(DBU) was purchased from Wako Pure Chemicals, Tokyo, Japan. N,N-Dimethylformamide (DMF) was dried with molecular sieve 4A, followed by distillation under reduced pressure. Poly(ethylene glycol)–poly(β-benzyl L-aspartate) block copolymer (PEG–PBLA) was synthesized by ring-opening polymerization of benzyl L-aspartate N-carboxy anhydride from a terminal primary amino group of α-methyl-ω-aminopoly(oxyethylene) as described in our previous paper (Yokoyama *et al.*, 1992). Codes of PEG–PBLA were based on molecular weight of the poly(ethylene glycol) chain (PEG) and polymerization degree of BLA units; for example, 5-27 means a block copolymer composed of the PEG block of M.W. of 5000 and the PBLA block possessing 27 units of BLA. PEG–PBLA 5-27 and PEG–PBLA 12-26 were used. Other chemicals were of reagent grade and used as purchased.

Alkaline Hydrolysis of PEG–PBLA Block Copolymer

Poly(ethylene glycol)–poly(aspartic acid) block copolymer (PEG–P(Asp)) was obtained by alkaline hydrolysis of poly(ethylene oxide)–poly(β-benzyl L-aspartate) block copolymer (PEG–PBLA) as reported previously (Yokoyama *et al.*, 1992). Briefly, PEG–PBLA was dispersed in a measured volume of 0.5 N NaOH that contained 1.5 mol equivalents NaOH to the benzyl aspartate residue of PEG–PBLA. With stirring at room temperature, the solution became homogeneous approximately in 15 min. Then, 6 N HCl was added (10 mol equivalents of HCl to the benzyl aspartate residue) to the solution and this solution was dialyzed against 0.1 N HCl, followed by dialysis against distilled water using a Spectrapor 6 dialysis membrane (molecular weight cut-off: 1000). PEG–P(Asp) block copolymer was obtained by freeze-drying of the dialyzed solution. The molecular weight of the PEG block was 5000 and the average number of the benzyl aspartate units were 27. It was known that main chain degradation did not occur by this hydrolysis procedure. It was also known that approximately 75% of the aspartic acid residues of the P(Asp) chain were converted to the β-amide form in this procedure (Yokoyama *et al.*, 1992).

Esterification of Aspartic Acid Residue of PEG–P(Asp)

PEG–(Asp) block copolymer was dissolved in DMF and added by a halogen compound (benzyl bromide, n-butyl bromide, n-butyl iodide, lauryl bromide, or 2-(bromomethyl)naphthalene) and DBU. The reaction mixture was stirred at 50°C for 14.6–18.1 h. Then, the reaction mixture was poured into a ten-fold volume excess diethyl ether and the precipitated polymer was collected by filtration, followed by washing with diethyl ether and drying. In order to remove contaminating DBU in polymer products, polymers were dissolved in DMSO and added by 6 N HCl that was much excess equivalents to the aspartic acid residue of the block copolymer. Then, this solution was dialyzed against distilled water and followed by freeze-drying.

Incorporation of Camptothecin (CPT) to Polymeric Micelles

CPT incorporation to polymeric micelles was examined by three methods; dialysis, emulsion and evaporation method.

(1) Dialysis Method

A block copolymer (5.0 mg) and CPT (0.5 mg) was dissolved in 1.0 ml of dimethyl sulfoxide (DMSO). This solution was dialyzed against distilled water using a dialysis membrane (Spectrapor 4, molecular weight cut-off: 12,000–14,000). After overnight dialysis, the solution in the dialysis membrane was collected and filtrated through a No. 1, 2 or 5A filter (Toyo Roshi, Tokyo, Japan).

(2) Emulsion Method

A block copolymer (5.0 mg) and CPT (0.5 mg) was dissolved in 0.5 ml of methylene chrolide(CH_2Cl_2). This solution was poured into 2.0 ml of distilled water with vigorous stirring using a magnetic stirrer. Methylene chloride was evaporated by overnight stirring at room temperature. The remaining aqueous solution was filtrated through a No. 5A filter.

(3) Evaporation Method

A block copolymer and CPT was dissolved in chloroform in a glass tube. This solution was stirred by a magnetic stirrer in a N_2 gas flow. Chloroform was completely evaporated at room temperature. Distilled water was added in this glass tube, followed by sonication for 2 min using a probe type sonicator model VC 100 (Sonics & Materials Inc., Connecticut, USA) equipped with a standard 6 mm probe in a cycle of sonication for 0.5 s and standby for 0.5 s at 4°C. The obtained solution was treated by the following two ways.

1. The solution was filtrated using a 5A filter paper.
2. The solution was centrifuged at 3900 rpm for 10 min. The supernatant was collected and filtrated through a nylon membrane filter with 1 μm pore (Puradisc 25NYL,6751-2510, Whatman, Clifton, New Jersey, USA).

General Procedure

^{1}H-NMR, UV-VIS, HPLC, and light scattering measurements ^{1}H-NMR were obtained with 1% solutions in 6D-DMSO added by 3% trifluoroacetic acid using a Varian UNITY INOVA NMR spectrometer at 400 MHz. Gel-filtration chromatography was carried out using a Tosoh HPLC system SC-8010 equipped with a Tosoh TSKgel G3000PWXL column. Distilled water was used as eluent

at a flow rate of 1.0 ml/min at 40°C. Fifty microlitter of sample solutions were injected into the column. The detection was performed by absorption at 351 nm using a Tosoh UV-8010 detector.

The CPT amount incorporated to polymeric micelles was determined by UV–VIS absorption spectroscopy. This measurement was done by two ways; in distilled water and in a mixture of DMSO and distilled water (volume ratio of DMSO/water = 9 : 1). In distilled water, the CPT amount was calculated from a value obtained by subtracting absorbance at 600 nm from absorbance at 370 nm to compensate background raise due to the presence of large aggregates. The ε (molar absorptivity) value of 19,900 from Merck Index twelfth edition was used. For determination in the DMSO/water mixture, the CPT amount was calculated from a value obtained by subtracting absorbance at 600 nm from absorbance at 365 nm using $\varepsilon = 20,860$ (This value was obtained with CPT solutions in this mixture.). For Fig. 4, UV–VIS absorption spectra was recorded in absorbance between 0.30 and 0.91 at a peak around 370 nm and their peak heights were normalized in order to compare background between 430 and 600 nm in which CPT or block copolymers have no absorption.

Proportions of a lactone form and an open form of CPT incorporated to polymeric micelles were determined by reversed-phase HPLC (Shenderova *et al.*, 1999), which allows separation of the two CPT forms within a single chromatographic run. CPT was extracted from polymeric micelles by adding DMSO and diluted with an aqueous buffer described below. The 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. A Waters C_{18} 3.9 × 150 mm reverse phase column 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 pH = 6). The detection was performed using a fluorescence detector with an excitation wavelength of 360 nm and emission wavelength of 430 nm.

Laser scattering measurements were carried out using a Photal dynamic laser scattering spectrometer DLS-7000 (Otsuka Electronics Co. Ltd., Tokyo, Japan) with a neon laser beam.

