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# Preparation and Biological Characterization of Polymeric Micelle Drug Carriers with Intracellular pH-Triggered Drug Release Property: Tumor Permeability, Controlled Subcellular Drug Distribution, and Enhanced *In Vivo* Antitumor Efficacy

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A novel intracellular pH-sensitive polymeric micelle drug carrier that controls the systemic, local, and subcellular distributions of pharmacologically active drugs has been developed in this study. The micelles were prepared from self-assembling amphiphilic block copolymers, poly(ethylene glycol)-poly(aspartate hydrazone adriamycin), in which the anticancer drug, adriamycin, was conjugated to the hydrophobic segments through acid-sensitive hydrazone linkers. By this polymer design, the micelles can stably preserve drugs under physiological conditions (pH 7.4) and selectively release them by sensing the intracellular pH decrease in endosomes and lysosomes (pH 5–6). *In vitro* and *in vivo* studies show that the micelles have the characteristic properties, such as an intracellular pH-triggered drug release capability, tumor-infiltrating permeability, and effective antitumor activity with extremely low toxicity. The acquired experimental data clearly elucidate that the optimization of both the functional and structural features of polymeric micelles provides a promising formulation not only for the development of intracellular environment-sensitive supramolecular devices for cancer therapeutic applications but also for the future treatment of intractable cancers with limited vasculature.

## INTRODUCTION

The selective augmentation of drug concentrations in avascular tumor tissues is the most challenging issue of current cancer chemotherapy using macromolecular drug conjugates (1–3). Most anticancer drugs are pharmacologically effective but limited in their clinical applications due to serious toxicity and low water solubility; thereby, the altered biodistribution of these drugs has an important meaning not only to reduce the toxicity but also to improve therapeutic effects (4–6). For these reasons, interest has centered on the creation of drug carriers that safely and precisely deliver the appropriate amounts of active drugs to solid tumors (7–10). Indeed, several macromolecular drug carriers are under clinical trials or used practically, which include water-soluble polymer–drug conjugates (11), liposomal carriers (12), and polymeric micelles (13). However, even though these carriers have made significant advancements in cancer therapy, recent studies point out their antitumor activities are subject to change according to the cancer species with pathological, pharmacological, and biochemical differences (14).

There are three major reasons why the present macromolecular drug carriers have difficulties in clinical use. First, the carriers injected into the body encounter *in vivo* barriers such as nonspecific systemic accumulation and phagocytotic clearance by the host defense system (15). Second, even after the carriers accumulated in solid tumors avoiding these *in vivo* barriers, they still have to overcome the heterogeneous tumor microenvironments that are characterized by insufficient blood supply, disordered vasculatures, and diffusion-limited interstitium (16). Third, the carriers that successfully accessed the inside of tumor tissues should release the loaded drugs back into active forms in order to exert the antitumor effect (17). Among these reasons, poor permeability of the carriers inside the tumor tissues and low concentrations of active drugs throughout solid tumors become particularly serious problems (18, 19). For these reasons, understanding the correlation between the physicochemical properties of drug carriers and their behaviors in the body is very important, and the combination of these two features is required for the design of ultimate carriers (20).

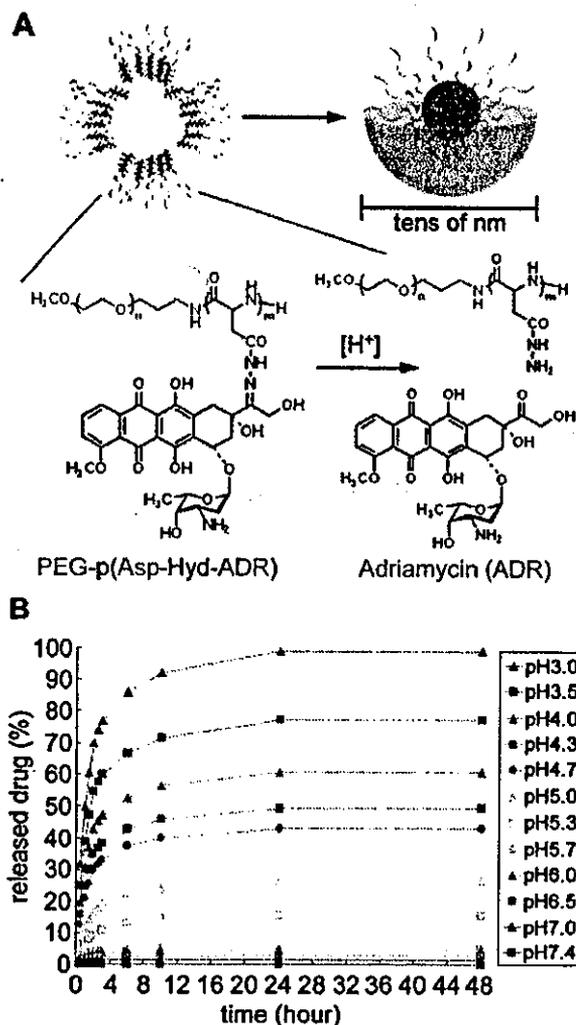
In this article, we will report that such tantalizing problems may be overcome by a novel tumor-infiltrating drug carrier, the pH-sensitive polymeric micelle, whose structural and functional features were optimized for the intracellular drug delivery (Figure 1A). The micelle is a nanosized supramolecular assembly from the self-assembling amphiphilic block copolymers, poly(ethylene glycol)-poly(aspartate hydrazone adriamycin) [PEG-p(Asp-Hyd-ADR)]. The anticancer drug, adriamycin (ADR), is

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**Figure 1.** Preparation of tumor-infiltrating polymeric micelles with intracellular pH-sensitivity. (A) Micelles with tens of nm size diameter were prepared from self-assembling amphiphilic block copolymers, PEG-p(Asp-Hyd-ADR), in which the anticancer drug, adriamycin (ADR), was conjugated through acid-sensitive hydrazone linkers. (B) The micelles released the loaded drugs under acidic conditions below pH 6.0 corresponding to intracellular space, but remained stable under the conditions of vascular and extracellular space (pH 7.4–7.0).

conjugated to the core-forming segments through the hydrazone linkers that are stable under physiological conditions (pH 7.4) but cleavable under acidic intracellular environments in endosomes and lysosomes (pH 5–6). This carrier design allows the micelle to safely protect hydrophobic drugs from the host defense system in the body and to selectively exert cytotoxicity due to intracellular pH-triggered drug release, improving both the delivery effect and therapeutic efficacy of the drugs (21). Therefore, the characteristic *in vitro* and *in vivo* behaviors of the micelles would offer intriguing information taking into account the future design and development of bioresponsive supramolecular carrier systems for the intracellular trafficking of biologically active molecules.

