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What are determining factors for stable drug incorporation into polymeric micelle carriers? Consideration on physical and chemical characters of the micelle inner core

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Abstract

Partially benzyl-esterified poly(ethylene glycol)-*b*-poly(aspartic acid) (PEG-P(Asp(Bzl))) having different hydrophobic inner-core structure were synthesized and analyzed. We obtained two types of the block copolymers for formation of polymeric micelle drug carriers; one had an amide-bond ratio of 1:3 (α/β) in the poly(aspartic acid) residues through alkaline hydrolysis, and the other one had 100% of the α-amide through acid hydrolysis. Subsequently, we prepared partially benzyl-esterified block copolymers with an esterification degree of 40 to 100% in the aspartic acid residue. Regarding camptothecin (CPT) incorporation into polymeric micelles, we evaluated effects that block copolymers' inner hydrophobic block structures have on CPT behavior. Regarding CPT-incorporation stability, PEG-P(α,β-Asp(Bzl) block copolymers with the α and β-amides were found to exhibit higher CPT-incorporation stability. Using fluorescent probes, we evaluated the properties of inner-core blocks such as hydrophobicity and mobility/rigidity, and the findings implied that stable CPT incorporation could be obtained by an adequate balance between the micelle inner core's hydrophobicity and the micelle inner core's rigidity or between the micelle inner core's hydrophobicity and steric configuration of the hydrophobic block chain.

Keywords: Polymeric micelle; Camptothecin; Incorporation; Inner core; Hydrophobicity

1. Introduction

Polymeric micelles have attracted much attention as a nano-sized drug carrier in drug delivery system (DDS) owing to their advantages such as very small size in a range of 10–100 nm and high structural stability [1–4]. The enhanced permeability and retention (EPR) effect [5,6] enables polymeric micelles to deliver various drugs selectively to solid tumor sites. For a successful achievement of this tumor targeting, stable drug incorporation into the inner core of the micelle is very important. In general, for targeting by intravenous injection, the targeting is unsuccessful if the drug-incorporation stability is low. Owing to low incorporation stability, a drug is too quickly released from the carrier, resulting in non-specific supply of drug in the bloodstream. Especially for the EPR mechanism's tumor targeting, stability is essential because effective targeting needs a considerably long period (e.g., 10 to 48 h) in which to function. Recently, a large number of studies on polymeric-micelle carriers have appeared; however, only a limited number of studies succeeded in tumor targeting *in vivo* [7,8]. It is expected that one reason for the unsuccessful targeting is unstable drug incorporation in *in vivo* circumstances, since targeting is unsuccessful if most drug is released before the carrier system reaches the target. The other important factor related to drug incorporation into polymeric micelles is efficiency. High drug-incorporation efficiency is preferable both in basic research and in clinical developments.

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The first successful example of the tumor targeting with a polymeric micelle carrier was adriamycin-incorporated polymeric micelle system [8,9]. In this system, pharmacologically active adriamycin was physically incorporated into the micelle's hydrophobic inner core, which formed from the adriamycin-conjugated poly(aspartic acid) polymer blocks. The stable drug incorporation was achieved by a high concentration of the total adriamycin molecules (both the chemically conjugated ones and the physically entrapped ones) in the micelle inner core. This study suggested that not only hydrophobic interactions but also $\pi-\pi$ interactions were important for the stable incorporation because the adriamycin molecules are rich in π-electrons. This adriamycin system is considered to be in a special case owing to the use of the chemically conjugated drug molecules as the source of cohesive interactions for drug incorporation as well as for micelle formation. In order to develop a more universal methodology that can be applied for various drugs, we have developed polymeric micelle systems that do not use drug molecules as the micelle's hydrophobicity source. For this type of the polymeric micelle system, we have reported polymeric micelles containing KRN-5500 [10] and camptothecin [11–13].

In the previous report, we reported successful incorporation of camptothecin (CPT) into polymeric carriers that did not use CPT molecules as the micelle's hydrophobicity source. CPT is a water-insoluble antitumor chemical whose action mechanism is inhibition of DNA topoisomerase I [14]. Its clinical trial failed because of a strong side effect at the bladder; however its two derivatives (CPT-11 and Topotecan) were approved in 1990s. This fact indicates the high potency of CPT and CPT derivatives as anticancer drugs. If CPT and CPT derivatives can facilitate tumor targeting, a great contribution to the cancer chemotherapy is feasible. We achieved stable drug incorporation, long circulation in the bloodstream, and a considerably high level of tumor targeting by optimizing the inner-core-forming block of the PEG-P(aspartate) block copolymer [11–13]. In this optimized block copolymer, the benzyl group was introduced in ca. seventy molar percent of the aspartic acid residue; the benzyl group functioned as the hydrophobic source of the micelle inner core. With regard to drug targeting, this benzyl ester content (60 to 70%) proved to be the best; both drug-incorporation stability and targeting efficiency were lowered in the case of the lower benzyl content and in the case of the higher (100%) benzyl content. This is very interesting because the more-hydrophobic polymer block (the 100%-benzyl-content case) was unfavorable irrespective of the fact that hydrophobic interactions are a main cohesive interaction working both for micelle formation and for drug incorporation.

