 5-27 Lau-43 showed very low incorporation stability and these values of peak area/[CPT] were lower than those formed from 5-27 n-Bu-47 and 5-27 Bz-44, as shown in Fig. 3b. The insert chart shows the results detected by GPC of CPT-loaded micelles formed from benzyl, *n*-butyl, and lauryl ester block copolymers.

Subsequently, the effects of the esterification degree on the CPT incorporation were evaluated using benzyl ester. As shown in Fig. 4a, the incorporation efficiency increased with an increase in benzyl ester degree from 44% to 69%. For 5-27 Bz-69, the CPT yield was very high, over 92% for 5% to 20% initial drug use to the block copolymer. However, a block copolymer with higher benzyl content (5-27 Bz-75) showed a decrease from 5-27 Bz-69. As regards the CPT incorporation stability (Fig. 4b), high benzyl contents tended to raise the stability. The highest stability value was exhibited by 5-27 Bz-75 with initial CPT content of 20%. In contrast, the incorporation stability of *n*-butyl ester and lauryl ester block copolymers did not increase when *n*-butyl and lauryl contents were increased (data not shown).

Following this, the effect of chain lengths in both the hydrophilic segment (PEG) and the hydrophobic segment [P(AspR)] were evaluated for CPT incorporation efficiency and the stability of CPT-loaded micelles. When the benzyl ester degree was identical (approximately 60–70%), 5-27 Bz-69 block copolymers showed higher CPT incorporation than 12-50 Bz-63, 12-26 Bz-64, and 5-52 Bz-67 block copolymers (Fig. 5). The CPT-loaded micelles reached approximately 90% in 5-27 Bz-69, depending on the initial drug used in the preparation. Although 5-52 Bz-67 showed the highest CPT-loaded micelle stability, it showed the lowest incorporation efficiency (less than 25% loading). Among the three block copolymers with higher CPT incorporation, 5-27 Bz-69 showed higher drug-loaded micelle stability than 12-50 Bz-63 and 12-26 Bz-64.

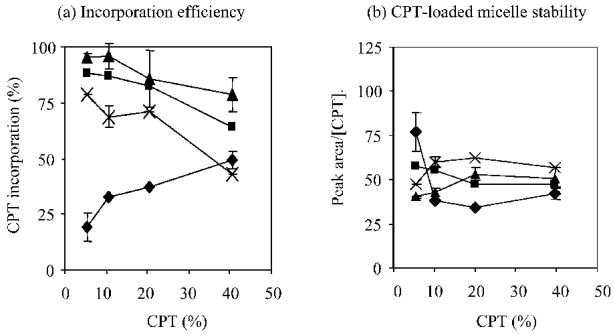

Fig. 4. Effect of benzyl ester contents of PEG-P(Asp) block copolymers on the (a) CPT incorporation efficiency and (b) the micelles stability by ratio of peak area/CPT concentration (♦, 5-27 Bz-44; ■, 5-27 Bz-57; ▲, 5-27 Bz-69; X, 5-27 Bz-75). Data are plotted in the mean ± SD of two measurements for 5% and 40% CPT of 5-27 Bz-44, all CPT contents of 5-27 Bz-69, and 10% CPT of 5-27 Bz-75. The other plots represent values of single measurement.

Micelle Drug Release

The CPT release from 5-27 benzyl ester-substituted block copolymer micelles initially loaded with 40% CPT is shown in Fig. 6. It was observed that the CPT release was well correlated with the benzyl ester degree and the initial concentration of CPT-loading micelles; the release rate increased when the micelles at CPT initial concentration of 300–700 μg/ml were diluted to 70 and 7 μg/ml. This implied that a critical micelle concentration of the micelle affected the CPT release. As the benzyl ester degree increased up to 75%, the release rate was retarded. A greater retarded release was

