Delivering Anticancer Agents

Design of Environment-Sensitive Supramolecular Assemblies for Intracellular Drug Delivery: Polymeric Micelles that are Responsive to Intracellular pH Change**

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The recent development of biomolecular devices that function within the living body has required the integration of capabilities for sensing in vivo chemical stimuli, generating detectable signals, and effecting suitable responses into a single molecule or molecular complex.[1] In particular, biopharmaceutical systems which interact with intracellular components or events such as ions, proteins, enzymes, and pH changes are becoming important for implementing programmed functions that respond to signatures of the body.[2–6] Supramolecular chemistry is attracting attention as it offers methods for assembling different constituents capable of structural and dynamic changes into single molecules.[7] Herein we demonstrate the intracellular localization of a pH-sensitive supramolecular assembly that changes its structure and fluorescences when activated to induce mortality of malignant cells.

There are many difficulties in the clinical use of some biomolecular devices, these problems include phagocytic clearance during blood circulation, systemic spread causing toxic side effects, and exclusion from the cell by membrane transporters. In general, the cells selectively permeable membranes prevent the access of biomolecular devices that have not been appropriately designed. Therefore, the creation of biomolecular devices that are sensitive to the intracellular environment has been suggested as a method to overcome these physiological bottlenecks.[8,9]

From self-assembling acid-sensitive amphiphilic block copolymers we have prepared a polymeric micelle that is activated by the intracellular pH value (Figure 1). The polymeric micelle is a supramolecular assembly with characteristic properties, such as a core-protecting double-layer structure that is tens of nanometers in diameter, low toxicity in the human body, and has a prolonged circulation in the blood owing to its high water-solubility, thus avoiding phagocytic and renal clearance.[10] In addition, the functionality of the micelles can be modified simply by changing the chemical structures of the block copolymers,[11] and materials such as drugs,[12–14] proteins,[15] and DNAs,[16–18] can be selectively delivered to solid tumors in the body.[19,20] Site-specific tumor targeting in the body is achieved by the enhanced permeability and retention (EPR) effect, proposed by Maeda and Matsumura.[21] According to their report, solid tumors have abnormal blood vessels with loose junction and insufficient lymphatic drainage, so that the micelles easily escape from the blood vessel and accumulate in tumor tissues but they hardly return to the blood stream again. In general, cells take up large materials, such as the micelles, by folding the cell membrane inwardly, surrounding the materials to be ingested. The material is then engulfed in small bubble-like endocytic vesicles. This is called the endocytosis process that allows supramolecular assemblies to sneak into intracellular regions avoiding the cell-membrane transporters. After the micelles are taken up to the cell interior through endocytosis, the substance transport occurs. The endocytic vesicles change from early and late endosomes and finally to lysosomes in which the proton concentration is 100-times lower (pH 5.0) than the physiological condition (pH 7.4), which is an important in vivo chemical stimuli that can be used to trigger functional biomolecular devices.[22]

An amphiphilic block copolymer, poly(ethylene glycol)–poly(aspartate–hydrazone–adriamycin) (PEG–p(Asp–Hyd–ADR)), was synthesized using the aspartic acid of poly(ethylene glycol)–poly(β-benzyl-L-aspartate) (PEG–PBLA) as a convenient template (Scheme 1). PEG–PBLA was synthesized from the ring-opening polymerization of β-benzyl-L-aspartate *N*-carboxy-anhydride (BLA–NCA). Poly-