#### EXPERIMENTAL PROCEDURES

**Materials.**  $\beta$ -Benzyl-L-aspartate was from Sigma and  $\alpha$ -methoxy- $\omega$ -amino poly(ethylene glycol) (PEG; MW =

12 000) was from Nippon Oil & Fats, Japan. PEG was purified using an ion-exchange gel column (CM-Sephadex C-50, Amersham Pharmacia Biotech) prior to the synthesis of the block copolymers. Adriamycin hydrochloride (ADR-HCl) was from Nippon Kayaku, Japan, and its purity was checked by reversed phase liquid chromatography (RPLC). Sephadex LH-20 gel was from Amersham Pharmacia Biotech, Sweden.

**Cell Lines and Animals.** A human small cell lung cancer cell line SBC-3 and murine colon adenocarcinoma 26 (C26) cells were from the National Cancer Center Research Institute, Japan, and cultured in a medium (DMEM, Sigma, St. Louis, MO) containing 10% fetal bovine serum in a humidified atmosphere with 5% CO<sub>2</sub> at 37 °C. CDF-1 mice (female, 6 weeks old) were from Charles River, Japan. The animals were cared for and all experiments were performed in compliance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

**Preparation of the pH-Sensitive Polymeric Micelles.** The self-assembling amphiphilic block copolymer, PEG-p(Asp-Hyd-ADR), was synthesized as reported elsewhere (22). Briefly, poly(ethylene glycol)-poly( $\beta$ -benzyl-L-aspartate) (PEG-PBLA) was synthesized from the ring-opening polymerization of  $\beta$ -benzyl-L-aspartate *N*-carboxyanhydride using PEG as a macro initiator, followed by substitution of the benzyl groups of PEG-PBLA with hydrazone groups for drug binding (see also Supporting Information). Unbound ADR was completely removed using Sephadex LH-20 gel, and the obtained polymers were redissolved in dimethylacetamide to prepare the micelle by a dialysis method.

**Evaluation of Acid-Sensitive Drug Release from the Micelles.** Reversed phase liquid chromatography (RPLC) analysis, using a  $\mu$ -Bondasphere 5  $\mu$ m C4-300A column (Nihon Waters, Japan), was used to assess the pH sensitivity of the micelle. The micelle with a 10 mg/mL concentration was incubated under various buffered conditions from pH 7.4 to 3.0 [20 mM phosphate buffer (pH 7.4–6.0), 20 mM acetate buffer (pH 5.8–3.0)], and time- and pH-dependently released drugs were measured from the peak intensity by a UV detector (485 nm).

**Observations on Intracellular Drug Release and Localization of the Micelles.** Multicellular tumor spheroid (MCTS) was prepared from a C26 cell line using a spheroid culture plate, Sumiloncettight (Sumitomo Bakelite, Japan); 200  $\mu$ m size MCTS were sorted and used for the experiments. Fluorescence images were observed using a confocal laser scanning microscope (LSM 510, Carl Zeiss, Germany) with a 20 $\times$  objective (Plan-Apochromat, Carl Zeiss, Germany) and a 63 $\times$  objective (C-Apochromat, Carl Zeiss, Germany) at excitation wavelengths of 488 nm (Ar laser) and 364 nm (UV laser) for ADR and Hoechst 33258, respectively. The concentrations of the micelles in the medium were adjusted to 10  $\mu$ g/mL (ADR equivalent). All images were acquired and processed with the accompanying software.

**In Vitro Growth Inhibition Assay.** A tetrazolium dye method, called the MTT assay, was used to evaluate the growth-inhibitory effect of the micelle. Using 96-well culture plates, exponentially growing SBC-3 cells were seeded (2000 cell/well) and preincubated for 24 h, followed by coinubation with ADR and the micelle samples. After exposure for 3, 10, and 24 h, the medium was discarded and each cell was reincubated in fresh medium for another 24 h. The cells were then counted using a Bio-RAD Microplate Reader 550 (Bio-Rad Laboratories Inc.).

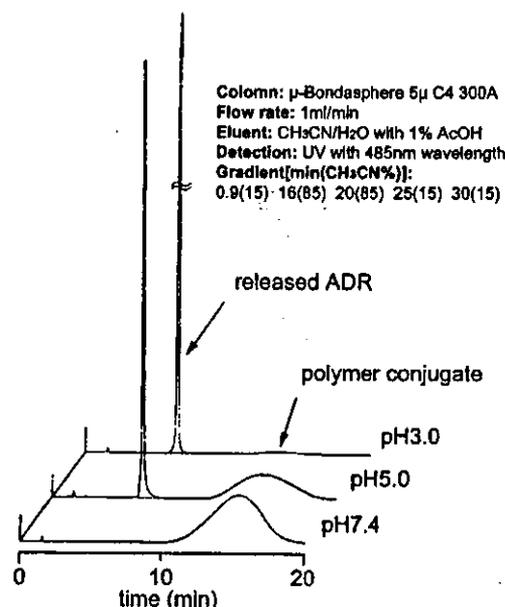
**In Vivo Antitumor Activity and Body Weight Change of Mice.** The antitumor activity of the micelles was evaluated with tumor bearing, 7-week-old, female SPF CDF1 mice ( $n = 6$ , Charles River, Japan). After implanting C26 cells 10 days earlier, injection of the samples took place using a volume of 0.1 mL/10 g body weight. The regimens of the micelles were scheduled by changing the administration dose (20, 40, and 60 mg/kg) three times with a 4-day interval, based on the optimized regimens of ADR as a control. However, in the case of ADR, only limited doses (5, 10, and 15 mg/kg) were applied to the mice due to the drugs toxicity. The mice were monitored daily, and tumor growth and body weights were measured at 2-day intervals. Tumor volume is calculated as follows:  $\text{volume} = 1/2 \times L \times W^2$  ( $L$  is the long diameter and  $W$  is the short diameter of a tumor).