In this paper, we analyze the effects that the chemical structures of the inner core-forming hydrophobic block have on CPT incorporation, and we focus on aspects other than hydrophobic interactions. Through this analysis, we will establish a strategy to design block copolymers that enable anticancer drugs to efficiently target tumors. For this purpose, we prepared two types of PEG-P(aspartate) block copolymers; one whose aspartate amide bond was a mixture of α-and β-types and the other one whose amide bond consisted only of the α-type. Using fluorescent probes, we measured properties of the inner core, hydrophobicity, and mobility/rigidity, and we analyzed these results in relation to drug-incorporation efficiency and drug-incorporation stability.

2. Materials and methods

2.1. Materials

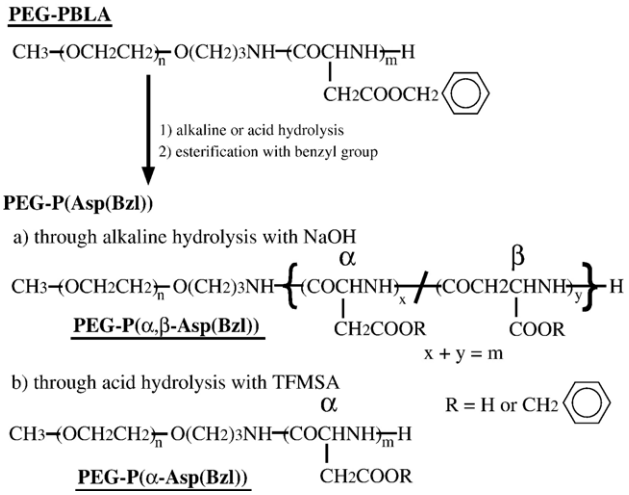
(s)-(+)-Camptothecin was purchased from Aldrich Chem. Co. (Milw., WI, USA). Poly(ethylene glycol)-*b*-poly(β-benzyl L-aspartate) (PEG-PBLA) was synthesized, as reported previously [15]. Trifluoromethanesulfonic acid (TFMSA) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) were purchased from Wako Pure Chemical (Tokyo, Japan). Thioanisole and m-cresol were purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). 1,3-Bis(1-pyrenyl)propane (dipyrene) was purchased from Dojindo Laboratories (Kumamoto, Japan). Other chemicals were of reagent grade.

2.2. Synthesis of partially benzyl-esterified poly(ethylene glycol)-b-poly(aspartic acid) (PEG-P(Asp(Bzl)))

As shown in Scheme 1, we synthesized partially benzyl-esterified poly(ethylene glycol)-*b*-poly(aspartic acid) with two types of the poly(aspartic acid) block. First, we hydrolyzed the poly(ethylene glycol)-*b*-poly(β-benzyl L-aspartate) (PEG-PBLA) by using two methods that brought about two types of the poly(aspartic acid) block in the block polymers: the first method was alkaline hydrolysis, which yielded the α-amide and β-amide mixture type; and the second method was acid hydrolysis, which yielded the α-amide pure type. On the second step, we formed benzyl ester at the aspartic acid residues of the PEG-P(Asp) block copolymers.

2.2.1. Synthesis of poly(ethylene glycol)-b-poly(β-benzyl L-aspartate)(PEG-PBLA)

We synthesized poly(ethylene glycol)-*b*-poly(β-benzyl L-aspartate) (PEG-PBLA) by ring-opening polymerization of β-benzyl L-aspartate *N*-carboxy anhydride (BLA-NCA) from the



Scheme 1. Synthetic scheme of two different amide bond types of block copolymers.

terminal primary amino group of α-methyl-ω-aminopropoxy poly(ethylene glycol), as reported previously [15]. We use a codename indicating a molecular weight of the poly(ethylene glycol) block and the unit number of the poly(amino acid) block. For example, 5-27 PEG-PBLA indicates that the molecular weight of the PEG block is 5000, and the unit number of the Asp residue in the PBLA block is 27.