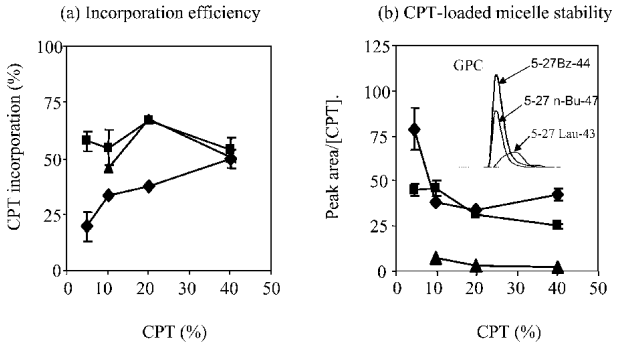

Fig. 3. Effect of esterified groups of PEG-P(Asp) block copolymers on the (a) CPT incorporation efficiency and (b) the CPT-loaded micelles stability by using the ratio of peak area/CPT concentration (♦, 5-27 Bz-44; ■, 5-27 n-Bu-47; ▲, 5-27 Lau-43). Data are plotted in the mean ± SD of two measurements for 5% and 40% CPT of 5-27 Bz-44 and 5%, 10%, and 40% CPT of 5-27 n-Bu-47. The other plots represent values of single measurement.

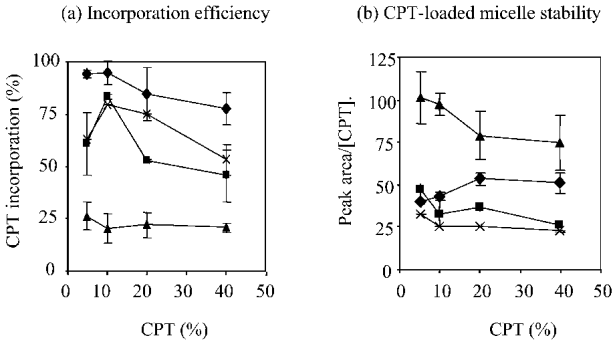

Fig. 5. Effect of chain lengths of benzyl esterification of PEG-P(Asp) block copolymers on the (a) CPT incorporation efficiency and (b) the micelles stability by ratio of peak area/CPT concentration (♦, 5-27 Bz-69; ■, 12-26 Bz-64; ▲, 5-52 Bz-67; X, 12-50 Bz-63). Data are plotted in the mean ± SD of two measurements except 5%, 10%, and 20% of 12-50 Bz-63 that are values of single experiment.

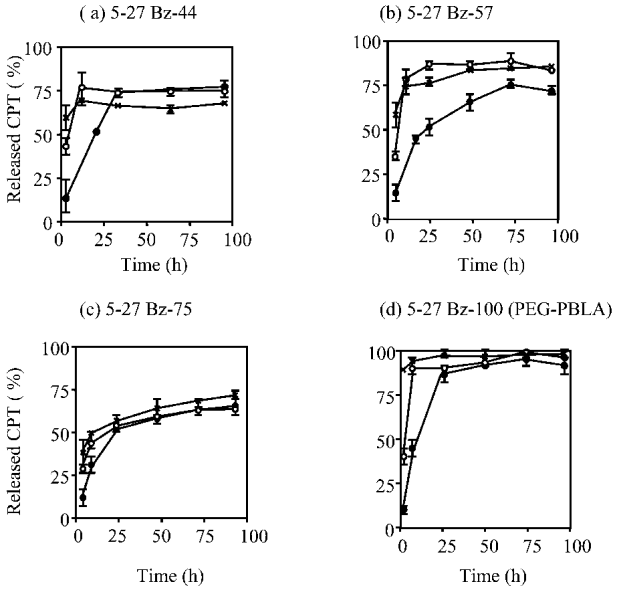

Fig. 6. Effect of benzyl esterification contents of PEG-P(Asp) block copolymers on the CPT release from the micelles forming from (a) 5-27 Bz-44, (b) 5-27 Bz-57, (c) 5-27 Bz-75, and (d) 5-27 Bz-100 or 5-27 PEG-PBLA at the initial CPT-loaded micelles concentration of 7 μg/ml (X); 70 μg/ml (○); 300–700 μg/ml (●).

exhibited by 5-25 Bz-57 block copolymers than by 5-27 Bz-44. For 5-27 Bz-75, the CPT release was the slowest, and its release rate was independent of the initial concentrations. For PEG-PBLA, that corresponds to 100% Bz, the CPT release from the micelles was the fastest providing most CPT release in 24 h (Fig. 6d).