**Biodistribution and Pharmacokinetics.** The CDF1 mice ( $n = 6$ ), when the tumor volume reached ca. 100 mm<sup>3</sup>, were injected with ADR and the micelles in a volume of 0.1 mL/10 g body weight for the experiments. The dose was either 10 mg/kg for ADR or the micelles (ADR equivalent). After the injection, blood, tumor, and major organs (heart, kidney, liver and spleen) were collected at 0.5, 1, 3, 6, 9, 24, and 48 h, followed by HPLC analysis (see Supporting Information for the detailed protocol).

**Fluorescence Microscopic Observations of Solid Tumors and Their Peripheral Blood Vessels.** The tumor-bearing mice were sacrificed at 24 h after the injection of the micelles with a 10 mg/kg dose. The intact tumor tissues with their peripheral blood vessels were harvested for macroscopic observations using a fluorescence microscope (Axiovert 200, Carl Zeiss, Germany) equipped with a 2.5 $\times$  objective (Plan-Neofluar, Carl Zeiss, Germany) and a filter set15 (BP546/12, FT580, LP590, mercury lamp excitation, Carl Zeiss, Germany).

## RESULTS

**Preparation of the Intracellular pH-Sensitive Polymeric Micelles.** The polymer backbone of PEG-p(Asp-Hyd-ADR) consisted of poly(ethylene glycol) (PEG) with a molecular weight of 12 000 g/mol for the hydrophilic shell-forming segment and 37 repeating units of polyaspartate (PAsp) for the core-forming segment (MW = 28 679), which were determined by gel permeation chromatography and <sup>1</sup>H NMR measurements. The 28 side chains in the PAsp block were replaced by hydrazide groups for the binding of the drugs. Adriamycin (ADR) was then conjugated to the polymer backbone through hydrazone bonds between the carbonyls at the C13 of ADR and the hydrazide groups of the PEG-p(Asp-Hyd) block copolymer. Even though the biological and chemical background of binding ADR to polymer backbone through hydrazone linkages has been delineated in our previous work (22) as well as many studies (23–26), it must be noticed that the hydrazone linkage is the most popularly used as pH-sensitive linkers due to the fact that this bond is quite stable at pH 7.4 but hydrolyzes under mild acidic conditions with pH 5–6. Therefore, to ensure that drug release from the micelles occurs only when acid-labile hydrazone linkers are cleaved, any unbound free ADR were completely removed in this study. These were confirmed by a reversed phase liquid chromatography (RPLC) analysis that is generally used for separating and purifying materials due to the differences in their hydrophobic properties (further details are described below). RPLC analysis showed the drug loading content of PEG-p(Asp-Hyd-ADR) was 42.5 wt % with respect to a single



**Figure 2.** Acid-sensitive cleavage of drug-binding hydrazone bonds evaluated by reversed phase liquid chromatography (RPLC) analysis. RPLC separates free drugs and polymer conjugates due to the differences in their hydrophobic properties, demonstrating absence of free drugs at pH 7.4 possibly binding to polymer conjugates in a physical way. On the other hand, it clearly shows that the amount of released drugs increases under acidic conditions in which the drug-binding hydrazone linkers can be cleaved.

block copolymer chain. The obtained PEG-p(Asp-Hyd-ADR) block copolymers self-assembled into micelles in aqueous solutions; the prepared micelles had a 65 nm diameter, which was confirmed by dynamic light scattering measurements.

**The Micelles Selectively Release the Loaded Drugs by Sensing a pH Decrease.** To confirm the acid-sensitive drug release profile, the micelles were incubated under various pH conditions from 7.4 to 3.0, and the released drugs were measured using RPLC analysis. Figure 1B shows that the micelles released the loaded drugs time- and pH-dependently as the external pH decreased, while they were stable under physiological conditions at pH 7.4 for over 48 h. The results indicate the micelles should release the loaded drugs in the intracellular acidic regions (pH 5–6) such as endosomes and lysosomes in which the drug-binding hydrazone linkers can be cleaved most effectively. Chemical evidence for absence of free drugs and acid-sensitive cleavage of hydrazone linkers to induce drug release was also presented by RPLC analysis (Figure 2). As described above, ADR is conjugated to amphiphilic block copolymers through pH-sensitive hydrazone linkers. If the drugs were simply entrapped in the micelles by physical interaction instead binding to polymers via chemical linkers, a sharp peak corresponding to the free drug should be separated from drug-polymer conjugates by RPLC and appeared in the physiological condition (pH 7.4) where the acid-labile hydrazone bond remains stable. However, the peak from the free drug was not shown at pH 7.4 but gradually increased in acidic conditions while the broad peak from polymer conjugates decreased. Eventually, almost 100% of the drugs were released at pH 3.0, and on the basis of this, we calculated the drug loading content of polymer conjugate described above. These results demonstrate that acid-sensitive cleavage

**Table 1. Growth Inhibitory Effects of the Micelles against Cancer Cells<sup>a</sup>**

sample	exposure time (hour)	IC <sub>50</sub> <sup>b</sup> (μg/mL ± SD)	relative index <sup>c</sup>
ADR	3	0.041 ± 0.035	1.05
	10	0.048 ± 0.026	1.23
	24	0.039 ± 0.025	1
micelle	3	1.08 ± 0.12	27.69
	10	0.45 ± 0.061	11.54
	24	0.27 ± 0.038	6.92