2.2.2. Synthesis of poly(ethylene glycol)-b-poly(aspartic acid) (PEG-P(Asp))

We removed the benzyl ester protecting group from PEG-PBLA to obtain poly(ethylene glycol)-*b*-poly(aspartic acid) block copolymers. To carry out this debenzylation, we used the following two methods: (1) alkaline hydrolysis with NaOH and (2) acid hydrolysis with TFMSA [16,17]. Conducting a ^{1}H NMR analysis, we observed no benzyl group in the products obtained by either of the two hydrolysis methods.

2.2.2.1. Alkaline hydrolysis of the PEG-PBLA block copolymer. We deprotected the benzyl ester by conducting alkaline hydrolysis that featured NaOH. To 3.97 g of 5–27 PEG-PBLA, we added a 0.5 N NaOH aqueous solution (30.0 mL, 1.5 equivalent mol. with respect to the aspartic acid group). The reaction mixture was stirred at room temperature for 2 h. The solution became transparent during this period. Then, we added 6 N HCl (10 equivalent mol. to the Asp unit) to the solution, which we purified by performing dialysis. To this end, we used a Spectra/Por 6 dialysis membrane (molecular weight cut-off is 1000) in water, followed by lyophilization. We obtained as white powder the poly(ethylene glycol)-*b*-poly(aspartic acid) (PEG-P(Asp)). To determine the ratio between the α-amide and the β-amide of the aspartic acid unit, we used ^{1}H NMR spectroscopy (methine proton of the α-amide at 4.68 ppm and methine proton of the β-amide at 4.47 ppm in D_2O at alkaline pH (adjusted with NaOD) [18].

2.2.2.2. Acid hydrolysis of the PEG-PBLA block copolymer. Alternatively, we subjected PEG-PBLA to acid hydrolysis by using trifluoromethanesulfonic acid (TFMSA) [16,17]. We dissolved PEG-PBLA (3.02 g) in 53 mL of trifluoroacetic acid (TFA), and then, we added 8.9 mL of thioanisole, 8.0 mL of m-cresol, and 6.6 mL of trifluoromethanesulfonic acid (TFMSA) to the solution, which was in an ice bath. The reaction mixture was stirred at 0 °C for 2 h. The color of the reaction mixture became green. To achieve reprecipitation, we poured the solution dropwise into 1500 mL of diethylether at 0 °C. The precipitate was collected and dried. The obtained precipitate was dissolved in dimethyl sulfoxide (DMSO), and the solution was dialyzed (we used a Spectra/Por 6 dialysis membrane, and the molecular weight cut-off was 1000) against water. We then performed lyophilization. To determine the purity of the α-amide form of the Asp unit, we used ^{1}H NMR spectroscopy in the same way as that for the alkaline hydrolysis stated above.

2.3. Esterification of PEG-P(Asp) with benzyl group

For the esterification of the PEG-P(Asp) block copolymer with the benzyl group, we used a nucleophilic-substitution reaction that occurred between benzyl bromide and carboxyl groups of the Asp block, as reported previously [11]. We dissolved PEG-P(Asp) in DMF. Then, we added benzyl bromide and DBU to the solution at various molar ratios both of benzyl bromide and DBU to the Asp unit, as summarized in Table 1. The reaction mixture was stirred at 50 °C for 16 h. The solution was added dropwise to diethylether at 0 °C, and then the precipitate was collected and dried. The obtained precipitate was dissolved in DMSO, and 6 N HCl (a molar equivalent to the molar of DBU) was added to the solution. The solution was dialyzed (a Spectra/Por 6 dialysis membrane was used), molecular weight cut-off was 1000) against water. We then performed lyophilization. To analyze the obtained block copolymers, we used ^{1}H NMR spectroscopy in DMSO-d_6 containing 3 v/v% trifluoroacetic acid. To determine the content of the benzyl group of the polymers, we identified a peak area ratio between the methylene protons of the benzyl group and the methylene protons of the PEG block. Table 1 lists the benzyl ester content of the block copolymers.

2.4. Incorporation of CPT into polymeric micelles

We incorporated the CPT into polymeric micelles by performing an evaporation method as reported previously

Table 1
Esterification conditions of PEG-P(Asp) block copolymers

Code	In feed						Obtained	
	Block copolymer		Benzyl bromide		DBU		Yield (mg)	Benzyl ester content per Asp unit
	Weight (mg)	Asp (mmol)	Weight (mn)	Molar ratio to Asp	Weight (mg)	Molar ratio to Asp		
PEG-P(α,β-Asp(Bzl 84%))	224.2	0.70	239.6	2.00	192.1	1.80	255.6	84%
PEG-P(α,β-Asp(Bzl 61%))	505.5	1.58	270.2	1.00	240.0	1.00	521.2	61%
PEG-P(α,β-Asp(Bzl 50%))	507.9	1.59	217.5	0.80	193.0	0.80	535.5	50%
PEG-PBLA	–	–	–	–	–	–	–	100%
PEG-P(α-Asp(Bzl 63%))	504.6	1.57	269.2	1.00	238.7	1.00	480.5	63%
PEG-P(α-Asp(Bzl 55%))	210.8	0.66	73.2	0.65	65.7	0.65	193.3	55%
PEG-P(α-Asp(Bzl 41%))	219.0	0.68	59.4	0.51	51.8	0.51	193.0	41%