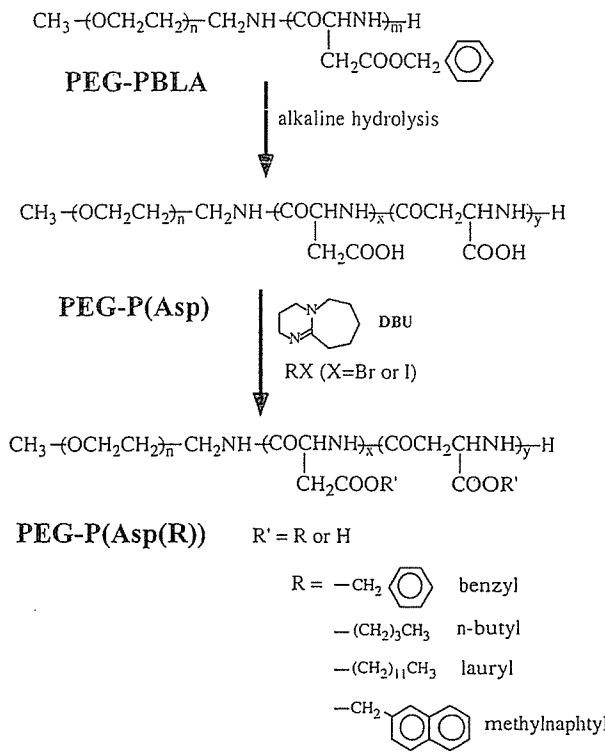

FIGURE 1 Synthetic scheme of block copolymers.

RESULTS

Esterification of Aspartic Acid Residue of PEG–P(Asp)

Various poly(ethylene glycol)–poly(aspartate ester) block copolymers were successfully synthesized from PEG–P(Asp) 5-27 in a synthesis scheme shown in Fig. 1. The obtained block copolymers are coded by a name of hydrophobic group and esterification degree as summarized in Table I. For example, benzyl-44 means 44% of the aspartic acid residue was converted to benzyl

TABLE I Esterification of PEG–P(Asp) block copolymer

Code	PEG–p(Asp) 5-27 (mg)	Halogen Species	Halogen mg (equivalent*)	DBU mg (equivalent*)	DMF (mL)	Reaction† time(h)	Yield (mg)	Esterification‡ (%)
Benzyl-25	198.6	Benzyl-Br	51.0(0.51)	57.0(0.56)	2.5	16.2	144.0	25
Benzyl-44	500.5	Benzyl-Br	236.8(0.83)	212.2(0.83)	5.0	16.0	520.0	44
Benzyl-61	200.0	Benzyl-Br	116.9(1.02)	103.9(1.02)	2.7	14.8	160.2	61
Benzyl-69	200.2	Benzyl-Br	227.0(1.98)	202.8(1.99)	2.0	14.6	154.1	69
n-butyl-47	200.8	n-butyl-Br	91.9(1.00)	101.4(0.99)	2.0	14.8	146.5	47
n-butyl-66	200.0	n-butyl-I	123.3(1.00)	101.4(1.00)	2.0	14.8	157.2	66
Lauryl-45	200.6	Lauryl-Br	165.8(0.99)	102.1(1.00)	2.0	15.6	143.7	45
Methylnaphtyl-53	200.4	Methylnaphtyl-Br	146.6(0.99)	101.2(0.99)	2.0	18.1	188.8	53
Methylnaphtyl-64	1400.1	Methylnaphtyl-Br	1034.7(1.00)	709.6(1.00)	14.0	15.3	1684.7	64

*Mol equivalents to aspartic acid residue of PEG–P(Asp) block copolymer.
†Reaction temperature was 50°C.
‡With respect to aspartic acid residue measured by ^{1}H-NMR spectra.

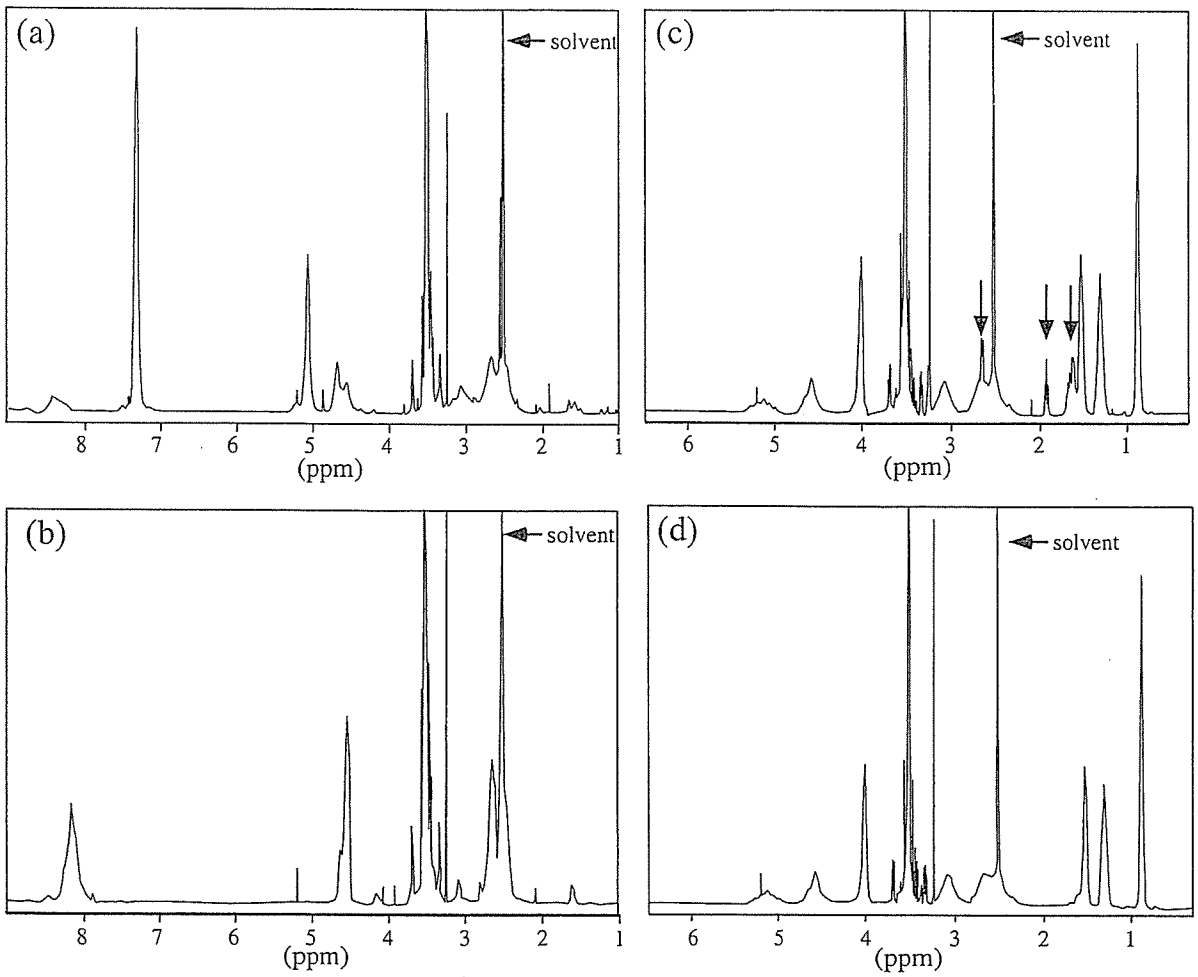

FIGURE 2 Proton NMR spectra of block copolymers. (a) benzyl-44, (b) PEG–P(Asp) 5-27, (c) n-butyl-47 without an acid treatment and (d) n-butyl-47 after an acid treatment. These spectra were obtained with 1% solutions in 6D-DMSO added by 3% trifluoroacetic acid.