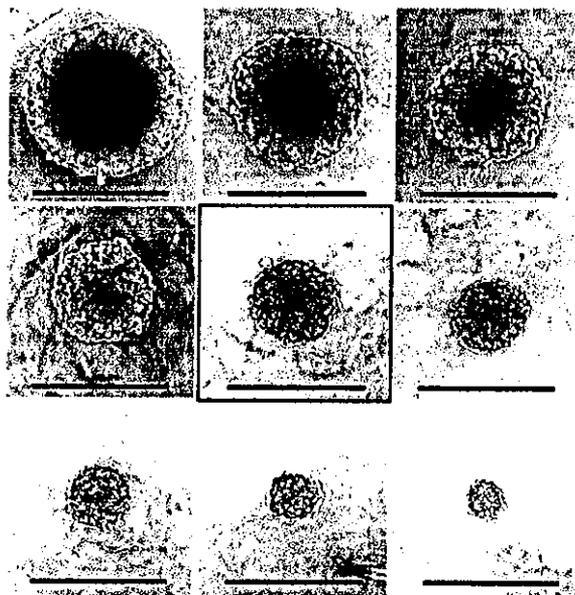
<sup>a</sup> Eight independent experiments were carried out using a human small cell lung cancer cell line SBC-3 ( $n = 8$ ). <sup>b</sup> IC<sub>50</sub> denotes the inhibitory concentration of the drugs required for 50% reduction in cell population. Concentrations of the micelles are calculated with free ADR equivalents. <sup>c</sup> Relative index denotes the ratio between a control and the object for comparison. Here, we evaluated the growth inhibitory effect of the micelles by converting their concentrations with respect to ADR after a 24 h incubation as the control.

of hydrazone linkers between drugs and polymer chains obviously induced drug release.

**Regenerated Drugs Were Pharmacologically Active Inhibiting Cell Growth in Vitro.** To verify whether the released drugs are pharmacologically active, an in vitro growth-inhibition test was carried out. The test revealed that the cytotoxic activity of the micelles was as high as 1/7-fold with respect to that of the free drugs after a 24 h exposure time (Table 1). Interestingly, the micelles showed delayed cytotoxicity that was drastically changed depending on the incubation time, which reflects that the drug release from the micelles took place and correlated with the cell metabolism.

**The Micelle Infiltrates into the Avascular Tumor Model Multicellular Tumor Spheroids.** Recently, it has been reported that the fluorescence quenching effect of the micelles provides a useful tool to observe the intracellular behaviors of the micelles (22). The fluorescence intensity of ADR, playing a role not only as an anticancer drug, but also as a fluorescence probe in this study, is quenched due to the locally increased high concentration in the micelle core; however, the fluorescence becomes detectable again as the micelles start to release ADR under acidic conditions. Consequently, the micelles emit intense fluorescence signals with the release of the entrapped ADR; a series of processes such as intracellular trafficking, drug release, and localization were able to be directly monitored in live cells accompanying the structural change. In the meantime, the multicellular tumor spheroid (MCTS) was used as an in vitro tumor model for the experiments because it is the most similar to avascular tumor regions of practical solid tumors in vivo that are characterized by limited accessibility of cell subpopulations (Figure 3). For example, MCTS reproduces adverse microenvironmental conditions of solid tumors in vivo such as hypoxia and nutritional depletion, and the extracellular matrix between tumor cells instead of intratumoral normal cell populations (27–28).

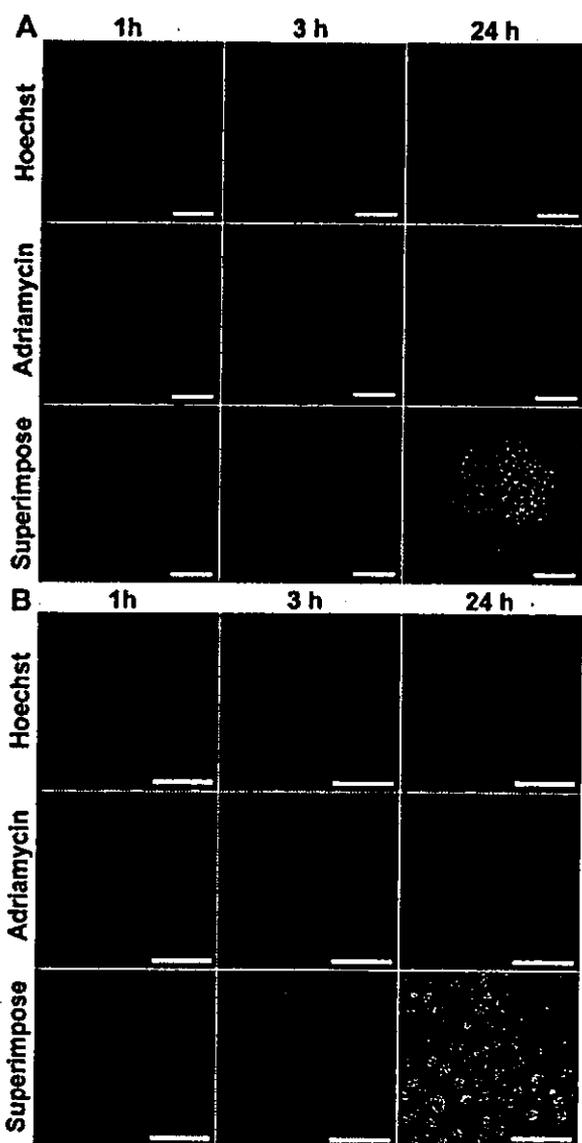
For these reasons, the intracellular behaviors of the micelles were observed within solid tumors using fluorescence quenching effect and the MCTS. The micelles were coincubated with the MCTS, and the change in the fluorescence intensity was monitored using a confocal laser scanning microscope (CLSM). The three-dimensional CLSM images demonstrated the time-dependent change in the fluorescence intensity of the micelle systems and their distributions throughout the MCTS with a 200 μm diameter depending on the incubation time (Figure 4A). The 200 μm diameter of MCTS was