[19]. A block copolymer and CPT were dissolved in a mixture of chloroform and acetonitrile (3:2, v/v) at various CPT-to-polymer weight ratios, then the solution was stirred at ca 40 °C under a nitrogen gas flow. The solvent was completely evaporated, and water (3 mL) was added to the residue. Then, we sonicated the solution by using a probe-type sonicator (vibra-cell VCX-750, Sonics & Materials Inc., Connecticut, USA) at 80 °C for 2 min in a cycle of sonication for 0.5 sec and standby for 1.0 sec. The solution was filtered through a 1 μm filter (Puradisc 25NYL, Whatman, USA). The polymeric micelle incorporating CPT was obtained in this filtrate.

2.5. Characerization of CPT-incorporated polymeric micelles

Our task, now, was to determine the amount of CPT that was incorporated into the polymeric micelles. Using a UV–Vis spectrometer (V-550 UV–Vis spectrometer, Jasco, Tokyo, Japan), we performed an absorbance measurement at 370 nm in a mixture of DMSO and water (9:1, v/v).

We evaluated the incorporation stability of the CPT in the polymeric micelles by performing gel-permeation chromatography, for which we used an HPLC system (LC-2000 series, Jasco, Tokyo, Japan) equipped with a TSK-gel G4000 PW_{XL} column. Water functioned as an eluent at a flow rate of 1.0 mL/min at 40 °C. We detected CPT by measuring absorbance at 351 nm [11]. We evaluated the stability by using a peak area of the CPT-incorporated micelle. We obtained a ratio of the CPT peak area/CPT concentration. We judged that the stability was higher when this ratio was larger.

2.6. Evaluation of inner core hydrophobicity and mobility/rigidity

We then set out to determine two things: first, the hydrophobicity of the polymeric micelles' inner core and, second, a critical micelle concentration of block copolymers. To make these determinations, we used fluorescence spectroscopy (FP-6500, Jasco, Tokyo, Japan) and used pyrene as a fluorescent probe. We used the evaporation method to prepare the polymeric micelles, and concentrations of their aqueous solutions varied from 0.03 μg/mL to 5 mg/mL. We added 5 μL of a pyrene solution in acetone to the 4 mL of the polymeric-micelle solution; then, we evaporated the acetone by stirring the solution overnight at room temperature while the concentration of pyrene was 6.0×10^{-7} M. The measured wavelength of emission was fixed at 383 nm, and band widths of both excitation and emission were 5 nm. Excitation spectra were recorded at micelle concentrations ranging from 0.03 μg/mL to 5 mg/mL. To determine the critical micelle concentration (CMC), we plotted the ratios of the excitation spectra's fluorescent intensities at 339 nm and 334 nm (I_{339}/I_{334}). This fluorescent intensity ratio was also used as a parameter indicating the hydrophobicity of the inner core at a high concentration (2 mg/mL) of block copolymers. This concentration was much greater than CMCs of the micelles.

We evaluated the mobility/rigidity of the polymeric micelles' inner core by using fluorescence spectroscopy of 1,3-bis(1-pyrenyl)propane (dipyrene) in the same experimental protocol as that for pyrene. For the fluorescent measurements with dipyrene, a concentration of dipyrene was 2.2×10^{-7} M. The wavelength of excitation was fixed at 333 nm. The band widths of both excitation and emission were 5 nm. From the emission spectra, we measured fluorescent intensity ratios between 480 nm (excimer complex) and 398 nm (pyrene monomer) when a polymer concentration was 2 mg/mL.

3. Results and discussion

3.1. Synthesis of partially benzylated PEG-P(Asp) block copolymers

Poly(ethylene glycol)-*b*-poly(aspartic acid) (PEG-P(Asp)) containing different amide-bonds in the P(Asp) block were successfully synthesized. For structures of copolymers see Scheme 1. We obtained two different PEG-P(Asp) block copolymers through two hydrolysis methods, alkaline hydrolysis and acid hydrolysis.