aspartate residue. Ester formation at the side chain of the P(Asp) block was confirmed by ^{1}H-NMR spectrum measurements. As shown in Fig. 2(a), benzyl ester formation was observed for benzyl-44 block copolymer by methylene protons (CH_2) and phenyl protons (C_6H_5) of the benzyl ester group at 5.1 and 7.3 ppm, respectively. These protons were absent in a spectrum of PEG–P(Asp) block copolymer as shown in Fig. 2(b). No benzyl bromide was contaminated in the obtained polymer because no peak of methylene protons (CH_2) of benzyl bromide was observed at 4.50 ppm. From a peak area ratio between the methylene protons of the benzyl ester group and methylene protons of the PEG block at 3.50 ppm, esterification degree was determined. As summarized in Table I, the esterification degree was well controlled in a range of 25–69% by adjusting molar ratio of benzyl bromide and DBU to the aspartic acid residue of the PEG–P(Asp). By increasing the molar ratios up to 2.0, a high benzyl ester content such as 69% was obtained.

n-Butyl ester was also successfully introduced to PEG–P(Asp). By using 1.0 equivalent mol of n-butyl bromide, 44% of the aspartic acid residues were converted to n-butyl aspartate. A ^{1}H-NMR spectrum of this butyl case is shown in Fig. 2(c). Butyl ester content was determined by a peak area ratio between the terminal methyl protons (CH_3) at 0.87 ppm of the butyl group and the PEG methylene protons. This ester content was lower than that of the benzyl ester case that provided 61% with 1.0 equivalent mol benzyl bromide. This difference was considered to result from higher reactivity of benzyl bromide than butyl bromide. No contaminated n-butyl bromide was seen in the NMR spectrum. However, a considerable amount of DBU was seen as shown by arrows in the NMR chart. DBU is basic and is expected to be contaminated in the product by interacting with a carboxyl group of the aspartic acid residue of the block copolymer. In order to remove this contaminated DBU, 52.5 mg of the obtained polymer was dissolved in 0.5 ml of DMSO, added by 25 μl of 6 N HCl and dialyzed against distilled water, followed by freeze-drying. After this acid treatment, the contaminated DBU was completely removed from the polymer as shown by disappearance of the DBU peak in Fig. 2(d). No change in the ester content by this acid treatment was confirmed by ^{1}H-NMR spectroscopy. Therefore, this acid treatment was done for all block copolymers to remove

DBU from products. The n-butyl ester content was increased from 47 to 66% by using n-butyl iodide on behalf of benzyl bromide.

Both lauryl and methylnaphtyl ester were also successfully introduced to the aspartic acid residue of the block copolymer using corresponding bromide compounds as well as benzyl and n-butyl cases. Block copolymers were obtained with 45% of lauryl ester and 53 and 64% of methylnaphtyl ester coded as lauryl-45, methylnaphtyl-53 and methylnaphtyl-64, respectively. A considerable difference in the esterification degree was found between methylnaphtyl-53 and methylnaphtyl-64 even though both runs were obtained with the same halogen and DBU molar ratios to the aspartic acid residue of PEG–P(Asp). This is considered to result from a difference in loss amounts during precipitation and purification processes, particularly due to relatively large loss in a small scale of synthesis of methylnaphtyl-53 case. All obtained block copolymers were found to be well dissolved in DMSO, chloroform and methylene chloride and therefore, incorporation of CPT to polymeric micelles forming from these block copolymers were examined by three methods using these three organic solvents.

Incorporation of Camptothecin (CPT) to Polymeric Micelles

CPT incorporation to polymeric micelles was examined by three methods: dialysis, emulsion and evaporation method.

(1) Dialysis Method

Table II summarizes results of CPT incorporation to polymeric micelles by a dialysis method. After dialysis, a precipitate was observed in a transparent solution. After filtration, the precipitate was removed and only very small amounts of CPT was found in the transparent solutions. All examined block copolymer was revealed to provide very small yields of CPT less than 5% by measuring absorbance at 370 and 600 nm ($\varepsilon = 19,900$). Absorbance at 600 nm was subtracted from absorbance at 370 nm to compensate possible background raise due to the presence of large aggregates. This quantification method is considered to a little underestimates incorporated CPT amounts since the ε value was obtained in organic solvent, not in distilled water where measurements in this experiment was done. This underestimation was found to be less than 50% by comparing data with measurements in a mixture of DMSO/water = 9 : 1. Therefore, the fact of the low incorporation yields by the dialysis method was not changed even by this underestimation of CPT.

TABLE II CPT incorporation to polymeric micelles by dialysis method

Run	Polymer	Incorporation conditions	Yield of CPT/μg (%)*
1	PEG–PBLA (5-27)	Standard†,	4 (1)
2	PEG-PBLA (12-27)	0.5 ml DMSO‡	8 (2)
3	Benzyl-25	Standard	5 (1)
4	Benzyl-61	Standard	8 (2)
5	Benzyl-69	Standard	10 (2)
6	n-butyl-47	Standard	6 (1)
7	Lauryl-45	Standard	11 (2)
8	Methylnaphtyl-53	Standard	18 (4)
9	Methylnaphtyl-53	0.5 ml DMSO‡	8 (2)
10	PEG–PBLA (5-27)	0.5 ml DMSO + sonication	228 (45)
11	PEG–PBLA (12-27)	0.5 ml DMSO + sonication	187 (37)
12	Methylnaphtyl-53	0.5 ml DMSO + sonication	37 (7)

*Estimated by (absorbance at 370 nm)—(absorbance at 600 nm) in water using $\varepsilon = 19,900$.
†Polymer 5.0 ± 0.1 mg, CPT 0.50 mg, DMSO 1.0 mL
‡Only volume of DMSO was different from the standard conditions.

Among the examined block copolymers, methylnaphtyl-53 provided the largest CPT yield. By reducing an initial DMSO amount, the CPT yield was lowered from 4 to 2%. PEG–PBLA 12-26 provided the same CPT yield in the reduced DMSO conditions as methylnaphtyl-53. In order to raise the CPT yield, dialysis conditions such as amounts of block copolymer and CPT, amount of DMSO and organic solvent (DMF and N,N-dimethylacetamide) were varied using PEG–PBLA 12-26. No increase in the CPT yield, however, was obtained (data not shown). These low incorporation yields were considered to result from CPT precipitation alone or with block copolymers during the dialysis procedure.