The effect of chain lengths on the CPT release is shown in Fig. 7. When the benzyl ester degree was identical (approximately 60–70%), 5-27 Bz-69 block copolymers exhibited more retarded release than 12-50 Bz-63 at the highest initial micelle concentration and than 12-26 Bz-64 at all the three initial concentrations. The current work demonstrated that the molecular weight of PEG affected not only the CPT incorporation but also the stability and release behavior of the micelles. PEG MW 5000 provided more stable micelles than PEG MW 12000, and the shorter hydrophobic segments provided more retarded release at the highest initial concentration. This result indicated that the balance between the hydrophobic and hydrophilic chains affected the formation of stable micelles.

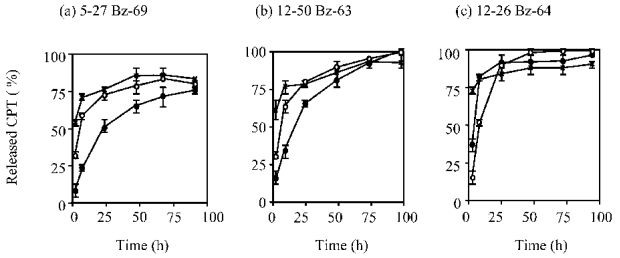

Fig. 7. Effect of chain lengths of benzyl-esterified PEG-P(Asp) block copolymers on the CPT release from the micelles forming from (a) 5-27 Bz-69, (b) 12-50 Bz-63, and (c) 12-26 Bz-64 at the initial CPT-loaded micelles concentration of 7 μg/ml (X); 70 μg/ml (○); 300–700 μg/ml (●). Data are plotted in the mean ± SD of two measurements.

Critical Micelle Concentration

In order to determine the CMC of polymeric micelles, fluorescence measurements were carried out using pyrene by a reported method (32). Pyrene is a widely used fluorescence probe because its fluorescence spectrum is sensitive to polarity of the atmosphere. With an increase in block copolymers concentrations, the total fluorescent intensity increased, and fluorescence spectrum changed. 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. 8. The CMC value can be determined at a polymer concentration of onset of the I_{337}/I_{334} ratio increase. The CMC values with different benzyl content block copolymers are shown in Table II. It was observed that the CMC values increased with a decrease in the benzyl content. These results indicated that the lower benzyl content block copolymers (5-27 Bz-44) exhibited difficulties in the formation of micelles, which was in accordance with the release results.

The association of amphiphilic block copolymers into micelles is a well-known phenomenon. However, micelles are in a dynamic equilibrium with single polymer chains. For low molecular weight surfactants, the relaxation time ranged from milliseconds to minutes. In the case of polymeric micelles, the relaxation time may be longer than low molecular weight surfactants. The relaxation time is a very important factor for the lifetime and stability of polymeric micelles as a drug carrier in the body. In this study, we established the methods for studying the relaxation time of polymeric micelles by measuring the CMC values on days 1, 3, 5, and 7 after micelle dilution. The results showed that polymeric micelles had the same CMC values for 7 days. Furthermore, they revealed that the relaxation time of the micelles was not as long as the day order, and indicated that these polymeric micelles at a concentration higher than the CMC value were stable. In addi-