The alkaline hydrolysis with sodium hydroxide brought about the PEG-P(Asp) block copolymer whose amide-bonds were a mixture of the α-amide and the β-amide. It is known that α-amide bonds of the PBLA block converted to β-amide bonds in an alkaline hydrolysis procedure [18]. We found that the ratio of the α-amide and the β-amide was 25:75 (α/β) by analyzing ^{1}H NMR spectrum in D_2O. We successfully removed the benzyl ester protecting group from 5–27 PEG-PBLA block copolymer. The complete deprotection was proven by the absence of the benzyl proton peaks in a ^{1}H NMR spectrum (data not shown).

We performed the acid hydrolysis on the 5–27 PEG-PBLA block copolymer by using TFMSA. By doing this, we obtained the PEG-P(Asp) block copolymer where the PBLA block completely retained the α-amide structure. We obtained the PEG-P(Asp) block copolymer whose amide bonds were composed of 100% of the α-amide. We confirmed this α-amide purity by measuring a ^{1}H NMR spectrum in D_2O.

We performed partial esterification of the copolymers' P(Asp) block. The esterification was successfully achieved for both the two types of PEG-P(Asp) block copolymers obtained through the alkaline hydrolysis and the acid hydrolysis. We successfully controlled the content of the P(Asp) block's benzyl group. The benzyl group content ranged from 40 to 80%. To control the benzyl group content, we varied molar ratios both of benzyl bromide and of DBU to the Asp units. Consequently, we obtained two types of block copolymers having different amide-bonds, PEG-P(α,β-Asp(Bzl)) and PEG-P(α-Asp(Bzl)). To determine the contents of the block copolymers' benzyl group, we used ^{1}H NMR spectroscopy by using DMSO-d_6 containing 3 v/v% trifluoroacetic acid as a solvent. Table 1 summarizes these results of the block copolymer syntheses.

3.2. Incorporation efficiency and stability of camptothecin (CPT) into polymeric micelles

We incorporated camptothecin (CPT) into polymeric micelles. To do this, we used an evaporation method and varied the weight ratios of CPT to the block copolymers (5, 10, 20 and

40 wt.%). Fig. 1a shows plots of CPT incorporation efficiency against CPT/polymer weight ratios. With the exception of a few plot points, all block copolymers clearly exhibited a reduction in the incorporation efficiency where there was an increase in the CPT/polymer ratio. This behavior may be due to the capacity limit of the hydrophobic inner core for drug incorporation. Even with this capacity limit, the micelle's drug contents were very high. For PEG-P(α-Asp(Bzl 55%)) at 40 wt.% of the CPT/polymer ratio, the drug content in the micelle was 25 wt.%. This value is high, and we calculated it by assuming that there was no loss of the block copolymer throughout the incorporation process. When we examined differences of the incorporation efficiency between the α and β-amide mixture type and the α-amide pure type, we observed no clear tendency. Fig. 1b illustrates this observation by showing the plots of the 10 wt.% of the CPT/polymer ratio.

In contrast, we observed pronounced and interesting differences in incorporation-stability between the two types of block copolymers. Fig. 2a shows the CPT-incorporation stability evaluated by using gel-permeation chromatography (GPC). In this aqueous GPC analysis, the stably incorporated CPT in the polymeric micelles can elute at a gel-exclusion

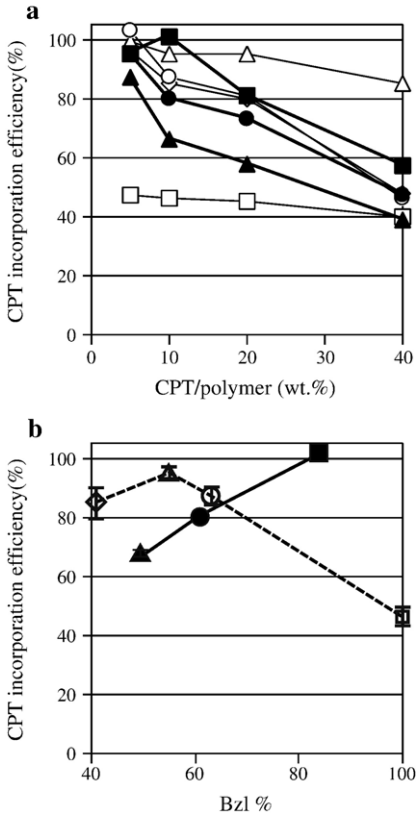


Fig. 1. Incorporation efficiency of CPT-incorporated polymeric micelles. Polymeric micelles form from α-amide and β-amide mixture type block copolymer, PEG-P(α,β-Asp(Bzl)) (■: Bzl 84%, ●: Bzl 61%, ▲: Bzl 50%) and α-amide type block copolymer, PEG-P(α-Asp(Bzl)) (□: Bzl 100% (PEG-PBLA), ○: Bzl 63%, △: Bzl 55%, ◇: Bzl 41%). Incorporation efficiencies are plotted as a function of a) weight ratios of CPT to the block copolymers in feed and b) benzyl (Bzl) contents at 10 wt.% of the CPT weight ratio to the block copolymer. Solid line: PEG-P(α,β-Asp(Bzl)), and dotted line: PEG-P(α-Asp(Bzl)). Data are plotted by the mean ± standard deviation of two experiments (b).