**Figure 3.** Preparation of multicellular tumor spheroid (MCTS). MCTS with diameter ranging from 100 to 500 μm was prepared from a C26 cell line. Among them, MCTS with a 200 μm diameter (red-edged) was used as an in vitro tumor model because it has the most suitable size for reproducing in vivo avascular tumor regions that are characterized by limited accessibility of cell subpopulations (bar = 500 μm).

determined as the most suitable size for the experiments, considering the fact that the maximum distance between the capillary blood vessels within avascular solid tumor is 200 μm or less (29). The fluorescence of the micelle system remained quenched at 1 h after incubation, but it was detected at 3 and 24 h. It is notable that most cell nuclei remained blue at 3 h. The images, therefore, suggest that the micelles began to intracellularly release the drugs but the released drugs were still localized in the cytoplasm. However, the intense fluorescence of ADR was eventually detected in most of the cell nuclei after 24 h. These results reflect that the micelles would access every cell in the avascular region of tumor tissues in vivo to release the drugs.

**The Micelles Enter the Cell Interior and Release the Drugs.** To get a better understanding of the intracellular distributions of the micelles and their released drugs, further observations of the MCTS coincubated with the micelles under high magnification were carried out using a 63× objective (Figure 4B). These images show clear evidence of the intracellular drug release from the micelles and accumulation of the released drugs in the cell nuclei. The localization of the ADR fluorescence in the cytoplasm after 3 h incubation is supporting our speculation that the micelles internalized the cells releasing the drugs. After additional incubation up to 24 h, the presence of the drugs both in the cytoplasm and cell nuclei was confirmed. When the same experiments were carried out with free ADR, all the cell nuclei in the MCTS became red within 1 h because the ADR with a low molecular weight rapidly penetrated into each cell, and we were not able to observe this unique time-dependent fluorescence change in intensity and its distribution. Therefore, it is obvious that the micelles are precisely functioning in the intracellular regions as we designed, and the possible drug release from the micelles in the extracellular regions is negligible.



**Figure 4.** Observation of tumor permeability and intracellular drug release behaviors of the micelles. (A) CLSM observations showed the time-dependent change in the fluorescence intensities of ADR in the micelle system in MCTS. The images showed that the micelles can access the inside of the MCTS and release the loaded drugs (bar = 100  $\mu\text{m}$ ). (B) The intracellular drug release and localization of the micelles in each cell of MCTS were observed in detail using a high-magnification 63 $\times$  objective. The images clearly demonstrated that the micelles internalized into the cells and released drugs, and that the released drugs eventually accumulated in the cell nuclei (bar = 50  $\mu\text{m}$ ).

**The Micelles Suppress Tumor Growth in Mice with Enhanced Therapeutic Efficacy and Lowered Toxicity.** The animal tests revealed that the micelles exerted an effective antitumor activity over a broad range of injection doses to suppress tumor growth in mice, showing some of the clear comparisons with ADR (Figure 5). In the case of ADR, tumor growth was suppressed with a 10 mg/kg dose, but the mice treated with a 15 mg/kg dose were dead due to the drug's toxicity. This corresponds well to the fact that the lethal dose of ADR killing 50% of the test animals within a designated period, called LD<sub>50</sub>, is generally 12.7–13.2 mg/kg. On the contrary, the micelles were safely injectable up to a 40

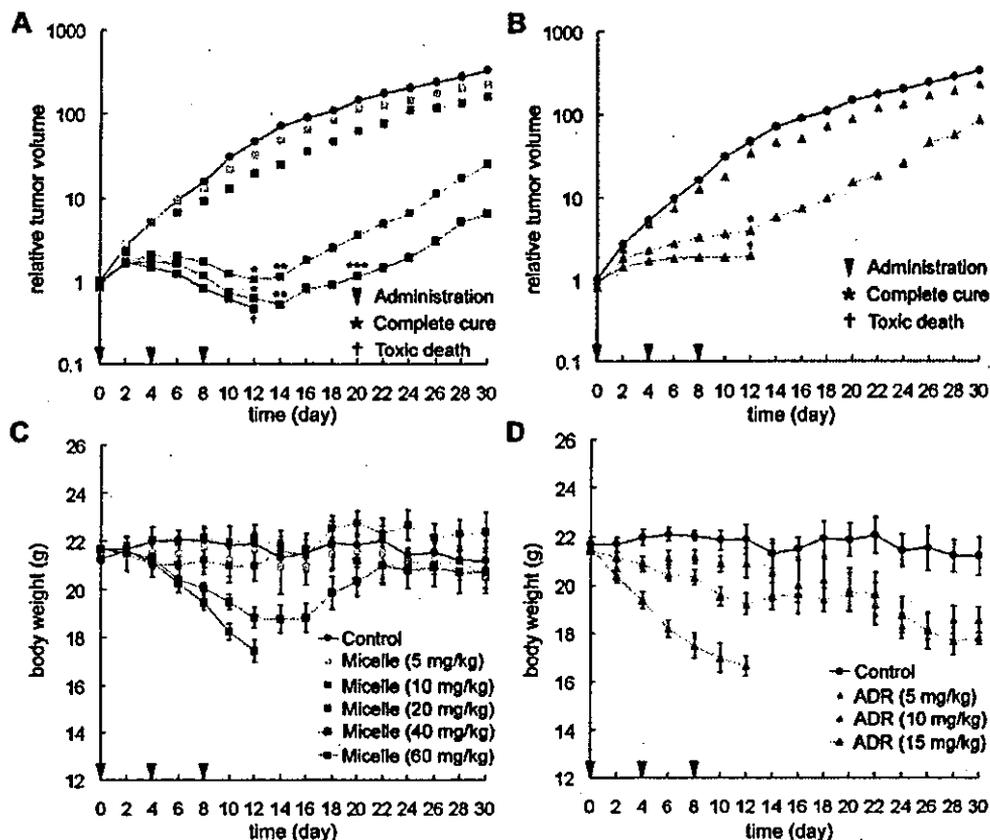
**Table 2.** In Vivo Antitumor Activity of the Micelles against C26 Tumor-Bearing Mice