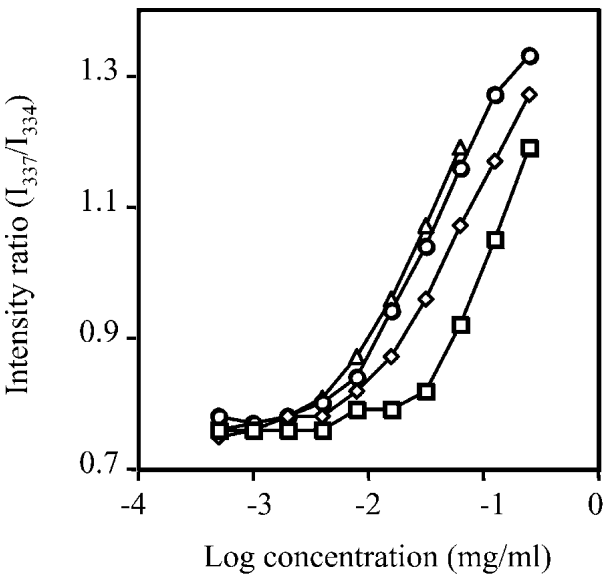

Fig. 8. I_{337}/I_{334} band intensity ratio of pyrene as a function of logarithm concentration of micelles forming from (□) 5-27 Bz-44, (◇) 5-27 Bz-57; (○) 5-27 Bz-75, and (Δ) 5-27 Bz-100. Data are plotted in the mean ± SD of two measurements.

Table II. The Estimation of CMC Values for PEG-P(Asp(R)) Copolymers

Polymer	CMC (μg/ml)
5-27 Bz-44	40
5-27 Bz-57	9
5-27 Bz-75	5
5-27 PEG-PBLA	3

CMC, critical micelle concentration.

tion, we observed the CMC value with 4 and 16 times dilution of the pyrene concentration. The CMC values did not change with this dilution for all benzyl-esterified block copolymers, which indicated that this determination brought about CMC, but not micelle capacity for pyrene incorporation.

Effect of Micelles on Lactone Ring Protection

Because carboxylate conversion limits the bioavailability and efficacy of CPT, maintenance of the lactone structure is a prerequisite for an improved therapy using polymeric micelles. 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. The micelle structure was found to greatly contribute toward keeping CPT in this biologically active lactone form. Micelles protected the lactone ring after 24 h with 85% in PBS and 72% in serum, respectively (Fig. 9). On the other hand, free CPT dissolved in PBS or in serum significantly exhibited ring opening. Only 20% and 35% of the lactone ring remained in serum and in PBS, respectively, after 2.3 h.

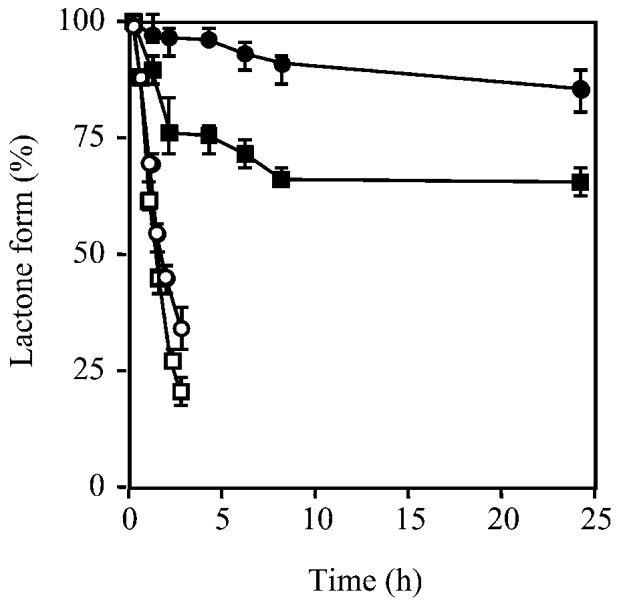

Fig. 9. Stability of CPT-lactone form of free drug and CPT loading in the polymeric micelles forming from 5-27 Bz-69 block copolymers vs. rapid hydrolysis in simulated physiologic environment. Free drug (10 μg/ml) incubated with PBS buffer pH 7.4 (○) and serum (□); and CPT-loaded in micelles (75 μg/ml) incubated with PBS buffer pH 7.4 (●) and 50% serum (■). Data are plotted in the mean ± SD of two measurements.