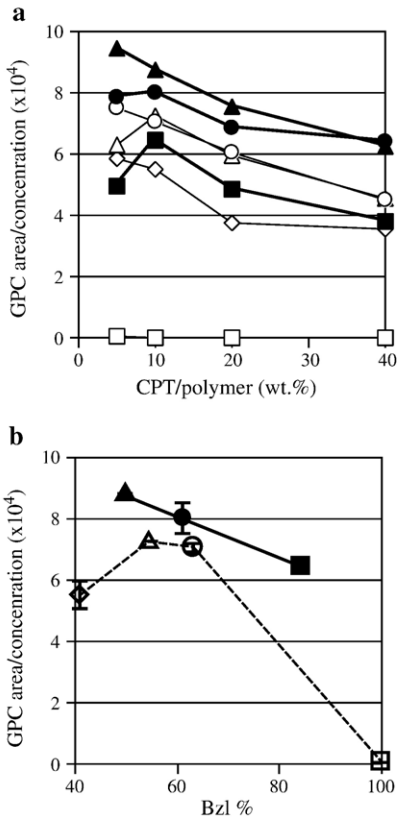


Fig. 2. Incorporation stability of CPT-incorporated polymeric micelles. Polymeric micelles form from α-amide and β-amide mixture type block copolymer, PEG-P(α,β-Asp(Bzl)) (■: Bzl 84%, ●: Bzl 61%, ▲: Bzl 50%) and α-amide type block copolymer, PEG-P(α-Asp(Bzl)) (□: Bzl 100% (PEG-PBLA), ○: Bzl 63%, △: Bzl 55%, ◇: Bzl 41%). The stability is evaluated by peak area of CPT-incorporated micelle normalized by the CPT concentration. Data are plotted as a function of a) weight ratios of CPT to the block copolymers, or b) as a function of benzyl (Bzl) contents at 10 wt.% of the CPT to the block copolymer. Solid line: PEG-P(α,β-Asp(Bzl)), and dotted line: PEG-P(α-Asp(Bzl)). Data are plotted by the mean ± standard deviation of two experiments (b).

volume that is the elution position of the micelles while unincorporated CPT and unstably incorporated CPT adhere to the GPC column by undergoing hydrophobic interactions. Therefore, we found that the incorporation stability was higher when there was a higher peak-area/CPT-concentration ratio. In fact, we reported that these ratios' increases closely related to enhancement in *in vivo* anti-cancer activity for an adriamycin-incorporated polymeric micelle system [20,21]. As Fig. 2a shows, the PEG-P (α,β-Asp(Bzl)) block copolymers that featured the α and β-amides exhibited higher CPT incorporation stability than did the corresponding block copolymers that featured the α-amide. PEG-P(α,β-Asp(Bzl 61% and 50%)) exhibited higher GPC-Area/CPT-concentration values than did PEG-P(α-Asp(Bzl 63%, 50% and 41%)) in all the CPT/polymer weight ratios from 5 to 40%. Both α-amide and β-amide are isomers. Therefore, in general, the two isomers do not differ from each other in terms of physical properties such as hydrophobicity. Therefore, the observed stability differences between the two isomer types suggest that steric configuration or physical factors other than hydrophobicity influenced the CPT-incorporation stability. Interestingly, the block copolymers

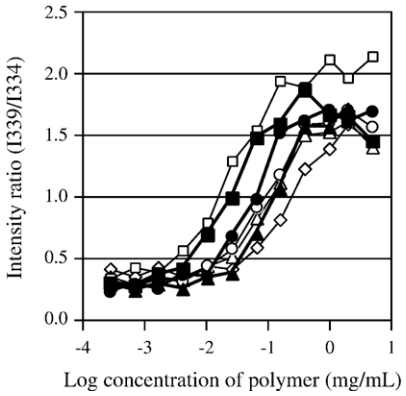


Fig. 3. Fluorescent intensity ratio of I_{339}/I_{334} of pyrene as a function of logarithmic concentrations of block copolymers. The α-amide and β-amide mixture type block copolymer, PEG-P(α,β-Asp(Bzl)) (◆, Bzl 84%; ■, Bzl 61%; ▲, Bzl 50%) and α-amide type block copolymer, PEG-P(α-Asp(Bzl)) (◇, Bzl 100% (PEG-PBLA); □, Bzl 63%; △, Bzl 55%; ○, Bzl 41%) are plotted.

