Analysis of Micelle Formation of an Adriamycin-Conjugated Poly(Ethylene Glycol)–Poly(Aspartic Acid) Block Copolymer by Gel Permeation Chromatography

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Received August 19, 1992; accepted November 26, 1992

The micelle-forming behavior of a drug–block copolymer conjugate {adriamycin-conjugated poly(ethylene glycol)–poly(aspartic acid) block copolymer; PEG-P[Asp(ADR)]} was analyzed by gel permeation chromatography (GPC). Four compositions of the conjugates were observed to form micellar structures in aqueous media, and their micelle-forming behavior was found to be dependent on the composition and media. These micelles did not reach equilibrium within short time periods like low molecular weight surfactants. One composition formed stable micelles in the presence of serum.

KEY WORDS: polymeric micelle; drug delivery system; drug targeting; adriamycin; block copolymer; poly(ethylene glycol).

INTRODUCTION

Synthetic polymers have been studied as carriers for selective drug delivery (1,2); however, the low water solubility of drug-polymer conjugates often causes problems in their synthesis (3–5) and in their injection into the bloodstream (6). Since most drugs are hydrophobic, drug conjugation with a polymer may lead to precipitation.

To circumvent this difficulty in polymeric carriers, a micellar architecture can be utilized. An AB-type block copolymer composed of hydrophilic and hydrophobic components can form a micellar structure as illustrated in Fig. 1. The hydrophobic drug-binding segment forms the hydrophobic core of the micelle, while the hydrophilic segment surrounds this core as a hydrated outer shell. With a core–shell structure, polymeric micelles may maintain their water solubility by inhibiting intermicellar aggregation of the hydrophobic cores irrespective of the high hydrophobicity of the inner cores. Furthermore, the functions that are required for drug carriers can be shared by the structurally separated segments of the block copolymer. The outer shell is responsible for interactions with the biocomponents such as proteins and cells. These interactions may determine pharmacokinetic behavior and biodistribution of drugs. Therefore, *in vivo* delivery of drugs may be controlled by the outer shell independently of the inner core of the micelle.

Until now, few studies were done to focus on the application of polymeric micelles as drug carriers (7,8). We obtained the first successful example of a micelle-forming polymeric drug, adriamycin-conjugated poly(ethylene glycol)–poly(aspartic acid) block copolymer {PEG-P[Asp(ADR)]}[4] with enhanced *in vivo* activity of the drug as well as confirmation of the micellar structure under physiological conditions (9–11). For effective delivery of the micelles, it is important to analyze the relationship between micelle stability and composition of the conjugate. Chain lengths of the constituents in both segments and content of the hydrophobic anticancer drugs determine micelle size and stability. With chemical and physical properties of the segment for the outer shell, these two physicochemical factors (size and stability) may determine biodistribution and pharmacokinetic behavior of the micelles.

In this report, micelle-forming behavior was analyzed by gel permeation chromatography (GPC) with four compositions of PEG-P[Asp(ADR)], two of which were known to form a micellar structure and express *in vivo* anticancer activity (9), and stability of the micelles in the presence of serum was evaluated.

MATERIALS AND METHODS

Materials

Adriamycin hydrochloride (ADR·HCl) was purchased from Sanraku Inc., Yatsushiro, Japan. Other chemicals were of reagent grade and used as purchased. Rabbit serum was prepared from blood taken from the femoral artery of male white rabbits (2.5–4.0 kg).

Synthesis of Drug–Block Copolymer Conjugate

PEG-P[Asp(ADR)] was synthesized by the reported procedure (9). The chemical structure is shown in Fig. 1. ADR was bound to poly(ethylene glycol)–poly(aspartic acid) block copolymer [PEG-P(Asp)] by amide bond formation. Eighty mole percent of aspartic acid residues of the poly(aspartic acid) chain were converted to β-amides (9). The content of ADR in the conjugate was determined by measuring the absorbance at 485 nm in distilled water on the assumption that ϵ_{485} of the ADR residue bound to the polymer was the same as that of free ADR ($\epsilon_{485} = 1.07 \times 10^4$). The ADR content was expressed as mole percent with respect to the aspartic acid residues. Compositions of the conjugates are summarized in Table I. The code for the conjugates is based on the molecular weight of the PEG chain, number of Asp units, and ADR content. For example, 43-17(30) means the

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[4] Abbreviations used: PEG, poly(ethylene glycol); P(Asp), poly(aspartic acid); PEG-P(Asp), poly(ethylene glycol)–poly(aspartic acid) block copolymer; PEG-P[Asp(ADR)], adriamycin-bound poly(ethylene glycol)–poly(aspartic acid) block copolymer; ADR, adriamycin; ADR·HCl, adriamycin hydrochloride; PBS, phosphate-buffered solution; PBSa, phosphate-buffered saline; GPC, gel permeation chromatography.