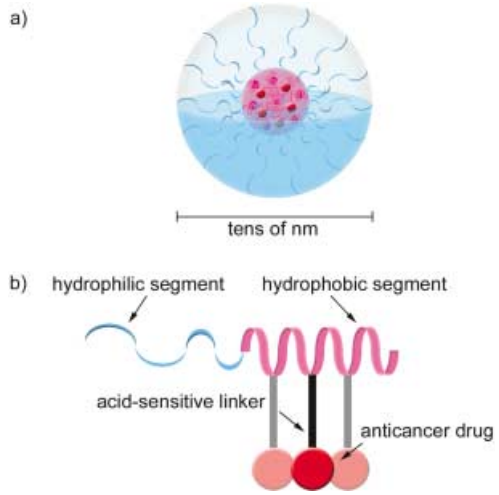

Figure 1. The polymeric micelles (a), were prepared from self-assembling acid-sensitive amphiphilic block copolymers (b), in aqueous solution. A supramolecular structure of the micelles has the advantage of site-specific targeting in the body, protecting reactive functional moieties with the hydrophilic outer shell during blood circulation.

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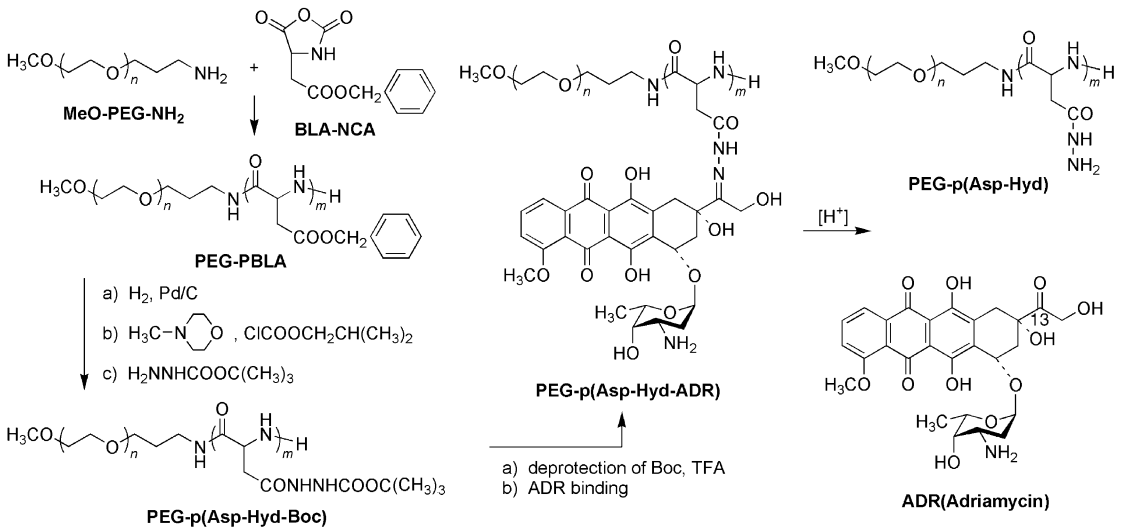

Scheme 1. Synthesis of PEG–p(Asp–Hyd–ADR) block copolymers. The Schiff base formed between the C13 ketone of ADR and the hydrazide groups of the PEG–p(Asp–Hyd) block polymer are most effectively cleavable under acidic conditions around pH 5.0, which correspond to that of the lysosomes in the cells. Boc = *tert*-butoxycarbonyl, TFA = trifluoroacetic acid.

merization of BLA–NCA was initiated by the terminal primary amino group of α-methoxy-ω-amino poly(ethylene glycol) under argon atmosphere in distilled dimethylformamide. After deprotection of the benzyl groups of PEG–PBLA, hydrazide groups were attached to the end of the aspartate side chains of the block copolymer by an acid anhydride reaction which is a modification of the synthetic method suggested by T. Kaneko et al.[23] ^{1}H NMR measurements in [D$_6$]DMSO at 80 °C reveal that the numbers of repeating units of P(Asp) block and hydrazide groups were 37 and 28, respectively (see Supporting Information). Adriamycin (ADR) was then conjugated to the polymer backbone through an acid-labile hydrazone bond between C13 of ADR and the hydrazide groups of the PEG–p(Asp–Hyd) block copolymer. Subsequently, the polymeric micelles were prepared by a dialysis method which brought the organic components into an aqueous environment. The micelles were about 65 nm in diameter and of uniform size, as confirmed by dynamic light-scattering measurements (DLS; Supporting Information). ADR is an anticancer agent and suppresses cell growth by binding with DNA strands in the cell nucleus. Despite its efficacy, ADR use is frequently accompanied by toxic side effects. However, its activity is suspended by binding to materials such as polymers, antibodies, and molecular complexes.[24] In addition, the detectable fluorescence of ADR allows it to be used as a fluorescence probe in this study.