PEG-P(α,β-Asp(Bzl 84%) and PEG-P(α-Asp (Bzl 100%)), which were significantly hydrophobic, exhibited lower CPT-incorporation stability than did the corresponding block copolymers that had lower benzyl (Bzl) content. In particular, PEG-P (α-Asp(Bzl 100%) exhibited quite low stability values, even though this polymer was the most hydrophobic block copolymer. The above-stated behavior is clearer in the plots for 10 wt.% of the CPT/polymer ratio, as shown in Fig. 2b.

These results indicate that the CPT-incorporation stability is governed not only by hydrophobicity but also by other factors such as steric configuration and/or mobility/rigidity of the block polymer, even though hydrophobic interactions are the main adhesive force both for micelle formation and drug incorporation.

3.3. Fluorescent measurements of the polymeric micelle inner cores in relation to stable drug incorporation

We conducted fluorescent measurements of the micelle's inner core. Pyrene and dipyrene were incorporated into the polymeric micelles' inner cores. The purpose of our measurements was to evaluate two inner-core properties: hydrophobicity and mobility/rigidity, by the use of pyrene and dipyrene, respectively.

Pyrene is known to exhibit a peak shift in its excitation spectrum upon its incorporation into a hydrophobic inner-core [22]. Fig. 3 plots the fluorescent intensity ratios of I_{339}/I_{334} in excitation spectra. We determined the critical micelle concentration (CMC) of block copolymers. (Table 2) In two amide-types of block copolymers, PEG-P(α,β-Asp(Bzl) and PEG-P (α-Asp(Bzl)), CMC values gradually increased on a range from 4 μg/mL to 39 μg/mL, as the benzyl content decreased. We examined the most hydrophobic block copolymers in each amide-type block copolymer — PEG-PBLA (=PEG-P(α-Asp (Bzl 100%)) and PEG-P(α,β-Asp(Bzl 84%)). These copolymers' CMC values were the smallest of all: the 4 and 5 μg/mL, respectively.

To evaluate the inner core's hydrophobicity, we measured the fluorescent intensity ratios of I_{339}/I_{334}. These measurements were conducted at 2 mg/mL of the polymer concentration. This polymer concentration was much greater than CMC values of all the polymers. The larger values of the ratios indicate the more-hydrophobic inner cores. The most hydrophobic block copolymer, PEG-PBLA, exhibited a value (2.0) that was slightly higher than those of the others, while the other block copolymers exhibited similar values (1.6–1.7) irrespective of their various benzyl contents. These results imply that small amounts of pyrene probes did not reveal the total potential hydrophobicity of the micelle inner core, probably because pyrene probes were present in small sites where hydrophobic benzyl groups associated in the polymeric micelle core.

We also evaluated the mobility/rigidity of the polymeric micelles' inner core by using a fluorescent probe, dipyrene. Dipyrene forms an intramolecular excimer complex if the atmosphere surrounding the probe has low rigidity [23]. Therefore, when the inner-core mobility increases, the value of fluorescent intensity ratio between excimer complex and monomer increases. Table 2 summarizes fluorescent intensity ratios of I_{480}/I_{398}. All measurements were conducted at 2 mg/mL of the polymer concentration. This polymer concentration was much greater than CMC values of the polymers.

PEG-P(α-Asp(Bzl 100%)), PEG-PBLA, exhibited the lowest I_{480}/I_{398} value, 0.04. This value indicates that PEG-PBLA had the most rigid inner core. This block copolymer, however, did not exhibit stable drug incorporation at all, as shown in Fig. 2. The low stability indicates that PEG-PBLA did not obtain stable drug incorporation simply owing to the micelle inner core's rigidity. The α-amide and β-amide mixture type of block copolymesr, PEG-P(α,β-Asp(Bzl 50, 61 and 84%)), exhibited significantly lower values (0.10 to 0.11) than those (0.22 to 0.41) of the α-amide pure type polymers, PEG-P(α-Asp (Bzl 41%, 50% and 61%)). According to our findings, the former block copolymers that exhibited the greatest drug incorporation stability possessed more rigid inner cores than the corresponding later block copolymers.