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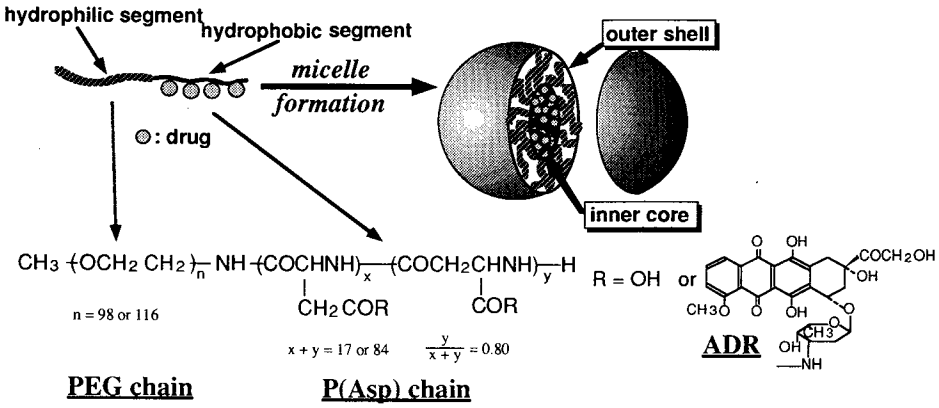

Fig. 1. Concept and chemical structure of micelle-forming polymeric drug.

conjugate with a PEG chain of 4300 MW, 17 Asp units, and 30 mole percent ADR content.

Gel Permeation Chromatography (GPC)

Samples were analyzed using an HPLC system (JASCO Intelligent HyPer LC System, Tokyo) equipped with a 802-SC system controller, two 880-PU pumps, an 880-50 degasser, an 851-AS autosampler, an 860-CO column oven, and an Asahipak GS-520 (50-cm-long) or GS-520H (25-cm-long) column at a flow rate of 1.0 mL/min. The gel-exclusion molecular weight of these columns is 300,000 based on the pullulan standard. Column temperature was 40°C unless otherwise stated. Detection was performed by absorption at 485 nm using an 870-UV UV/VIS detector. Analysis was done after sample incubation in several media at 20°C, 37°C, or room temperature. The media include distilled water, phosphate-buffered solution (PBS; 0.1 *M* + 0.3 *M* NaCl, pH 7.4), phosphate-buffered saline (PBSa; 0.15 *M*, pH 7.4), a 1:1 mixture of distilled water and cell culture medium RPMI 1640 containing 10% fetal calf serum (½ culture medium), and a 1:1 mixture of PBSa and rabbit serum.

Fractionation of the Conjugate with Sephadex LH-20

PEG-P[Asp(ADR)] [14.0 mL, 43-17(30) at 21.2 mg ADR·HCl equiv/mL] was applied to 100 mL of Sephadex LH-20 in methanol. The first fraction (23.7 mL) was collected and transferred into distilled water by evaporation of methanol and gel permeation with Sephadex G-25 (medium) in distilled water, followed by ultrafiltration using a PM-30 membrane (Grace Japan, Tokyo). The yield based on ADR moiety was 38.1%.

Table I. Composition of PEG-P[Asp(ADR)]

Code	MW of PEG	Number of Asp units	ADR content (mol%)[a]
43-17(30)	4300	17	30
43-17(17)	4300	17	17
51-84(34)	5100	84	34
51-84(13)	5100	84	13

[a] With respect to Asp residues.