When sonication was applied after the dialysis process, considerable increases in the CPT yield were observed, as shown by runs 10–12 in Table II. The increase was most prominent for PEG–PBLA 5-27 and its CPT yield reached 46%. The obtained solution, however, was cloudy due to the presence of aggregates that were much larger than polymeric micelles with 50–100 nm diameter as shown by UV–VIS absorbance spectrum. Figure 3 shows the spectra whose peak heights around 370 nm were normalized. In Fig. 3(a), considerable absorbance was observed for run 10 of Table II in a range from 430 to 600 nm. Since no chemical species were present for absorption in this range, this absorption resulted from light scattering of large aggregates. It is considered that absorption at 370 nm substantially included scattering phenomenon of the large aggregates due to a significant raise of absorbance from 600 to 430 nm. Therefore, the calculated CPT amount of run 10 in Table II was overestimated considerably.

All these results indicate that CPT incorporation was not obtained with high CPT yields and high solution clarity by the dialysis method.

(2) Emulsion Method

The obtained solution by the emulsion method were all cloudy due to the presence of aggregates larger than polymeric micelles. This cloudiness of the solutions was confirmed by considerably high values of absorbance at 600 nm. Incorporated CPT amount was calculated by subtracting absorbance at 600 nm from absorbance at 370 nm. A spectrum of benzyl-61 (run 5 in Table III) is shown in Fig. 3(b). Since base line from 430 to 600 nm

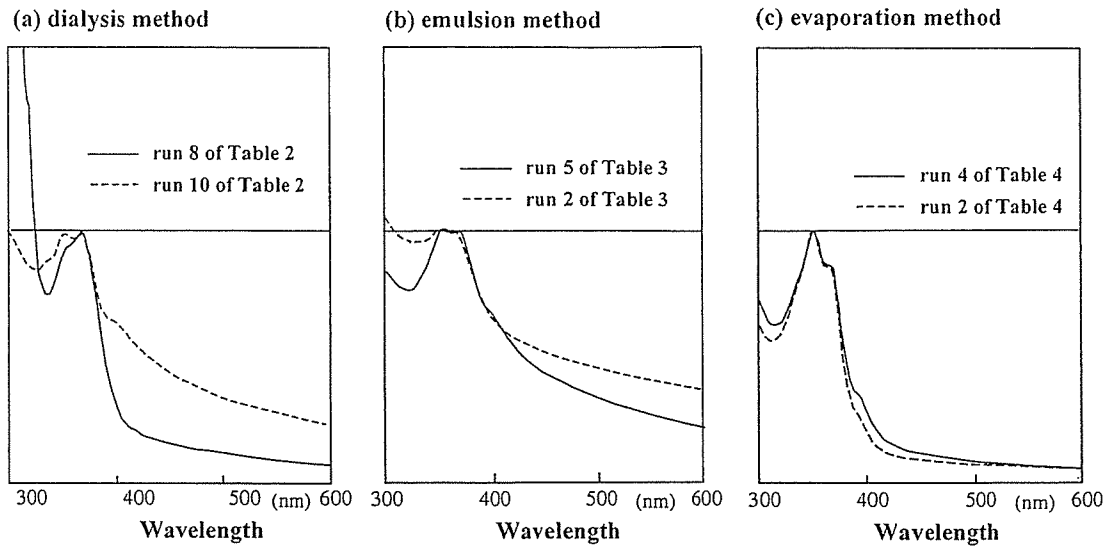

FIGURE 3 UV–VIS absorption spectra of aqueous polymeric micelles containing CPT. Peak heights of around 370 nm were normalized. (a) obtained by dialysis method, solid line: run 8 of Table II, dotted line: run 10 of Table II, (b) obtained by emulsion method, solid line: run 5 of Table III, dotted line: run 2 of Table III and (c) obtained by evaporation method, solid line: run 4 of Table IV, dotted line: run 2 of Table IV.

was not flat (CPT does not possess absorbance between 430 and 600 nm), the calculated CPT amount may be overestimated even after subtracting absorbance at 600 nm. Although the CPT incorporation amounts seem to be higher than those obtained by the dialysis method, incorporation to polymeric micelles was considered unsuccessful due to the presence of the larger aggregates. Sonication did not improve the CPT incorporation in this emulsion method. As shown in Table III, only a small increase in the incorporated CPT amount by sonication was seen for PEG–PBLA 5-27 (comparison between run 1 and 11 in Table III). The large aggregate was also present in the sonicated sample and the base line of UV–VIS spectrum was also high (data not shown). Even when incorporation conditions were varied in CH_2Cl_2 volume (from 0.25 to 1.0 ml), organic solvent ($CHCl_3$), CPT amount (250 μg) and aqueous medium (containing NaCl or acetic acid), the CPT incorporation behavior was not improved (data not shown). As a result, the CPT incorporation to polymeric micelles was not successful by the emulsion method.

TABLE III CPT incorporation to polymeric micelles by emulsion method

Run	Polymer	Incorporation conditions	Yield of CPT/ μg (%)*
1	PEG–PBLA (5-27)	Standard†	130 (26)
2	PEG–PBLA (5-27)	CH_2Cl_2 1.00 ml‡	151 (30)
3	PEG–PBLA (12-27)	Standard	94 (19)
4	Benzyl-25	Standard	81 (16)
5	Benzyl-61	Standard	184 (36)
6	Benzyl-69	Standard	68 (14)
7	n-Butyl-47	CH_2Cl_2 1.00 ml‡	84 (17)
8	n-Butyl-66	Standard	112 (23)
9	Lauryl-45	Standard	85 (17)
10	Methylnaphtyl-53	Standard	113 (23)
11	PEG–PBLA (5-27)	Sonication¶	186 (37)

* Estimated by (absorbance at 370 nm) – (absorbance at 600 nm) in water using $\varepsilon = 19,900$.
† Polymer 5.0 ± 0.1 mg, CPT 0.50 mg, CH_2Cl_2 0.50 ml, distilled water 2.0 ml.
‡ Only volume of CH_2Cl_2 was different from the standard conditions.
¶ Incorporation conditions were the same as those of standard except sonication.

(3) Evaporation Method

CPT incorporation was examined with PEG–PBLA 12-26 by an evaporation method. After compete evaporation of chloroform from the mixed solution of a block copolymer and CPT, 2.0 ml of distilled water was added and the solution was sonicated. The obtained solutions were revealed to contain large amounts of CPT after filtration through a 5A filter. As summarized in Table IV, the CPT yield determined by absorption in water (this assay method was the same as done in Table III) reached approximately 60% for run 1 and 2 with initial 0.25 and 0.50 mg CPT, respectively. These solutions were very transparent as confirmed by low background from 430 to 600 nm in UV–VIS absorption spectrum as shown in Fig. 3(c). An addition of n-hexane or CH3CN to chloroform did not change the CPT yield so much for PEG–PBLA 12-26. In order to measure the incorporated CPT amounts correctly, UV–VIS absorption spectra were measured in a mixture of DMSO and distilled water (DMSO/water = 9 : 1), since CPT was considered to be completely released from the micelle inner core in this mixed solvent. Absorption coefficient of CPT was obtained in this mixture. When the CPT amount was determined in this mixed solvent, it was found that CPT was incorporated quantitatively in polymeric micelles in a range of experimental error. This shows the evaporation method was much better method than the dialysis and emulsion methods for CPT incorporation to the polymeric micelles and that CPT was quantitatively incorporated by this method.

