Physical Entrapment of Adriamycin in AB Block Copolymer Micelles

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The entrapment of Adriamycin (ADR) in micelles composed of AB block copolymers (poly(ethylene oxide-co-β-benzyl L-aspartate) (PEO-PBLA)) was investigated. The loading process involved transfer of ADR and PEO-PBLA into an aqueous milieu from dimethylformamide (DMF) through a dialysis procedure. Evidence for the physical entrapment of ADR in the polymeric micelles was derived from fluorescence spectroscopy and gel permeation chromatography (GPC). The total fluorescence intensity of ADR was low, suggesting that the drug was self-associated in the micelles. In addition, quenching experiments, using a water-soluble quencher (iodide (I^-)), showed that the fluorescence of ADR present in micellar solutions was largely unaffected by I^-, whereas the fluorescence of free ADR was readily quenched. From Stern-Volmer plots, quenching constants (K_{SV}) of 2.2 and 17 M^{-1} were determined for ADR in micellar solutions and free ADR, respectively. As a result of the entrapment of ADR in the micelles, ADR binds only slightly serum albumin as evidenced by GPC. In contrast, ADR readily binds serum albumin in aqueous solutions. The findings suggest that ADR is stably entrapped in PEO-PBLA micelles. ADR entrapment in polymeric micelles is expected to affect markedly the pharmacokinetics of ADR.

KEY WORDS: Adriamycin; polymeric micelles; AB block copolymer; drug delivery systems.

INTRODUCTION

AB block copolymer micelles which form in aqueous systems have been the subject of much interest in recent study (1–5). In contrast with micelles formed from low-molecular weight surfactants, block copolymer micelles dissociate slowly to free polymeric chains (1,2), have a greater capacity for solubilizing aromatic molecules (3), and express lower critical micelle concentrations (4,5). Thus, AB block copolymer micelles may be useful as vehicles for hydrophobic drugs. To this end, we have prepared AB block copolymers composed of poly(ethylene oxide) (PEO) and poly(β-benzyl L-aspartate) (PBLA). PEO is the hydrophilic block which is non-toxic and as part of the micelles has the ability to inhibit interactions with proteins and cells. PBLA is the hydrophobic block which may be biodegradable. At certain compositions, PEO-PBLA form micelles in aqueous solutions, having spherical, core/shell structures with diameters of ca. 20–40 nm (5). PEO-PBLA micelles solubilized pyrene in their cores (6), the extent of which was linearly proportional to its concentration (pyrene is a polycyclic, aromatic molecule whose photophysical properties were used to study the solubilization process).

Herein, we report on the physical entrapment of Adriamycin (ADR) in PEO-PBLA micelles. A procedure was developed to entrap ADR in the polymeric micelles, and evidence for the loading of ADR in PEO-PBLA micelles was derived through photo-physical means, making use of the intrinsic fluorescence of ADR. In addition, the retention of ADR in the PEO-PBLA micelles in the presence of serum albumin was investigated by GPC/HPLC. The results will be discussed in regard to the differences between micelles formed from block copolymers and low-molecular weight surfactants, and implications for the delivery of ADR will be noted.

MATERIALS AND METHODS

The Physical Loading of ADR in PEO-PBLA Micelles

The synthesis of PEO-PBLA block copolymers is described in detail elsewhere (7). The molecular weights of the PEO and PBLA blocks for AB block copolymer were 12,000 and 4000 g/mol, respectively (7). The critical micelle concentration for this polymer, as determined by fluorescence probe technique, is ca. 10^{-6} M (5). The physical entrapment of ADR in PEO-PBLA micelles was carried out as follows: ADR · HCl (25 mg) was added to DMF (4.0 ml) and solubilized by the addition of 1.3 equivalents of triethylamine. PEO-PBLA (20 mg) was added, and the solution was stirred overnight in the dark. To form micelles and remove free ADR, the solution was dialyzed (Spectrapor, molecular weight cutoff 12–14,000 g/mol) against 1.0 L of 0.1 M acetate buffer, pH 5.5 and subsequently with 3 × 1.0 L of distilled water over 24 h. The amount of entrapped ADR was determined by measuring the UV absorbance of the micellar solution retained within the membranes after dialysis. 1 g/L of ADR has an extinction coefficient of 1.85 × 10 L/cm · g at 485 nm (Ubest 50, JASCO, Japan).