RESULTS

Micelle formation of PEG-P[Asp(ADR)] was confirmed by GPC. As shown in Fig. 2, conjugate 43-17(30) in PBSa eluted at the gel-exclusion volume (4.2 mL). The molecular

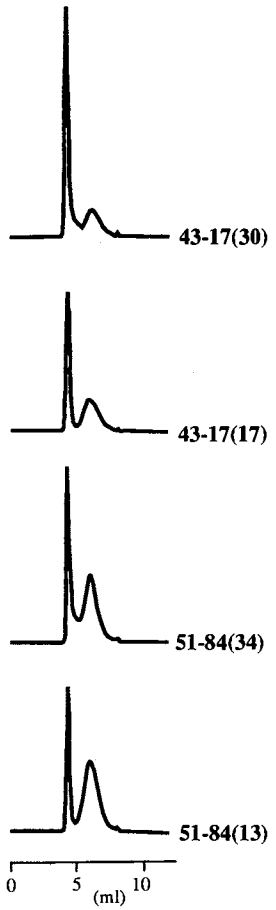

Fig. 2. Gel permeation chromatograms of the four compositions: 43-17(30) at 73.1 μg ADR·HCl equiv/mL, 43-17(17) at 69.0 μg ADR·HCl equiv/mL, 51-84(34) at 69.0 μg ADR·HCl equiv/mL, and 51-84(13) at 71.3 μg ADR·HCl equiv/mL. Column, Asahipak GS-520 H; eluant, 0.1 *M* PBS (pH 7.4, containing 0.3 *M* NaCl); sample, 100 μl of PBSa solution.

weight corresponding to the gel-exclusion volume was much higher than the molecular weight of this conjugate (ca. 9000), indicating micelle formation of the conjugate. The second peak is considered to correspond to single polymer chains (unimers) isolated from the micelles. The addition of 1% (w/v) sodium dodecyl sulfate decreased the area of the first peak and increased that of the second peak. This confirmed that this micelle was constructed by noncovalent interchain interactions. The total peak area of the first and the second peaks accounted for 70% of the injected amount by measuring the peak area obtained without any column; therefore, part of the conjugate was adsorbed onto the column. Micelle formation of this conjugate was observed at the minimum detection level (0.3 μg ADR·HCl equiv/mL; made by dilution of a sample containing 73 μg ADR·HCl equiv/mL and analyzed within several hours after dilution). This concentration corresponds to a 1×10^{-7} M concentration of the polymer chain. Micelle formation at such a low concentration is commonly observed in polymeric micelles (12).

The fractions of the first and second peaks of conjugate 51-84(13) were applied again to the same column immediately after fractionation. As shown in Fig. 3, each fraction was found to elute at the same place as before fractionation. These facts indicate that the peak ratio between the first and the second peaks did not result from an equilibrium achieved within short periods such as several minutes between micellar forms and unimers.

In Fig. 4, the effect of incubation on the GPC chromatogram is shown. When 43-17(30) was subject to gel filtration with Sephadex LH-20 in methanol, the first fraction at the gel-exclusion volume yielded only the micelle peak with an Asahipak GS-520 H. Peak area analysis showed quantitative (98%) recovery of the injected amount, indicating that a micelle fraction with a higher stability was obtained by fractionation. The second peak reappeared upon incubation in PBSa at room temperature within a day, reflecting a very low rate of dissociation of the polymeric micelles. Further, this behavior was affected by the incubation medium. No dissociation of the micelles was observed for the sample incubated in distilled water at 37°C for 2 days, while the second peak appeared upon incubation in PBSa (18% of the total peak area). The incubation in a mixture of distilled water and RPMI 1640 culture medium containing 10% fetal calf serum (1:1) resulted in a larger area (39%) of the second peak.