TABLE IV CPT incorporation to PEG–PBLA (12-26) polymeric micelles by evaporation method*

Run	CPT amount (mg)	Solvent	Yield of CPT/ μg (%) Method A†	Method B‡
1	0.25	$CHCl_3$ 0.25 ml	146 (58)	276 (109)
2	0.50	$CHCl_3$ 0.5 ml	297 (60)	544 (109)
3	0.51	$CHCl_3$ 0.5 ml + n-hexane 0.10 ml	350 (69)	540 (107)
4	0.50	$CHCl_3$ 0.5 ml + CH_3CN 0.5 ml	281 (56)	536 (108)

*Polymer 5.0 ± 0.1 mg.
†Method A: measured in distilled water and estimated by (absorbance at 370 nm) − (absorbance at 600 nm) using $\varepsilon = 19,900$.
‡Method B; measured in distilled DMSO/distilled water = 9:1 and estimated by (absorbance at 365 nm) − (absorbance at 600 nm) using $\varepsilon = 20,860$.

Characterization of Polymeric Micelles Incorporating CPT

Camptotecin (CPT) was incorporated to polymeric micelles forming from various block copolymers by the evaporation method and the obtained polymeric micelles were characterized. As summarized in Table V, 11 block copolymers were examined. All the polymers gave clear solutions after sonication. The CPT yields, however, were lower than those in Table IV due to a difference in filtration/centrifugation procedures. All the samples in Table V were measured spectroscopically after centrifugation and filtration through 1 μm filter. This more complete procedure to remove larger aggregates was considered to lower the CPT yield. The CPT yields were relatively low for methylnaphtyl–substituted block copolymer (methylnaphtyl-44) and low substitution contents of benzyl ester cases (benzyl-25, -44 and -61).

These obtained polymeric micelles containing CPT were characterized by two methods; gel-permeation chromatography (GPC) and laser light scattering. Several GPC charts are shown in Fig. 4 and their numerical results are summarized in Table V. All the samples were observed to form polymeric micelle structures by micelle peaks near the gel-exclusion volume that was much smaller than those corresponding molecular weights of the block copolymers (approximately 10,000–20,000). Micelle-forming behavior, however, turned out to be dependent on block copolymer structures by different peak area and elution volume. Peaks at the micelle position were very small for PEG–PBLA 5-27 and 12-26. The peak was detected by absorption at 341 nm, indicating that peak area stands for the incorporated CPT amount. These two PEG–PBLA block copolymers provided very small values of ratios of peak area / [CPT] of the injected sample such as 3.5 and 1.5. This means that most CPT was adsorbed to the GPC column by hydrophobic interactions due to unstable packaging of CPT in the micelles. The benzyl-25 micelles provided a little larger ratio of the peak area/[CPT], 6.1, however, peaks of later elution volume (5.15 and 6.57 ml) were accompanied. It is considered that a low content of benzyl ester resulted in loose packaging of the hydrophobic inner core and that such loosely packed inner core allowed hydrophobic interactions with the column to a considerable degree. By these interactions, elution could be delayed. A weight-average diameter of benzyl-25 was 389 nm, indicating the substantial presence of large aggregates. As the benzyl content increased, micelle peaks became larger and their elution became earlier. For benzyl-61, the elution volume was 3.62 ml and its ratio of peak area/[CPT] was 49.0. This ratio was also high for benzyl-69. Diameter of benzyl-61 and benzyl-69 micelles were 35 and 64 nm, respectively. These diameters

TABLE V CPT incorporation to polymeric micelles by evaporation method

Run	Polymer	CPT amount (mg)	(mg)	Solvent†	Yield of CPT/μg (%)‡	GPC analysis* Elution volume (mL)	Peak area¶ [CPT]	Micelle diameter§ (nm)
1	PEG–PBLA (5-27)	7.6	0.75	$CHCl_3$	507 (68)	3.33	1.5	80
2	PEG–PBLA (12-27)	7.5	0.75	$CHCl_3$	548 (73)	3.38	3.5	n.d.‖
3	Benzyl-25	7.5	0.77	$CHCl_3$	285 (37)	3.30,5.15	6.1,16.9	389
4	Benzyl-44	7.6	0.75	$CHCl_3$	154 (21)	3.60,4.78	23.0,15.8	n.d.‖
5	Benzyl-61	7.6	0.75	$CHCl_3$	275 (37)	3.62,4.30	49.0,38.1	35
6	Benzyl-69	7.5	0.75	$CHCl_3$	420 (56)	3.42	47.6	64
7	n-butyl-47	7.4	0.75	$CHCl_3$	464 (62)	3.30,4.42	14.7,3.6	>1000
8	n-butyl-66	7.6	0.75	$CHCl_3$	498 (66)	3.60,4.35	17.6,5.2	n.d.‖
9	Lauryl-45	7.5	0.75	$CHCl_3$	550 (74)	4.02	11.2	n.d.‖
10	Methylnaphtyl-53	7.6	0.75	$CHCl_3$	338 (45)	3.53	49.9	n.d.‖
11	Methylnaphtyl-64	7.4	0.75	$CHCl_3$	394 (52)	3.63	54.3	39
12	Methylnaphtyl-64	7.5	0.75	$CHCl_3 + CH_3CN$	570 (76)	3.67	59.4	n.d.‖
13	Methylnaphtyl-64	7.5	1.50	$CHCl_3$	734 (49)	3.62	50.7	n.d.‖
14	Methylnaphtyl-64	7.6	1.50	$CHCl_3 + CH_3CN$	1079 (72)	3.68	56.7	n.d.‖

*Column: tosoh TSKgel G3000PWXL, eluent: distilled water, temp.: 40°C
†$CHCl_3$: 1.0 ml per 1.0 mg of CPT, CH_3CN: 0.67 ml per 1.0 mg of CPT
‡ Measured in DMSO/distilled water = 9:1 and estimated by (absorbance at 365 nm) − (absorbance at 600 nm) using $\varepsilon = 20,860$.
¶A ratio of peak area/concentration of CPT (μg/ml).
§Weight-average diameter measured by dynamic light scattering.
‖n.d.: not determined.