sample	dose (mg/kg) <sup>a</sup>	body weight change on day 30 (%) <sup>b</sup>	toxic death	duration days of tumor growth <sup>c</sup>	complete cure
control	0	-2.18 $\pm$ 1.74	0/6	3.7	0/6
	5	-13.35 $\pm$ 0.59	0/6	4.2	0/6
	10	-16.84 $\pm$ 1.26	0/6	14.6	1/6
micelle	15	-	6/6	-	-
	5	-0.89 $\pm$ 1.68	0/6	3.9	0/6
	10	-4.51 $\pm$ 1.44	0/6	4.0	0/6
	20	3.13 $\pm$ 1.60	0/6	22.1	2/6
	40	-4.07 $\pm$ 0.92	0/6	27.9	3/6
	60	-	6/6	-	-

<sup>a</sup> Administrations were carried out three times with a 4-day interval, and doses were determined in free ADR equivalents. <sup>b</sup> Body weights were measured on day 30 after the first injection to compare the long-term toxicity between ADR and the micelles. Values are expressed as mean  $\pm$  SEM. <sup>c</sup> Duration time to reach 5-fold initial tumor volume.

mg/kg dose, while three of six mice were completely cured and there was no death among the treated mice. Notably, the body weights of the mice that slightly decreased during the micelle administration recovered, or even increased, on day 30 with respect to the controls (Table 2). Such behavior was not observed in the case of ADR, and the mice were emaciated with a 10 mg/kg dose that was the optimum dose for ADR to suppress tumor growth. Namely, the therapeutic efficacy of the micelles was significantly improved over that of ADR within this animal experiment setting, which distinguishes the micelles from ADR that has a narrow therapeutic window between 10 and 15 mg/kg. In the meantime, the tumor-suppressing antitumor activity of the micelles is shown from a 20 mg/kg dose. The micelles also extended the duration of tumor growth reaching a 5-fold initial tumor volume up to 22 and 28 days for the 20 and 40 mg/kg doses, respectively. These results indicate that the micelles achieved both enhanced therapeutic efficacy and a reduced toxicity of the loaded drugs, which are of great advantage to create effective and safe drug carrier systems.

**The Micelles Circulate for a Long Time in the Blood and Selectively Accumulate in Solid Tumors.** Effective antitumor activity and low toxicity imply that the micelles are stable in the blood without drug release (or leakage); therefore, their systemic and local distribution may dominate the tumor-suppressing antitumor activity. To demonstrate this, we investigated the in vivo dispositions of the micelles in detail using a biodistribution study. The levels of the micelles in the blood, tumor, and major organs, such as the heart, kidney, liver, and spleen, are expressed as percentage of each dose at specific times after the intravenous injection (Figure 6A). As summarized in Table 3, the micelles circulated in the blood for a prolonged time, and the area under the concentration curve (AUC) of the blood was 15-fold greater than that of ADR. In particular, it is noteworthy that the AUC values of the micelles in the heart and kidney decreased as compared to ADR, indicating that their tumor selectivity (AUC<sub>tumor</sub>/AUC<sub>organ</sub>) increased 6- and 5-fold higher with respect to the heart and the kidney, respectively. Such tumor-selective accumulation of the micelles may reduce the side effects of ADR such as cardiotoxicity and nephrotoxicity. In the meantime, the micelles showed a relatively low uptake in the liver and spleen despite the long residence time in the blood in comparison with tumors. These results suggest that the micelles may rapidly evacuate from these reticular



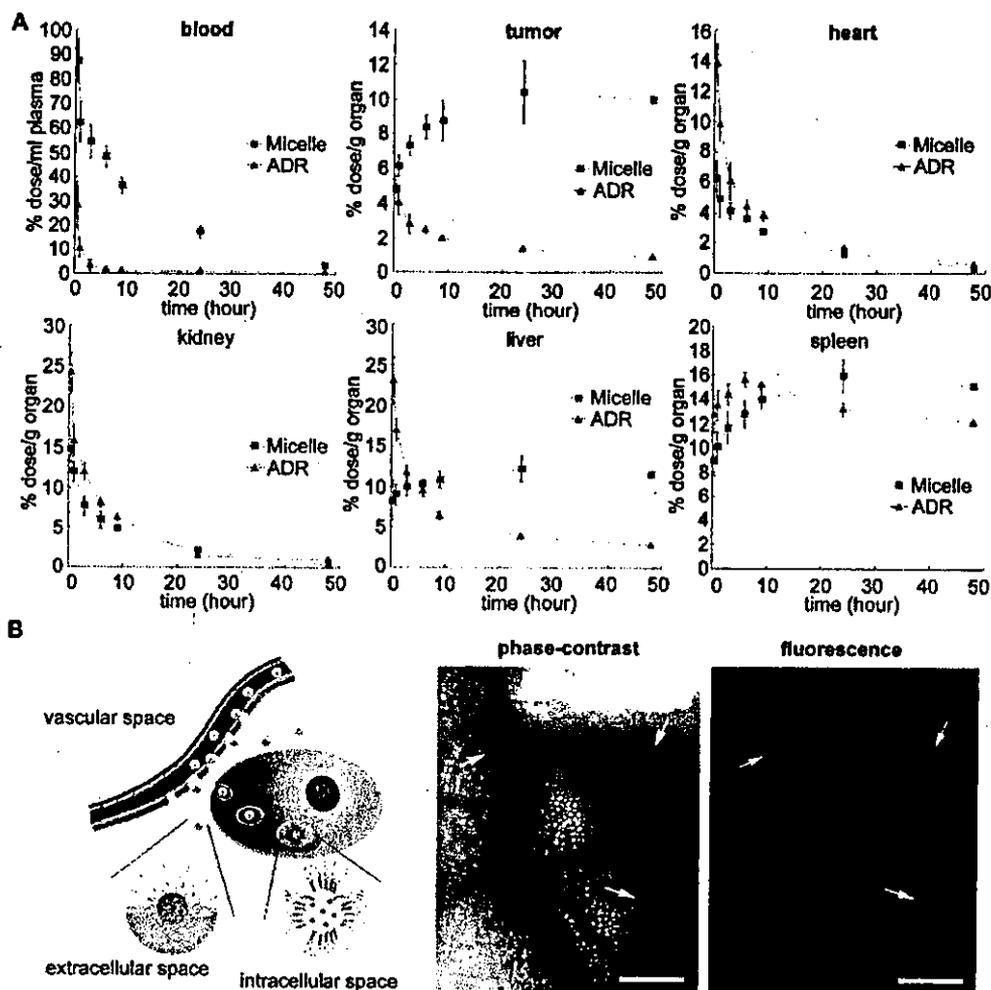
**Figure 5.** Tumor size and body weight changes of the treated mice. The figures show effective tumor-suppressing activity and low change in body weights over a broad range of injection doses of the micelles (A and C). To the contrary, ADR showed a narrow therapeutic window between 10 and 15 mg/kg doses avoiding toxic death (B and D). Administrations were carried out three times with a 4-day interval into tumor bearing CDF1 mice (female, 6-week-old,  $n = 6$ ). The micelle doses are shown as ADR equivalents. Values are expressed as mean and mean  $\pm$  SEM for the tumor volume and body weight, respectively.