Dynamic Light Scattering

The hydrodynamic diameter of the PEO-PBLA micelles, which had been prepared using the loading process, was determined by dynamic light scattering (DLS) (Photal, DLS-700, Otsuka Electronics, Japan) (5).

Fluorescence Studies

The fluorescence emission of ADR was measured using a fluorescence spectrometer (770F, JASCO, Japan). The excitation and emission bandwidths were both 10 nm. The solution of PEO-PBLA micelles and ADR (PEO-PBLA/ADR)

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was diluted with 0.10 M phosphate buffer solution (PBS) (11.50 g/l Na_2HPO_4, 2.28 g/l NaH_2PO_4), pH 7.4 to concentrations of 5–10 μg/ml ADR equivalents. The effects of sodium dodecyl sulfate (SDS) on the fluorescence of ADR were examined. The quenching of ADR by I^- (KI) was also investigated. The solutions were maintained at constant ionic strength by the addition of NaCl (ca. 0.60). An antioxidant, $Na_2S_2O_4$ (10^{-5} M), was used to prevent oxidation of iodide. All fluorescence experiments were carried out at 25°C.

Interaction of PEO-PBLA/ADR with Serum Albumin

PEO-PBLA/ADR was equilibrated in PBS, pH 7.4 with or without bovine serum albumin (BSA, Sigma Fraction V) (10 mg/ml) at ADR concentration of 10 μg/ml. ADR (10 μg/ml) was also equilibrated with BSA solutions (ADR in PBS, pH 7.4 binds to the HPLC columns and does not elute). The samples were analyzed immediately by GPC using HPLC (JASCO Intelligent HyPer LC system, Japan) equipped with an Asahipak 520H column. The separations were done at 40°C using a flow rate of 1.0 ml/min. Additional samples were stirred in the dark at 25°C for 5 hr and then subjected to GPC analysis.

RESULTS AND DISCUSSION

In PBS, pH 7.4, the direct physical entrapment of ADR in PEO-PBLA micelles does not proceed to any significant extent. ADR is a weak base (pK_a = 8.22) (8) and largely positively charged at physiological pH's. Thus, we hypothesized that by bringing ADR in its unionized form and PEO-PBLA into an aqueous milieu from a good solvent for both species (in this case DMF), ADR could be entrapped in polymeric micelles. And this was done using a simple dialysis procedure. As the DMF is removed, PEO-PBLA micelles form and have a prospect of incorporating ADR.

After the dialysis procedure, the PEO-PBLA/ADR solution retained within the dialysis membranes was observed to be a slightly dark, purple color (this is observed for ADR in alkaline solutions), whereas free ADR in the same aqueous buffer solutions had bright orange color, indicating ADR was in an unionized state in the micellar solutions. DLS measurements revealed PEO-PBLA micelles having a hydro-dynamic diameter of ca. 30 nm (PEO-PBLA micelles formed directly in water have diameters of ca. 20 nm). The amount of entrapped ADR in the PEO-PBLA micelles was estimated from absorbance measurements at 485 nm to be ca. 10% w/w of PEO-PBLA (8.0% of the initial amount of ADR).

Figure 1 shows the fluorescence emission spectra of PEO-PBLA/ADR and ADR in aqueous solutions. ADR displays its characteristic spectrum, whereas PEO-PBLA/ADR has a low total fluorescence intensity. Interestingly, the total fluorescence intensity of PEO-PBLA/ADR increased slightly as a function of incubation time in PBS (Figure 1). Even after 96 h, the total fluorescence intensity of PEO-PBLA/ADR was low compared with free ADR–this may reflect the self-association of ADR in the polymeric micelles and its gradual release from the micelles over time (self-association of ADR outside the polymeric micelles was discounted strongly by I^- quenching and GPC/HPLC results described shortly).

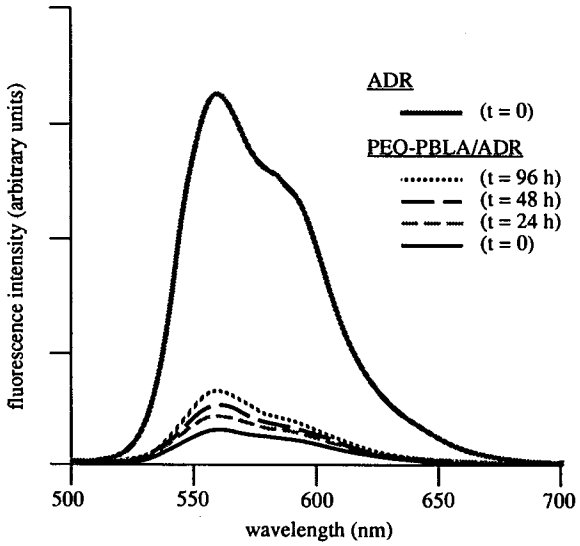

Fig. 1. Fluorescence emission spectra of ADR and PEO-PBLA/ADR micelles in PBS buffer (0.10 M, pH 7.4). The concentration of ADR is 10 μg/ml.