DISCUSSION

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. As shown in Fig. 3, benzyl and *n*-butyl ester showed higher CPT-loaded micelle stability in larger ratio of peak area/[CPT] than lauryl ester, as the lauryl block copolymer was more hydrophobic with a longer acyl chain (C12) than *n*-butyl (C4) and benzyl (C7). This implied that not only the hydrophobic interaction but also the rigidity of the hydrophobic inner core was crucial for stable CPT incorporation since the longer acyl chain was more flexible. For the benzyl ester block copolymers, the incorporation efficiency increased with an increase in the benzyl ester content from 44% to 69%, followed by a decrease in the efficiency at 75% in comparison with 69%, as shown in Fig. 4a. This suggested a large contribution of $\pi-\pi$ interaction between the aromatic groups of CPT molecules and the phenyl groups of these block copolymers. The GPC stability results were consistent with the release results. The release rate was lowered with an increase in the benzyl contents. For 5-27 Bz-75, the CPT release was the slowest, and its release rate was independent of the initial concentrations. This indicated that a certain quantity of hydrophobic component was essential to form stable CPT-loaded micelles, and particularly a benzyl content greater than 57% was enough for stable micelle formation with high CPT incorporation. In contrast, PEG-PBLA (100% benzyl ester) showed unstable CPT-loaded micelles, as determined by low peak area/[CPT] and fast CPT release. The possible reasons for this inversed relation between benzyl content and CPT-loaded micelle stability is the difference in the polymer main chain structure between 5-27 Bz-75 and PEG-PBLA. Three-quarters of the amino acid units of the 5-27 Bz-75 were β-amide units and racemic due to an alkaline hydrolysis procedure in the syntheses (28), whereas PEG-PBLA was composed of α-amide units and the L-configuration. These results suggested that the β-amide units in the racemic configuration could be advantageous for stronger interactions with CPT molecules. Furthermore, for 5-27 Bz-75, 25% Asp units lacked a benzyl group, which may provide the appropriate space required to insert CPT molecules, resulting in good interactions with the adjacent benzyl groups by hydrophobic interactions.

In chain lengths, 5-27 Bz-69 showed a higher CPT incorporation than 12-50 Bz-63, 12-26 Bz-64, and 5-52 Bz-67. Although 5-52 Bz-67 showed the highest CPT-loaded micelle stability by GPC, the incorporation efficiency was very low (less than 25% loading). This low incorporation made it unsuitable to be used as a drug carrier. Moreover, the release rate of 5-52 Bz-67 was faster than 5-27 Bz-69 (data not shown). Among the higher CPT incorporations, 5-27 Bz-69 showed higher CPT-loaded micelle stability than 12-50 Bz-63 and 12-26 Bz-64. This suggested that the balance between the hydrophobic and hydrophilic chains affected the stable formation of micelles.

Based on the fact that the delivery of the lactone form of CPT is crucial for anti-tumor activity, we postulated that an optimal controlled release preparation would exclusively release the lactone form of CPT. We have reported that the hydrophobic inner core functioned as a good reservoir of the drug by inhibiting drug inactivation reactions of adriamycin

(33). We observed that the lactone form remained within micelles >95% over a month. Under a simulated physiologic environment (50% serum), we found that more than 70% of CPT remained in the lactone form for over 24 h (Fig. 9). 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. It is expected that after the CPT-loaded polymeric micelles were incubated in the physiologic environment, only the released CPT was hydrolyzed to the carboxylate form, resulting in a decrease in the lactone form of CPT. It is well-known that at a physiologic pH more than 80% of CPT exists as the carboxylated form at equilibrium (34).

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. Further studies are in progress to determine the *in vivo* pharmacokinetics behavior of the CPT loaded micelles.

CONCLUSIONS

A water-insoluble anticancer agent, CPT, was successfully incorporated into polymeric micelles formed from esterified PEG-poly(aspartic acid) block copolymers by an evaporation method. For stable incorporation of this drug into micelles, the chemical structure of the hydrophobic chain of the block copolymer, drug content, and chain lengths were found to influence the incorporation efficiency and stability to a great extent. The benzyl ester moieties on the hydrophobic chain were the most suitable for stable micelles. This indicates the importance of the molecular design of the hydrophobic block chain to obtain preferable drug carrier properties for tumor targeting.