connective tissues without interacting with monocytes and macrophages that are responsible for engulfing and removing cellular debris, old cells, and unwelcome external invaders from the bloodstream. On the contrary, tumor tissues are characterized by a large vascular permeability and high interstitial diffusivity while a lack of lymphatic drainage is observed. This phenomenon is explained by the enhanced permeability and retention (EPR) effect, which significantly affects distributions of the macromolecules facilitating their access and accumulation in tumors (30, 31). Consequently, the micelles that accumulated in the solid tumors through the EPR effect can stay for a prolonged time. The biodistribution study, therefore, showed that the cytotoxicity of the micelles seems to depend on their retention time as well as in the accumulated amounts in each organ, which may be crucial in attaining both an effective antitumor activity and reduced toxicity in a single drug carrier system.

In vivo antitumor activity test showed that the micelles effectively suppressed tumor growth in mice over a broad range of injection doses while toxicity remained extremely low. On the other hand, the biodistribution study revealed that the micelles circulated in the blood for a long period of time and accumulated in normal organs as well as the tumors. These results are very interesting because the micelles showed organ-dependent differences in cytotoxicity. To elucidate this, we investigated the localization of the micelles in each organ by calculating the tissue-to-blood concentration ratio  $K_b$  (Table 4). The  $K_b$  value is defined as  $[K_b = C_{\text{tissue}}/C_{\text{blood}}]$  where  $C_{\text{tissue}}$  and

$C_{\text{blood}}$  denote the tissue concentration and the blood concentration of the micelles, respectively. Each  $K_b$  value indicates distribution of the micelles in vascular space ( $K_b < 0.1$ ), extracellular space ( $0.1 < K_b < 0.5$ ), and intracellular space ( $0.5 < K_b$ ) (32, 33). The data revealed that the micelles localized in the cell interior of tumor tissues but mainly distributed in the extracellular space of other organs after accumulation. It is in good accordance with our previous results published elsewhere (34, 35). We speculate that such drastic alterations might be due to the pathological differences in vasculatures and lymphatic drainages between organs. Consequently, even though the micelles accumulate in normal organs, they can be excreted from the body before releasing drugs. The organ-dependent cytotoxicity of the micelles, therefore, probably would depend on their retention time as well as the accumulated amounts in each organ.

**The Micelles Regulate the Local Drug Distribution within Solid Tumors.** In view of their tumor-specific accumulation and intracellular distribution, the micelles should release the drugs in solid tumors along with emitting fluorescence as we observed in the intracellular drug release experiment using MCTS. In contrast, if the micelles released the drugs slowly or not at all, the fluorescence of ADR would remain quenched. On the basis of this hypothesis, we observed the solid tumors and their peripheral blood vessels in mice after intravenous injection of the micelles using a fluorescence microscope (Figure 6B). The observations were carried out at 24 h after injection because the micelles needed to



**Figure 6.** Tumor-specific accumulation of the micelles and locally increased drug concentrations. (A) Biodistribution study revealed the prolonged circulation in the blood and tumor-specific accumulation of the micelles. (B) Fluorescence microscopic observations of the solid tumor and its peripheral regions at 24 h after micelle injection demonstrate that the drug concentrations in the tumor tissues selectively increased due to the tumor-specific accumulation and controlled drug release from the micelles (bar = 500  $\mu\text{m}$ ).