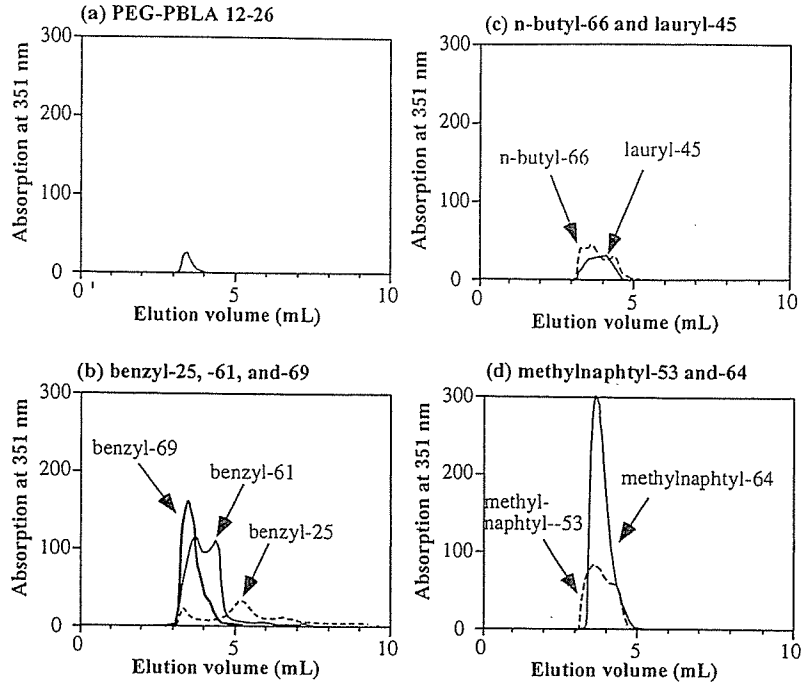

FIGURE 4 Gel-filtration chromatograms of polymeric micelles incorporating CPT. (a) PEG–PBLA 12-26 (run 2 of Table V), (b) benzyl-25, -61 and -69 (run 3, 5 and 6 of Table V, respectively) (c) n-butyl-66 and lauryl-45 (run 8 and 9 of Table V, respectively), (d) metylnaphtyl-53 and-64 (run 10 and 12 of Table V, respectively).

were typical values of polymeric micelles. This shows that a quantity of hydrophobic component was essential for stable CPT incorporation and particularly, benzyl-61 and benzyl-69 block copolymer micelles stably incorporated CPT without forming larger aggregates.

n-Butyl ester substituted block copolymers did not show as stable micelle formation as benzyl ester substituted block copolymers as summarized in Table V. For n-butyl-47, a micelle peak at 3.30 ml was accompanied by the second peak at a later elution volume (4.42 ml) and its peak area/[CPT] ratio value of the first micelle peak was small (14.7). Weight-average diameter was larger than 1000 nm due to large aggregate formation caused by CPT incorporation. This poor micelle stability was not improved by increasing butyl content up to 66% as shown in Table V and Fig. 4(c). Lauryl-45 brought about a high CPT yield such as 73%, however, micelle stability was found to be low by a little delayed elution at 4.02 ml and a small value of the peak area/[CPT] ratio.

In contrast to n-butyl and lauryl cases, methylnaphtyl-substituted block copolymers methylnaphtyl-53 and -64 showed high micelle stability upon the CPT incorporation even though these CPT yields were lower than those of n-butyl-47, n-butyl-66 and lauryl-45. Particle size of run 11 of Table V for metylnaphtyl-64 was 39 nm in diameter that was a typical value of polymeric micelles without larger aggregates. For this polymer, the CPT yield and the peak area/[CPT] ratio was found to increase by adding CH_3CN in the polymer-CPT mixture for both the CPT/polymer ratios (10 and 20 wt.%). Figure 4(d) shows two gel-filtration charts of run 10 and run 12 of Table II. Both these runs brought about typical micelle peaks around 3.6 ml in elution volume. However, run 12 brought about a sharper peak than run 10 which was accompanied by a shoulder around 4.3 ml. In run 14, CPT content was calculated at 13 wt.% in the polymeric micelle assuming no polymer was lost during the incorporation process. Such a high content indicates successful incorporation of water-insoluble drug to polymeric micelles with maintaining good water solubility.

It is known that biological activity of CPT is completely dependent on its forms; the intact lactone form shows cytotoxic activity while the other water-soluble open form does not. It is also known that the lactone forms exists in a pH-dependent equilibrium with the open carboxylate form and that most CPT exists in the inactive open form in human plasma (Burke and Mi, 1994). Therefore, it is preferable to incorporate CPT in the lactone form. By reversed-phase HPLC, 97 and 95% of the incorporated CPT was found to exist in the active lactone form for benzyl-69 (run 6 in Table V) and n-butyl-66 (run 8 in Table V), respectively. The hydrophobic atmosphere of the inner core is considered to contribute to keep CPT in the more hydrophobic lactone form.

DISCUSSION

Poly(ethylene glycol)–poly(aspartate) block copolymers were prepared with corresponding halides and DBU as catalyst. Ono *et al.* (1978) and Rao (1980) reported this esterification reaction for preparations of low molecular weight esters and this method was successfully applied to block polymer syntheses in this paper. For ester syntheses, condensation reaction between carboxyl and

hydroxyl groups with carbodiimides as catalyst is the most common way. However, an anhydride structure forming from two carboxyl groups is the active species reacting with a hydroxyl group to form an ester. Therefore, this condensation reaction is not considered to proceed for obtaining high degree of esterification of polymers because the anhydride formation becomes more difficult due to restricted freedom of the polymeric carboxyl groups as the esterification proceeds. In contrast, the esterification using halides and DBU shown in Fig. 1 is considered preferable for high degree of esterification of polymers, since the active species for esterification is carboxy anion ($-COO^-$) formed by interaction with low molecular weight compound DBU and this anion has nucleophilic attack on a halide to result in ester formation. In this reaction mechanism, intra or inter polymer reaction is not included and therefore, it is considered that high degree of esterification for polymeric carboxyl groups is possible by this synthetic method. In fact, a high esterification value, 69%, was obtained for benzyl ester. By optimizing reaction conditions such as temperature and solvent, a higher esterification degree may be obtained for the benzyl case and it is expected that various hydrophobic groups are conjugated to (PEG–P(Asp)) block copolymer to a considerably high substitution degree. This allows large freedom for block copolymer structures in order to optimize targeting of various kinds of hydrophobic anticancer drugs as well as for establishing strategy of drug incorporation to polymeric micelles.

Incorporation methods are expected to be very influential on incorporation behaviors of hydrophobic drug to polymeric micelles. However, little has been known concerning this influence. For doxorubicin incorporation to a block copolymer micelle with poly(D,L-alctide) inner core, an optimum water/organic solvent ratio was reported to be present as the starting medium of dialysis (Kohori *et al.*, 2002). This study revealed that incorporation method was very influential on incorporation behavior of CPT to polymeric micelles. In fact, choice of the incorporation method was critical for obtaining polymeric micelles containing CPT in a high yield of CPT and with high clarity of the solution. Lavasanifar *et al.* (2001) reported successful incorporation of amphotericin B to polymeric micelles by an evaporation method. By the evaporation method, polymeric micelles containing CPT was successfully obtained with considerably high CPT content such as 13 wt%. More detailed study, however, is required to elucidate the nature of drug incorporation to polymeric micelles and to predict an appropriate method for a new drug case. A hint for this purpose is present in an addition effect of CH_3CN in solvent of the evaporation method. In this incorporation method, solubility of drug and each block as well as interaction between drug and each block is considered to be a crucial role in the incorporation. Since many kinds of solvent can be examined as this additive, there is large freedom for optimization of the drug incorporation and also for analysis of incorporation mechanism.