When SDS was added to the solutions of PEO-PBLA/ADR, a dramatic increase in total fluorescence intensity was evident (Figure 2), indicating the disruption of the PEO-PBLA micelles containing ADR and the formation of mixed micelles. Although further studies are required to elicidate the nature of the PEO-PBLA/SDS interactions, the formation of mixed micelles has been proposed for PEO-poly(styrene) block copolymer/SDS systems (9). Free ADR, in contrast, showed only a slight change in its fluorescence emission spectrum upon the addition of SDS (Figure 2).

Further evidence for the entrapment of ADR in PEO-PBLA micelles was derived from fluorescence studies using I^- as a quenching agent. The molecule is negatively charged

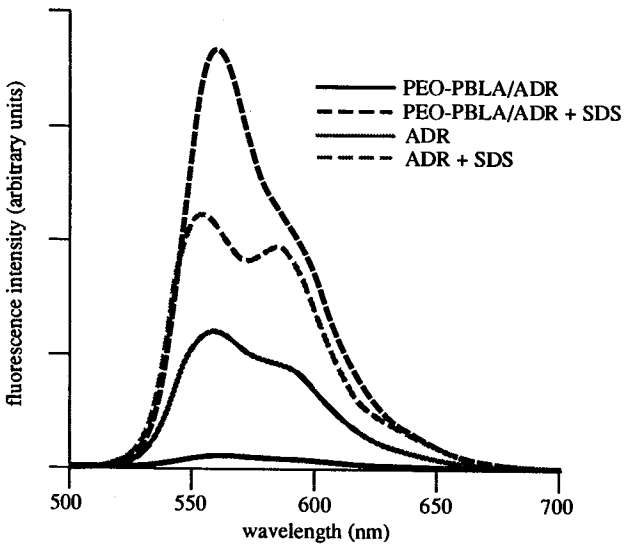

Fig. 2. Fluorescence emission spectra of ADR and PEO-PBLA/ADR micelles in PBS buffer (0.10 M, pH 7.4) with and without the addition of SDS (20 mg/ml). The concentration of ADR is 10 μg/ml.

and highly hydrated and cannot access hydrophobic microdomains (e.g., micellar cores). Thus, quenching studies using I^- are useful for providing insight on the proximity of ADR in solutions of PEO-PBLA micelles. As the concentration of I^- was increased from 0.10 to 0.60 M, the fluorescence of free ADR decreased substantially. In contrast, the fluorescence of PEO-PBLA/ADR diminished slightly as concentrations of I^- were raised. Assuming a single population of homogeneous fluorescent molecules, the following equation described dynamic quenching processes (10,11)

$$F_o/F = 1 + K_{SV}[I^-]$$

where F and F_o are the total fluorescence intensities of ADR with and without I^-. K_{SV} is the collisional quenching constant and is defined as

$$K_{SV} = kt_o$$

where k is the bimolecular collisional rate constant and t_o is the excited-state lifetime of ADR in the absence of quencher. Figure 3 shows the Stern-Volmer plots of the PEO-PBLA/ADR and ADR; linear plots were obtained for both cases. For PEO-PBLA/ADR solutions, this revealed that I^- quenched weakly a single population of ADR in micellar solutions, which may be ADR present near or at the interface of the core of the micelles and the palisade region of hydrophilic PEO chains. It also indicated negligible concentrations of free ADR in the solutions. K_{SV} of 2.2 and 17 M^{-1} were determined for PEO-PBLA/ADR and ADR, respectively. It is noted that the decrease in the K_{SV} values in micellar solutions may reflect changes to both k and t_o. ADR entrapped in the cores of PEO-PBLA micelles likely has diminished fluorescence lifetimes due to self-quenching. Without measurements of ADR lifetimes, individual contributions to K_{SV} cannot be discerned. In the case of small k or t_o, both are consistent with the entrapment of ADR in PEO-PBLA micelles.