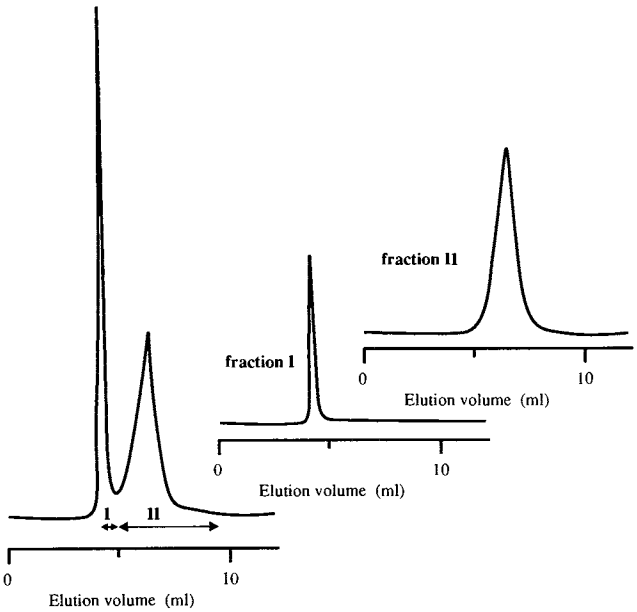

Fig. 3. Fractionation of peaks of 51-84(13). Concentration: 570 μg ADR·HCl equiv/mL. Other chromatography conditions were the same as for Fig. 2.

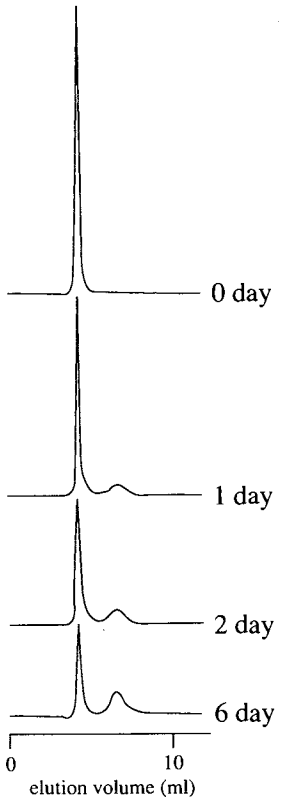

Fig. 4. Dissociation of micellar structure of fractionated 43-17(30) with Sephadex LH-20 in methanol. Incubation at room temperature in PBSa at 11 μg ADR·HCl equiv/mL. Other chromatography conditions were the same as for Fig. 2.

In Fig. 2, chromatograms of the four compositions studied are compared. All samples were applied to the column within a few hours after preparation of the samples from stock solutions at 20 mg ADR·HCl equiv/mL (stock solutions were kept in frozen). Micelle stability of the samples was observed to be strongly dependent on the composition. 43-17(30) provided the largest area of the micelle peak. A decrease in the ADR content resulted in the smaller first peak as seen for 43-17(17). On the other hand, a longer ADR-conjugated P(Asp) chain led to less stable micelles as judged by comparison between 51-84(34) and 43-17(30). A decrease in the ADR content resulted in less stability of the micelle structure also for the conjugate with the longer P(Asp) chains.

Micelle stability was evaluated with an Asahipak GS-520 in the presence of rabbit serum (in a 1:1 mixture of PBSa and rabbit serum) at room temperature with three concentrations. The micelle peak was observed to decrease over

time for all compositions and concentrations, as shown in Fig. 5. The most stable micelle formation was observed for 43-17(30). Even at a low concentration (9.1 μg ADR·HCl equiv/mL), the micelle peak area decreased by only 30% (with respect to the injected amount to the column) from 10 to 378 min. This high stability was significant in comparison with the low stability of conventional liposomes (13). As seen in the chromatograms of the samples in PBSa (Fig. 2), the conjugates with a longer P(Asp) chain having almost the same ADR content (34 mole percent) resulted in less stable micelle formation. Both the conjugates having lower ADR contents (17 and 13 mole percent) showed less stability of the micelles than the conjugates with high ADR contents. For 43-17(30), the concentration had little effect on stability, while the other three compositions became less stable as the concentration decreased.