In our previous report, a slight difference in micelle diameter was observed between two block copolymers with different hydrophobic block for water-insoluble drug incorporation (Yokoyama *et al.*, 1998). In this paper, the CPT incorporation behavior was analyzed with systematically varying both chemical structure and content of hydrophobic ester groups of PEG–poly(aspartate) block copolymers. Substantial effects of the chemical structure and the content on the incorporation stability were found. An interesting finding was that stability of the CPT incorporation was not attained simply by hydrophobicity of the block copolymers. In stability assay by the GPC, PEG–PBLA micelle provided much lower incorporation stability than benzyl-69 block copolymer. Since all the carboxyl groups are benzylated for PEG–PBLA, hydrophobicity of PEG–PBLA is higher than benzyl-69 block copolymer. PEG-PBLA could be handled as benzyl-100, since all the aspartic acid residues were benzylated. However, PEG-PBLA provided much lower CPT incorporation stability than benzyl-69 as judged by the peak/[CPT] ratio. Possible reasons for this inversed relation between benzyl content and the incorporation stability, are the following two.

(1) Difference in polymer main chain structure. Three-quarters of the amino acid units of the benzyl-25, -44, -61, and -69 were β-amide units due to an alkaline hydrolysis procedure in their syntheses, while PEG-PBLA was composed only of α-amide units. A β-amide unit is longer than an α-amide unit. Additionally, configuration of the benzyl-25, -44, -61, and -69 was almost racemic due to the alkaline hydrolysis procedure, while the amino acid units of PEG-PBLA is composed only of L-configuration. The β-amide units in racemic configuration could be advantageous for stronger interactions with CPT molecules.

(2) The aspartic acid residue may work for a good insertion site of CPT molecules. The aspartic acid is hydrophilic, therefore, cannot interact with CPT molecule by hydrophobic interactions as strongly as the benzyl aspartate unit. However, due to the lack of benzyl moiety, CPT molecule may be more accessible to the poly (aspartate) main chain than the benzyl aspartate unit because of the absence of possible steric hindrance of the benzyl group. If this inserted CPT molecule interacts well with adjacent benzyl groups by hydrophobic interactions, the inserted molecule may be more stably interacted with polymers than PEG-PBLA; a case that does not allow CPT to have an access to the main chain.

This fact implies that location of CPT molecules (e.g., deeply inserted to main chain or present between two polymer chain) and other interactions than hydrophobic interactions can contribute to stable CPT incorporation. It is of great interest to analyze detailed factors for stable micelle incorporation of hydrophobic drugs. Furthermore, in comparison between n-butyl-47 and lauryl-45, n-butyl-47 showed higher stability both in earlier elution and

bigger ratio of peak area/CPT concentration ratio. Lauryl-45 block copolymer is more hydrophobic with longer acyl chain (C_{12}) than n-butyl case (C_4). This implies that rigidity of the hydrophobic inner core is a key for stable CPT incorporation since a longer acyl chain is more flexible. The benzyl and methylnaphtyl block copolymers at high esterification degree provided high CPT incorporation stability shown by a sharp and big peak at a small elution volume. This suggests large contribution of $\pi-\pi$ interaction between aromatic groups of CPT molecules and phenyl/naphtyl groups of these block copolymers.

It was reported that the hydrophobic inner core worked as a good reservoir of drug by inhibiting drug inactivation reactions of adriamycin (Yokoyama *et al.*, 1990). Incorporation of CPT in the hydrophobic inner core is a bigger advantage of polymeric micelles for effective CPT delivery, since the hydrophobic atmosphere may work to keep CPT in the active lactone form at a high concentration for a long time period.

In vivo micelle stability, long-circulation properties and drug release behavior are important for CPT tumor targeting. It is also important to obtain correlations between these behaviors and physico-chemical properties evaluated in this paper. This paper opens a strategic way to optimize tumor targeting of anti-cancer drug by systematically changing chemical structures of the hydrophobic blocks for the micelle inner core.

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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.

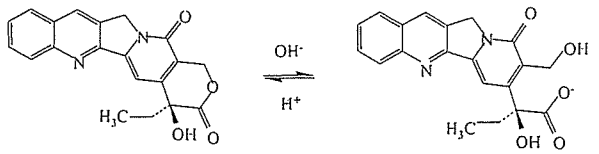

Fig. 1. Chemical structure of camptothecin.

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, WI, 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

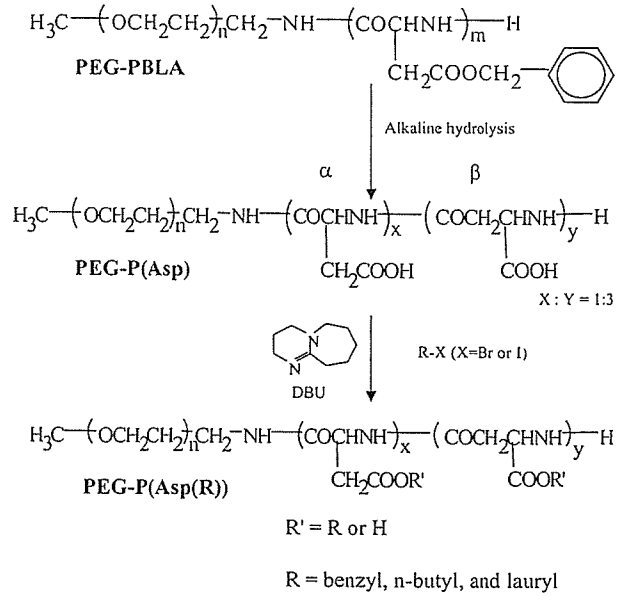

Fig. 2. Synthesis of esterified block copolymer PEG-P(Asp(R)) consisting of hydrophilic PEG and hydrophobic P(Asp(R)) from poly(ethylene glycol)-poly(aspartic acid) block copolymer (PEG-P(Asp)).

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

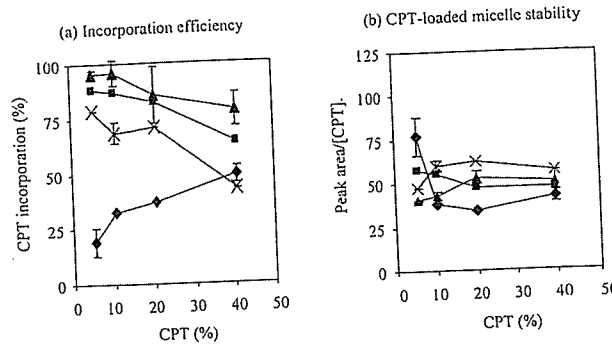

Fig. 4. Effect of benzyl ester contents of PEG-P(Asp) block copolymers on the (a) CPT incorporation efficiency and (b) the micelles stability by ratio of peak area/CPT concentration (♦, 5-27 Bz-44; ■, 5-27 Bz-57; ▲, 5-27 Bz-69; X, 5-27 Bz-75). Data are plotted in the mean ± SD of two measurements for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

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.