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REFERENCES

1. R. P. Hertzberg, M. J. Caranfa, and S. M. Hecht. On the mechanism of topoisomerase I inhibition by camptothecin: evidence for binding to an enzyme-DNA complex. *Biochemistry* **28**:4629–4638 (1989).
2. J. Fassberg and V. J. Stella. A kinetic and mechanistic study of the hydrolysis of camptothecin and some analogues. *J. Pharm. Sci.* **81**:676–684 (1992).
3. X. Liu, B. C. Lynn, J. Zhang, L. Song, D. Bom, W. Du, D. P. Curran, and T. G. Burke. A versatile prodrug approach for liposomal core-loading of water-insoluble camptothecin anticancer drugs. *J. Am. Chem. Soc.* **124**:7650–7661 (2002).
4. B. B. Lundberg. Biologically active camptothecin derivatives for incorporation into liposome bilayers and lipid emulsions. *Anticancer Drug Des.* **13**:453–461 (1998).
5. D. S. Chow, L. Gong, M. D. Wolfe, and B. C. Giovanella. Modified lactone/carboxylate salt equilibria in vivo by liposomal delivery of 9-nitro-camptothecin. *Ann. N. Y. Acad. Sci.* **922**:164–174 (2000).
6. W. Tong, L. Wang, and M. J. D'Souza. Evaluation of PLGA microspheres as delivery system for antitumor agent-camptothecin. *Drug Dev. Ind. Pharm.* **29**:745–756 (2003).
7. V. Kumar, J. Kang, and R. J. Hohl. Improved dissolution and cytotoxicity of camptothecin incorporated into oxidized-cellulose microspheres prepared by spray drying. *Pharm. Dev. Technol.* **6**:459–467 (2001).
8. B. Ertl, P. Platzer, M. Wirth, and F. Gabor. Poly(D,L-lactic-co-glycolic acid) microspheres for sustained delivery and stabilization of camptothecin. *J. Control. Rel.* **61**:305–317 (1999).
9. J. W. Singer, P. De Vries, R. Bhatt, J. Tulinsky, P. Klein, C. Li, L. Milas, R. A. Lewis, and S. Wallace. Conjugation of camptothecins to poly-(L-glutamic acid). *Ann N Y Acad Sci* **922**:136–150 (2000).
10. C. D. Conover, R. B. Greenwald, A. Pendri, and 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**:499–506 (1999).
11. S. S. Dharap, B. Qiu, G. C. Williams, P. Sinko, S. Stein, and T. Minko. Molecular targeting of drug delivery systems to ovarian cancer by BH3 and LHRH peptides. *J. Control. Rel.* **91**:61–73 (2003).
12. K. M. Tyner, S. R. Schiffman, and E. P. Giannelis. Nanobiohybrids as delivery vehicles for camptothecin. *J. Control. Rel.* **95**: 501–514 (2004).
13. M. Yokoyama, T. Okano, Y. Sakurai, H. Ekimoto, C. Shibazaki, and K. Kataoka. Toxicity and antitumor activity against solid tumors of micelle-forming polymeric anticancer drug and its extremely long circulation in blood. *Cancer Res.* **51**:3229–3236 (1991).
14. Y. Mizumura, Y. Matsumura, T. Hamaguchi, N. Nishiyama, K. Kataoka, T. Kawaguchi, W. J. Hrushesky, F. Moriyasu, and T. Kakizoe. Cisplatin-incorporated polymeric micelles eliminate nephrotoxicity, while maintaining antitumor activity. *Jpn. J. Cancer Res.* **92**:328–336 (2001).
15. M. Yokoyama, T. Okano, Y. Sakurai, S. Fukushima, K. Okamoto, and K. Kataoka. Selective delivery of adriamycin to a solid tumor using a polymeric micelle carrier system. *J. Drug Target.* **7**:171–186 (1999).
16. M. Yokoyama, A. Satoh, Y. Sakurai, T. Okano, Y. Matsumura, T. Kakizoe, and K. Kataoka. Incorporation of water-insoluble anticancer drug into polymeric micelles and control of their particle size. *J. Control. Rel.* **55**:219–229 (1998).
17. V. P. Torchilin, A. N. Lukyanov, Z. Gao, and B. Papahadjopoulos-Sternberg. Immunomicelles: targeted pharmaceutical carriers for poorly soluble drugs. *Proc. Natl. Acad. Sci. USA* **100**:6039–6044 (2003).
18. V. P. Torchilin. PEG-based micelles as carriers of contrast agents for different imaging modalities. *Adv. Drug Deliv. Rev.* **54**:235–272 (2002).
19. J. Liaw, S. F. Chang, and F. C. Hsiao. In vivo gene delivery into ocular tissues by eye drops of poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) polymeric micelles. *Gene Ther.* **8**:999–1004 (2001).
20. S. V. Vinogradov, T. K. Bronich, and A. V. Kabanov. Self-assembly of polyamine-poly(ethylene glycol) copolymers with phosphorothioate oligonucleotides. *Bioconjug. Chem.* **9**:805–812 (1998).
21. Y. G. Takei, T. Aoki, K. Sanui, N. Ogata, Y. Sakurai, and T. Okano. Temperature-modulated platelet and lymphocyte interactions with poly(N-isopropylacrylamide)-grafted surfaces. *Biomaterials* **16**:667–673 (1995).
22. Y. Kaneko, S. Nakamura, K. Sakai, A. Kikuchi, T. Aoyagi, Y. Sakurai, and T. Okano. Synthesis and swelling-deswelling kinetics of poly(N-isopropylacrylamide) hydrogels grafted with LCST modulated polymers. *J. Biomater. Sci. Polym. Ed.* **10**:1079–1091 (1999).
23. A. Harada and K. Kataoka. Pronounced activity of enzymes through the incorporation into the core of polyion complex micelles made from charged block copolymers. *J. Control. Rel.* **72**: 85–91 (2001).
24. Z. Tuzar and P. Kratochvil. Block and graft copolymer micelles in solution. *Adv. Colloid Interface Sci.* **6**:201–232 (1976).
25. K. Greish, J. Fang, T. Inutsuka, A. Nagamitsu, and H. Maeda. Macromolecular therapeutics: advantages and prospects with