Table 2
Evaluation of CMC and inner core properties by the use of fluorescent probes pyrene and dipyrene

Code	CMC (μg/mL)[a]	I339/I334 of pyrene excitation[b]	I480/I398 of dipyrene emission[b]
PEG-P(α,β-Asp (Bzl 84%))	5	1.7	0.10
PEG-P(α,β-Asp (Bzl 61%))	13	1.6	0.11
PEG-P(α,β-Asp (Bzl 50%))	28	1.7	0.10
PEG-PBLA	4	2.0	0.04
PEG-P(α-Asp (Bzl 63%))	15	1.7	0.41
PEG-P(α-Asp (Bzl 55%))	18	1.6	0.23
PEG-P(α-Asp (Bzl 41%))	39	1.6	0.22

[a] Obtained from Fig. 3 plot with pyrene.
[b] At 2 mg/mL of polymer.

The above-stated results imply that stable CPT incorporation derived from an adequate balance between micelle inner cores' hydrophobicity and micelle inner cores' rigidity. Alternatively, steric configuration of the inner core-forming polymer chain would be another factor that controls stability. This is so because the α- and β-amide mixture type block copolymers have one additional alkyl methylene chain (CH_2) in the β-amide unit in comparison with the α-amide chain. Accordingly, the α- and β-amide mixture type block copolymer can sandwich a hydrophobic drug molecule between two benzyl groups in a more favorable and stable manner than the α-amide pure type block copolymer.

In this paper, we reveal that hydrophobicity is not the single factor controlling the CPT-incorporation stability into the micelle inner core. In other words, other factor(s) over hydrophobicity can contribute to CPT-incorporation stability. We could not elucidate the other controlling factor(s), even though both the rigidity of the inner core and the steric configuration of the micelle-forming block's main chain were possible factors responsible for the stable incorporation.

Through its tumor-targeting effect, the adriamycin-incorporated polymeric-micelle system substantially enhanced *in vivo* antitumor activity. Concerning this system, we suggested that a factor other than hydrophobic properties contributed significantly to stable drug incorporation. And concerning the drug incorporation, the adriamycin-system exhibited the following three distinctive features: (1) two types of drug molecules chemically bound to the inner-core-forming polymer and drug molecules physically entrapped in the inner core arose hydrophobic interactions for micelle formation. (2) The drug content (both chemically bound and physically entrapped) determined the stable drug incorporation that resulted in high tumor-targeting efficiency. (3) The drug-content range in which polymeric micelle formed was very wide. In particular, we obtained a micelle that possessed very large adriamycin content (51 wt.%), and we did so without relying on the formation of a water-insoluble precipitate. (For the CPT case of this paper, the hydrophobic origin for micelle formation was not a drug molecule itself). It is well known that adiriamycin molecules preferentially form a non-covalent dimer complex in aqueous media in a similar manner to that of daunorubicin (an adriamycin analogue) [24]. The main contribution to this dimer formation comes from intermolecular π–π interaction. It is widely believed that the π–π interaction can contribute effectively to stable adriamycin incorporation because this interaction works in a specific manner, like ligand-receptor interactions. The specific interaction is expected to work preferentially for drug incorporation without producing precipitates that can be frequently seen in cases of non-specific hydrophobic interactions. As stated above, the study of the adriamycin micelle system suggested the importance of interactions (other than hydrophobic interaction) for the stable drug incorporation into polymeric micelles. In this paper, we pointed out clear evidence for the presence of stable-drug-incorporation factor(s) other than hydrophobic interactions, even though we could not elucidate the other important factor(s).

Elucidation of the factor(s) is a very important aspect of research on polymeric-micelle carrier systems because stable drug incorporation – in other words, very sustained release from a very small drug reservoir – is required for its drug targeting. It is known that the typical diameter of the hydrophobic inner core ranges from 3 to 10 nm [25,26]. In contrast to this very small size, sustained drug release study has concerned micron-sized carriers in the DDS research history. That is why exceptionally stable drug incorporation is required for polymeric micelle cases. In fact, Kwon et al. reported extremely low diffusion constants of drug molecules, such as 2.0×10^{-19} cm^2/s, in their polymeric micelle system [27]. Elucidation of the controlling factors is important for the establishment of a strategic polymer design in the polymeric micelle drug carrier systems.

4. Conclusion

We synthesized two different types of the block copolymer, PEG-P(α,β-Asp(Bzl)) and PEG-P(α-Asp(Bzl)), and we evaluated the behaviors of camptothecin (CPT) incorporation. For CPT incorporation, the block copolymer whose amide-bond type was a mixture of α-amide and β-amide showed higher CPT-incorporation stability. Furthermore, we used fluorescent probes to measure properties of the inner-core block such as the hydrophobicity and the mobility/rigidity of polymeric micelles. Fluorescent-measurement results imply that stable CPT incorporation could arise from either an adequate balance between hydrophobicity and rigidity or an adequate balance between hydrophobicity and the steric configuration of the polymeric micelle inner core. These results emphasize the high degree to which the exact design of the inner-core block is important for stable drug incorporation that is essential to tumor targeting.

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