Figure 4 shows the gel permeation chromatogram for PEO-PBLA/ADR just after equilibration in PBS pH 7.4. The detection of ADR was carried out by measuring its UV absorbance at 485 nm and fluorescence emission at 595 nm.

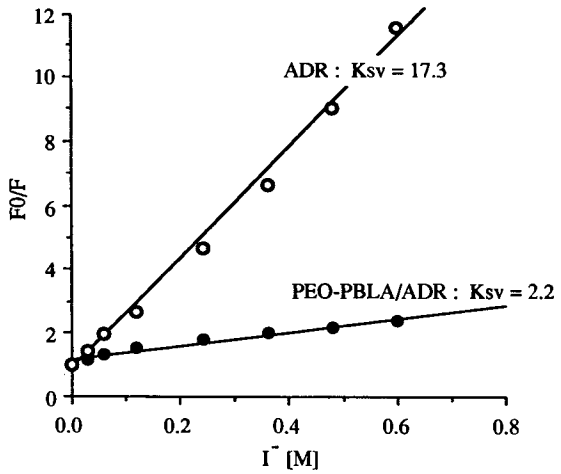

Fig. 3. Stern-Volmer plots for free ADR and ADR incorporated into PEO-PBLA micelles in PBS buffer (0.1 M, pH 7.4). The concentration of ADR is 5 μg/ml.

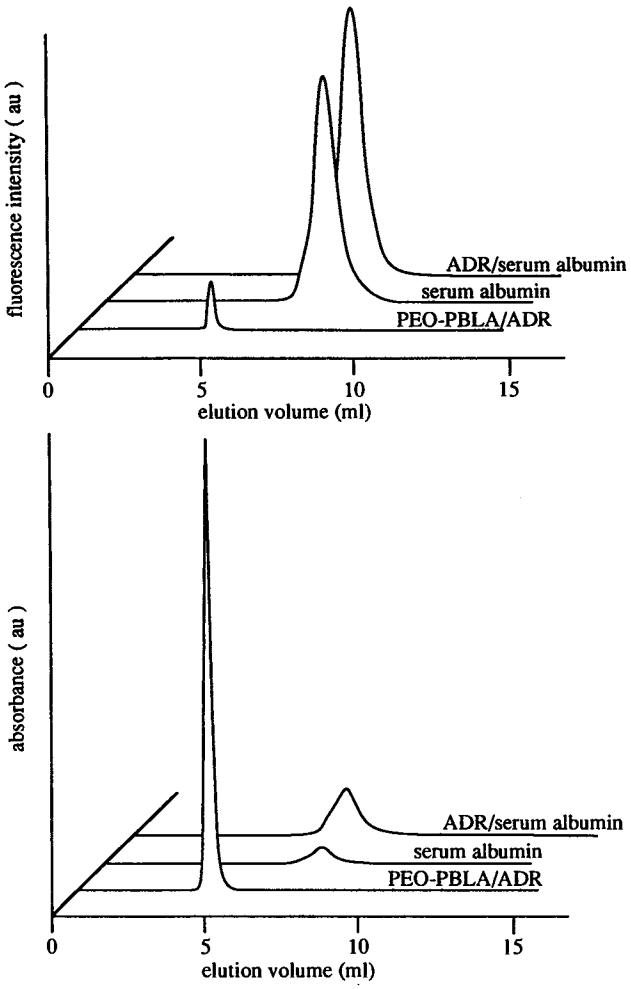

Fig. 4. Gel permeation chromatograms for PEO-PBLA/ADR micelles, serum albumin, and ADR equilibrate with serum albumin (10 mg/ml in 0.10 M PBS, pH 7.4). The concentration of ADR is 10 μg/ml. Column: Asahipak GS520H; eluent: 0.10 M PBS, pH 7.4; Detection of absorbance was carried out at 485 nm. The excitation and emission wavelengths for fluorescence detection of ADR are 471 and 595 nm, respectively.