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

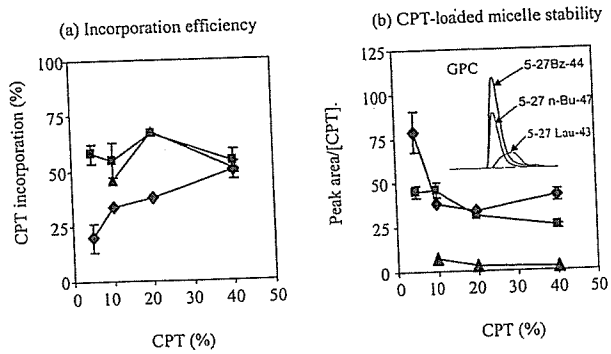

Fig. 3. Effect of esterified groups of PEG-P(Asp) block copolymers on the (a) CPT incorporation efficiency and (b) the CPT-loaded micelles stability by using the ratio of peak area/CPT concentration (♦, 5-27 Bz-44; ■, 5-27 n-Bu-47; ▲, 5-27 Lau-43). Data are plotted in the mean ± SD of two measurements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

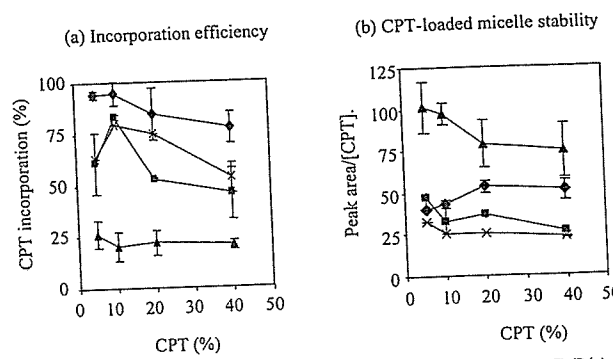

Fig. 5. Effect of chain lengths of benzyl esterification of PEG-P(Asp) block copolymers on the (a) CPT incorporation efficiency and (b) the micelles stability by ratio of peak area/CPT concentration (♦, 5-27 Bz-69; ■, 12-26 Bz-64; ▲, 5-52 Bz-67; X, 12-50 Bz-63). Data are plotted in the mean ± SD of two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

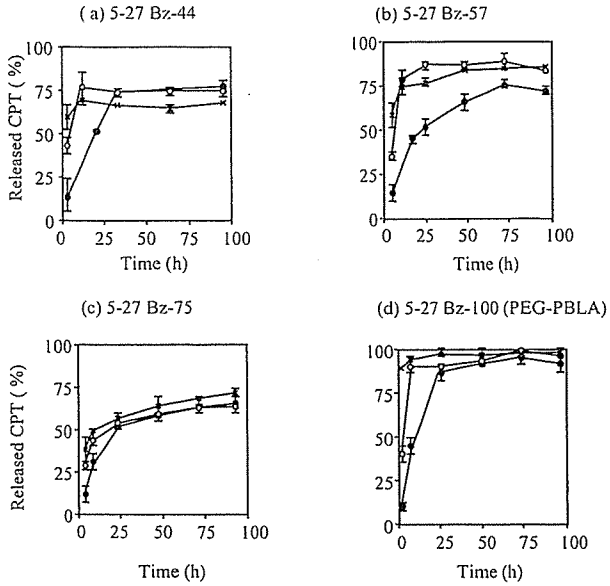

Fig. 6. Effect of benzyl esterification contents of PEG-P(Asp) block copolymers on the CPT release from the micelles forming from (a) 5-27 Bz-44, (b) 5-27 Bz-57, (c) 5-27 Bz-75, and (d) 5-27 Bz-100 or 5-27 PEG-PBLA at the initial CPT-loaded micelles concentration of 7 μg/ml (X); 70 μg/ml (O); 300–700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

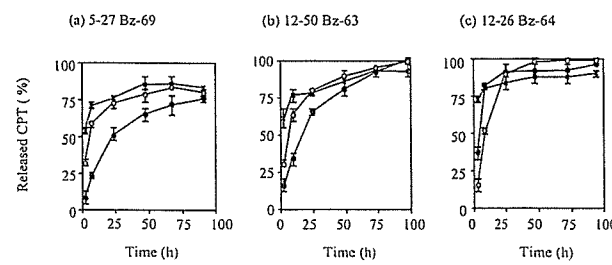

Fig. 7. Effect of chain lengths of benzyl-esterified PEG-P(Asp) block copolymers on the CPT release from the micelles forming from (a) 5-27 Bz-69, (b) 12-50 Bz-63, and (c) 12-26 Bz-64 at the initial CPT-loaded micelles concentration of 7 μg/ml (X); 70 μg/ml (O); 300–700 μg/ml (●). Data are plotted in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

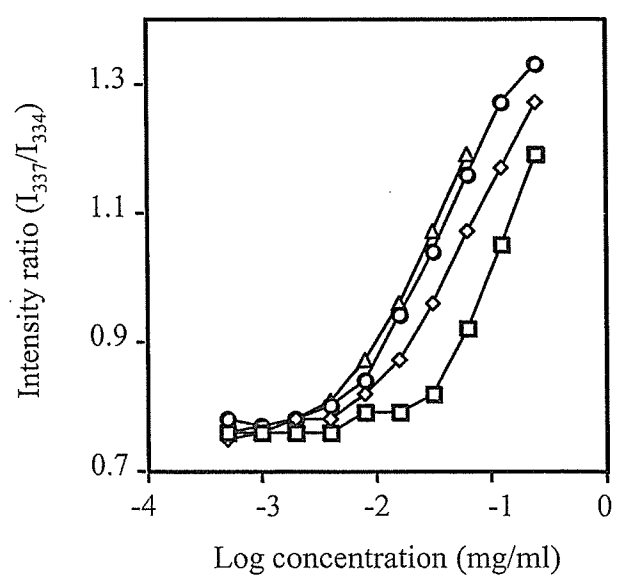

Fig. 8. I_{337}/I_{334} band intensity ratio of pyrene as a function of logarithm concentration of micelles forming from (□) 5-27 Bz-44, (◇) 5-27 Bz-57; (O) 5-27 Bz-75, and (Δ) 5-27 Bz-100. Data are plotted in the mean ± SD of two measurements.

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.

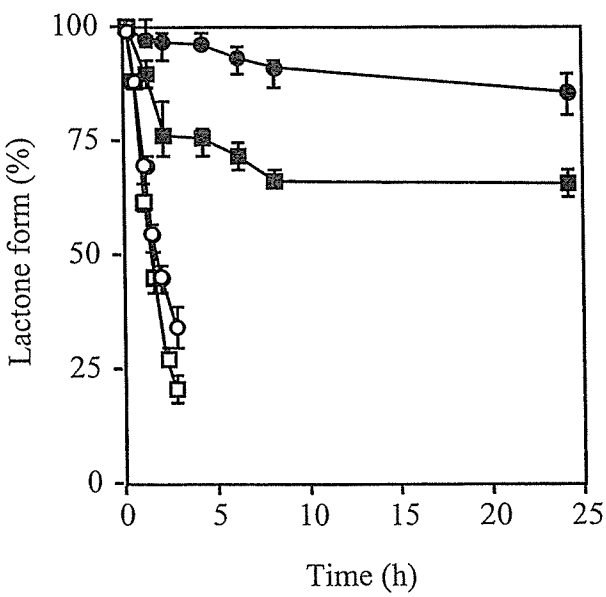

Fig. 9. Stability of CPT-lactone form of free drug and CPT loading in the polymeric micelles forming from 5-27 Bz-69 block copolymers vs. rapid hydrolysis in simulated physiologic environment. Free drug (10 μg/ml) incubated with PBS buffer pH 7.4 (○) and serum (□); and CPT-loaded in micelles (75 μg/ml)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

ACKNOWLEDGMENTS

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