**Table 3.** Tumor-Specific Accumulation of the Micelles

	AUC <sup>a</sup>		AUC ratio (AUC <sub>micelle</sub> /AUC <sub>ADR</sub> )	tumor selectivity <sup>b</sup> (AUC <sub>tumor</sub> /AUC <sub>organ</sub> )		tumor selectivity ratio [(AUC <sub>tumor</sub> /AUC <sub>organ</sub> ) <sub>micelle</sub> / (AUC <sub>tumor</sub> /AUC <sub>organ</sub> ) <sub>ADR</sub> ]
	ADR	micelle		ADR	micelle	
blood	58.86	858.54	14.59	—	—	—
tumor	49.59	210.21	4.24	—	—	—
heart	94.70	65.24	0.69	0.52	3.22	6.19
kidney	151.56	117.80	0.78	0.33	1.78	5.39
liver	176.15	261.17	1.48	0.28	0.80	2.86
spleen	341.38	329.66	0.97	0.15	0.64	4.27

<sup>a</sup> AUC denotes the area under a concentration curve that is obtained from the biodistribution study. Values are calculated based on the trapezoidal rule up to 24 h after intravenous injection. <sup>b</sup> Tumor selectivity of ADR and the micelles was determined by calculating the relative accumulated concentrations between the tumor tissues and each organ (AUC<sub>tumor</sub>/AUC<sub>organ</sub>). Their ratios indicate the change in the tumor selectivity of the micelles with respect to ADR.

accumulate in the tumor tissues enter the cells and release the drugs intracellularly as long as they were present in the blood. A phase-contrast image showed that the tumor blood vessels containing the micelles begin to leak into the extravascular compartment of the solid tumors (note the red color of the micelles). However, no fluorescence was detected at the corresponding part but in the limited regions of the solid tumor. Therefore, even though colloidal drug carriers are generally considered to localize in the limited peripheral regions of the solid

tumors due to poor accessibility, we have confirmed that the micelles can infiltrate into tumor tissues after accumulation, thus increasing the local drug concentration. This conclusion is in good accordance with the results from the permeability testing of the micelles into MCTS.

#### DISCUSSION

This paper has revealed a new potency of the polymeric micelle drug carrier systems that they can directly deliver drugs to the interior of targeted cells *in vivo* by infiltrat-

**Table 4. Tissue-to-blood Concentration Ratio ( $K_b$ ) in Each Organ\***

time (h)	organ				
	tumor	heart	kidney	liver	spleen
0.5	0.0545	0.0714	0.168	0.0946	0.1023
1	0.0976	0.0772	0.1903	0.1429	0.1617
3	0.134	0.0755	0.1402	0.1834	0.2136
6	0.1747	0.0764	0.1226	0.2141	0.2651
9	0.2412	0.0762	0.134	0.3014	0.3859
24	0.5992	0.0761	0.1162	0.7037	0.9158

\*  $K_b$  value is defined as  $[K_b = C_{\text{tissue}}/C_{\text{blood}}]$  where  $C_{\text{tissue}}$  and  $C_{\text{blood}}$  were the tissue concentration and the blood concentration of the micelles, respectively. Each  $K_b$  value indicates the distribution of the drugs in vascular space ( $K_b < 0.1$ ), extracellular space ( $0.1 < K_b < 0.5$ ), and intracellular space ( $0.5 < K_b$ ).

ing into solid tumors. These findings provided a clear answer to the questions whether the nanosized supramolecular drug carriers can access the cells in the avascular region of the solid tumors without structural and functional breakdown, which is also crucial to other intracellular environment-sensitive macromolecular bioconjugates that are injected through the vein for tumor targeting.

For the past several decades, natural and artificial macromolecules have evolved into a very useful class of drug delivery media (36–39). However, their *in vivo* applications are not always successful because limited accessibility inside solid tumors causes stagnation of drug carriers at the periphery of the tumor vasculatures, inducing low concentrations of active drugs in the targeted tumor tissues (40). Such a poor delivery efficiency of active drugs to the solid tumors is considered a serious problem facing recent cancer therapy using drug carriers. In this regard, we considered that the intrinsic characteristics of drug carriers may play a critical role in determining their *in vivo* behaviors, such as tumor permeability, drug release property, and anti-tumor activity. To confirm this, a suitable carrier model is required that controls the release of drugs while its structural features are optimized for a tumor-targeting drug delivery, and we prepared a new type of drug carrier in this study by adding an intracellular pH-triggered drug release property to the polymeric micelle drug carrier system whose chemical and biological properties are clearly identified (41).

As shown in the experimental results, the prepared micelle behaves not only as a biocompatible nanosized drug carrier with a high drug-loading content but also as a bioresponsive device with intracellular pH-sensitivity to control the drug release. These characteristic behaviors suggest that the nanosized core-shell structure of the micelles seems important to take the best advantage of the PEG shielding, imparting both stability and fragility into a single carrier system. The micelles safely protect the loaded drugs and functional linkers by providing a nanocompartment in the core that is completely segregated from the external environments; they maintain a high water-solubility with the hydrophilic shell that prevents the adsorption of proteins and the adhesion of cells so as to circumvent the uptake by reticuloendothelial systems during blood circulation (42, 43). In the meantime, intracellular trafficking of the micelles in the solid tumors is evident, which indicates that PEG shielding of the micelle may behave as a kinetic barrier that regulates the condition of the molecular affinity interaction between the micelles and cell membrane according to the exposure or retention time. As explained by the EPR effect, solid tumors feature a large

vascular permeability, high interstitial diffusivity, and poor lymphatic drainage; this is a fact that results in tumor-specific retention over a long period of time required for interacting with tumor cells. The retention time in each organ influences the intracellular trafficking of the micelles; thereby, any undesirable cytotoxicity to the normal organs is avoided. Consequently, the intracellular pH-triggered drug release property of the micelles should be the major reason for *in vivo* antitumor activity with low toxicity.

In conclusion, the intracellular pH-sensitive polymeric micelles exemplify the supramolecular drug carriers that control the systemic, local, and subcellular distribution of the active drugs. They show a higher bioavailability than free drugs, and therefore, the intracellular delivery of drugs would be the most effective and promising formulation for cancer chemotherapy with enhanced therapeutic efficacy and low toxicity. Moreover, the study reveals that the biocompatible structure and environment-sensitive functionality should be considered as a single event in order to realize the carrier systems that are related to these intracellular environments and material transports for the future treatment of cancers with avascular tumor tissue.

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**Supporting Information Available:** Synthetic scheme for PEG-p(Asp-Hyd-ADR) block copolymer. HPLC analysis protocol for biodistribution. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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