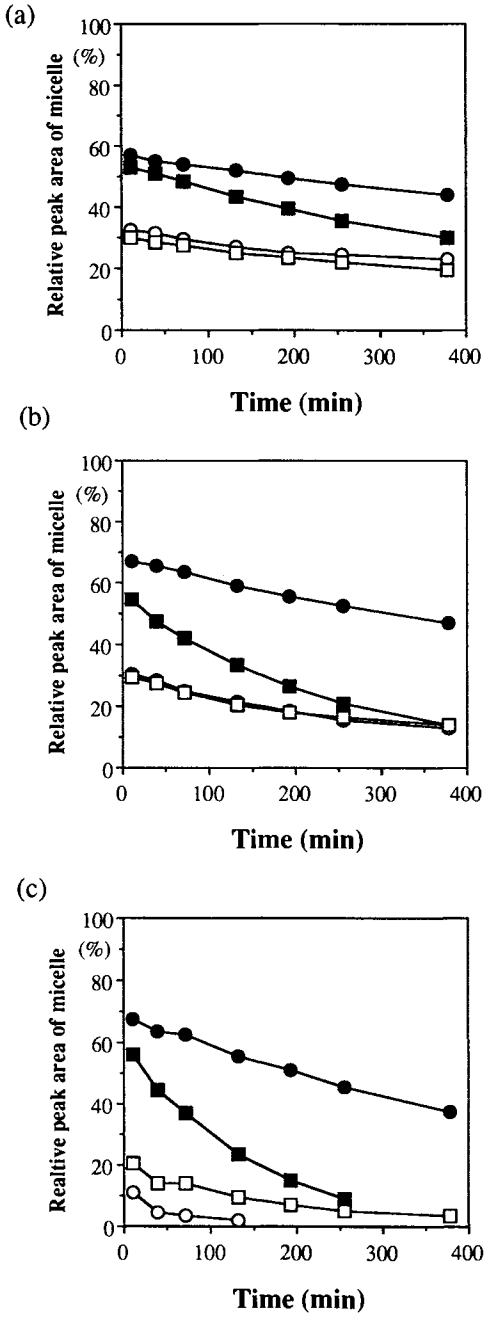

Fig. 5. Stability of micelles in the presence of rabbit serum: (a) high concentration; (b) medium concentration; (c) low concentration. Column, Asahipak GS-520; incubation in a 1:1 mixture of PBSa and rabbit serum at room temperature; column at room temperature. Other chromatography conditions were the same as for Fig. 2. Concentrations of the samples are expressed as ADR·HCl equivalents. (●) 43-17(30): (a) 585 μg/mL; (b) 73.1 μg/mL; (c) 9.1 μg/mL. (○) 43-17(17): (a) 552 μg/mL; (b) 69.0 μg/mL; (c) 8.6 μg/mL. (■) 51-84(34): (a) 552 μg/mL; (b) 69.0 μg/mL; (c) 8.6 μg/mL. (□) 51-84(13): (a) 570 μg/mL; (b) 71.3 μg/mL; (c) 8.9 μg/mL.

DISCUSSION

GPC analyses of the conjugate PEG-P[Asp(ADR)] showed that stability of the micellar structure depended on the composition of the conjugate. Polymeric micelles are considered to be more stable than those of low molecular weight compounds because of their low critical micelle concentration. For PEG-P[Asp(ADR)] 43-17(30), micelle formation was observed at a very low concentration (1×10^{-7} *M*). A more important factor for actual delivery in the physiological environments may be rates of association and disassociation of the micellar structure. The inner core of the micelle of this conjugate has solid characteristics because of the adriamycin moiety, which can induce strong hydrophobic and aromatic interactions with each other. Since polymeric micelles with solid-like cores are expected to equilibrate slowly with the polymer chain unimers (14), micellar structure may retain stable for long time periods in the bloodstream. Since the amide bond between ADR and the glutamic acid residue of the poly(L-glutamic acid) homopolymer was reported to be resistant to hydrolytic cleavage under physiological conditions (3), the slow decrease in the micelle peak shown in Fig. 6 is considered to result from a low dissociation rate of the micelles without a change in the amount of the bound ADR.

Conjugate 43-17(30) retained approximately 50% of the micelle peak area independent of its concentration (from 9.1 to 585 μg ADR·HCl equiv/mL) 255 min after mixing with serum. For this composition, it was reported that 33% of the injected amount remained in the bloodstream (76 μg ADR·HCl equiv/mL) 1 hr after an intravenous injection in ddy mice (10). The difference between the values for *in vitro* stability and the *in vivo* blood circulation suggests that cells, tissues, and organs contributed to uptake or destabilization of the polymeric micelles. Thus, the stability of the micellar structure in the presence of serum demonstrates its potential for drug delivery.

ACKNOWLEDGMENTS

The authors express their gratitude to Professor Tamotsu Kondo of the Science University of Tokyo for his valuable discussion and Dr. Glenn S. Kwon for his kind English correction. This study was supported by a Grant-in-Aid for Cancer Research and a young fellow grant from the Ministry of Education, Science and Culture, Japan, and a grant from the Japan Research Foundation for Clinical Pharmacology.

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