special emphasis on solid tumour targeting. *Clin. Pharmacokinet.* **42**:1089–1105 (2003).
26. Y. Matsumura, M. Yokoyama, K. Kataoka, T. Okano, Y. Sakurai, T. Kawaguchi, and 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**:122–128 (1999).
27. M. Yokoyama, P. Opanasopit, Y. Maitani, K. Kawano, and T. Okano. Polymer design and incorporation method for polymeric micelle carrier system containing water-insoluble anti-cancer agent camptothecin. *J. Drug Target.* (in press).
28. M. Yokoyama, G. S. Kwon, T. Okano, Y. Sakurai, T. Seto, and K. Kataoka. Preparation of micelle-forming polymer-drug conjugates. *Bioconjug. Chem.* **3**:295–301 (1992).
29. A. Lavasanifar, J. Samuel, and G. S. Kwon. Micelles self-assembled from poly(ethylene oxide)-block-poly(N-hexyl stearate L-aspartamide) by a solvent evaporation method: effect on the solubilization and haemolytic activity of amphotericin B. *J Control. Rel.* **77**:155–160 (2001).
30. M. Yokoyama, G. S. Kwon, T. Okano, Y. Sakurai, and K. Kataoka. Influenceing factors on in vitro micelle stability of adriamycin-block copolymer conjugates. *J. Control. Release* **28**: 59–65 (1994).
31. A. Shenderova, T. G. Burke, and S. P. Schwendeman. The acidic microclimate in poly(lactide-co-glycolide) microspheres stabilizes camptothecins. *Pharm. Res.* **16**:241–248 (1999).
32. C. Zhao, Y. Wang, M. A. Winnik, G. Riess, and M. D. Croucher. Fluorescence probe technique used to study micelle formation in water-soluble block co-polymer. *Langmuir* **6**:514–516 (1990).
33. M. Yokoyama, M. Miyauchi, N. Yamada, T. Okano, Y. Sakurai, K. Kataoka, and 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**:1693–1700 (1990).
34. J. Fassberg and V. J. Stella. A kinetic and mechanistic study of the hydrolysis of camptothecin and some analogues. *J. Pharm. Sci.* **81**:676–684 (1992).