PEO-PBLA/ADR eluted at the gel exclusion volume, consistent with the elevated molecular weights of the micelles which are probably greater than 10^6 g/mol (5) (molecules having molecular weights in excess of 10^5 g/mol elute at the gel exclusion volume). As noted earlier, in PBS, pH 7.4, free ADR adsorbed to the gel and did not elute from the column. It is noteworthy that the fluorescence of PEO-PBLA/ADR was highly quenched, consistent with prior fluorescence studies. Free ADR, in contrast, eluted with BSA (69,000 g/mol) and the total fluorescence intensity of the drug is much higher than the ADR in the presence of the polymeric micelles. Figure 5 shows the gel permeation chromatograms of PEO-PBLA/ADR samples in the presence of BSA. Again, most of the ADR eluted at the gel exclusion volume; a small peak was discerned at ca. 8.0 ml, indicating that some of the ADR had associated with BSA. After 5 h, a slight increase in absorbance of the second peak is noted.

The entrapment and retention of ADR in the PEO-PBLA micelles may be a result of their solid-like cores (i.e.,

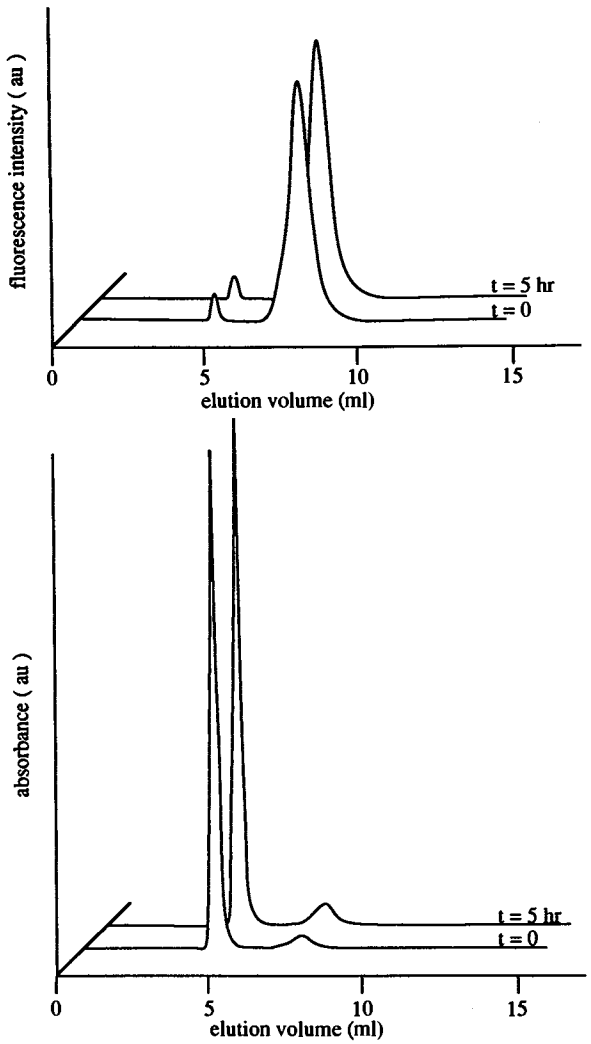

Fig. 5. Gel permeation chromatograms for PEO-PBLA/ADR micelles after incubation with serum albumin (10 mg/ml in 0.10 M PBS, pH 7.4). The concentration of ADR is 10 μg/ml. Asahipak GS520H; eluent: 0.10 M PBS, pH 7.4; Detection of absorbance was carried out at 485 nm. The excitation and emission wavelengths for fluorescence detection of ADR are 471 and 595 nm, respectively.

high microviscosity (5)), which was suggested from fluorescence probe techniques and ^{1}H NMR. The diffusion of ADR in the cores of the micelles may be highly constrained and ionization of ADR inhibited by the hydrophobicity of the micellar cores (i.e., low micropolarity) and ADR self-association. In this regard, studies of Urry *et al.* are noted which indicate that the pK_a's of ionizable groups can be shifted strongly by adjacent hydrophobic groups (12). Micelles based on low-molecular weight surfactants, on the other hand, have liquid-like cores (i.e., low microviscosities). And in this case, the molecules move freely and would not be constrained to their cores.

In summary, a simple procedure has been developed to entrap ADR in PEO-PBLA micelles. Our results, using ADR as a paradigm, indicate ionizable, hydrophobic drugs that are charged under physiological conditions can be entrapped in polymeric micelles. In light of our previous experience with micelle-forming PEO-poly(aspartate)-ADR conjugates (2,13,14), which have a similar core/shell structure with PEO forming the palisade region, and other's work with PEO-liposomes (15), we speculate that PEO-PBLA/ADR will show prolonged circulation times in blood and possibly higher uptake at solid tumor sites in comparison with free ADR. Subsequent studies will investigate the biodistribution of PEO-PBLA/ADR.

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