The acid-sensitivity of the micelles was evaluated by reversed-phase liquid chromatography (RPLC) (Supporting Information). As shown in Figure 2, the micelles release ADR both time- and pH-dependently as the pH value decreases from pH 7.4 to 3.0. The micelles were stable over 72 h in region A (Figure 2), which corresponds to physiological and early endosomal conditions. On the other hand, the release of ADR gradually increases and reaches equilibrium as the pH decreases in regions B and C. The ADR release profile in region B is notable considering that the pH values in

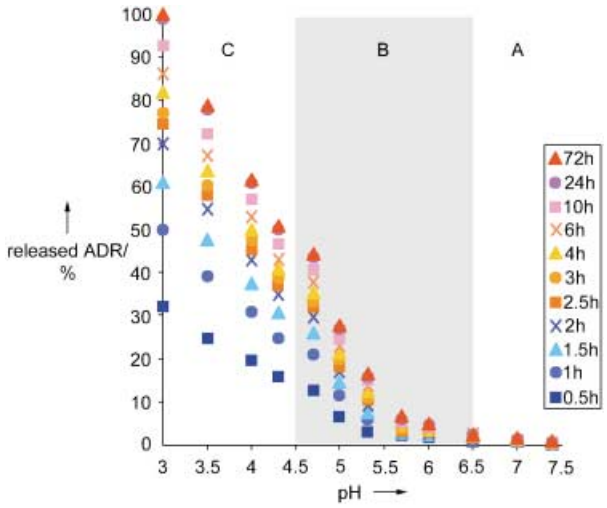

Figure 2. Time and pH-dependent ADR release profile of the micelles. The micelles selectively released ADR under the pH conditions of regions A and B which correspond to outer and intracellular conditions, respectively. The amount of loaded ADR on the micelles was calculated at pH 3.0 in region C, where the release was the maximum.

late endosomes and/or lysosomes in the cells are around 5.0 where the acid-sensitive hydrazone bonds can be cleaved most effectively. Because the formation of reversible hydrazone bonds is hindered by strong acidity, the loading content of ADR on the micelles was calculated from the maximum ADR release at pH 3.0 in region C (Supporting Information). The calculation revealed that the micelles consisted of the block copolymers containing ADR with 67.6 % mol substitution with respect to aspartate units of PEG–p(Asp–Hyd–ADR).

Measurement of fluorescence intensity reveals that the micelles are stable under physiological conditions and fluorescence only occurs when the ADR is released under acidic

conditions (Supporting Information). The micelles and free ADR were incubated in cell culture medium, Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum for 24 h. Ion and pH levels are controlled in DMEM, which is very similar to physiological condition in the body. Concentrations of ADR and the ADR bound in the micelles were adjusted to be equivalent (100 $\mu g\,mL^{-1}$). Samples were excited with the wavelength of 485 nm, and the fluorescence at 590 nm was monitored by a spectrofluorometer. Compared with the intense fluorescence of free ADR, the fluorescence intensity of the ADR-bound in the micelles remained low and no significant change in intensity was observed after 24 h monitoring. Like most fluorescence materials, the fluorescence of ADR is quenched in a high concentration in solution. This phenomenon also occurs in the micelle core where ADR molecules are confined at high local concentrations. The fluorescence remains quenched as long as the ADR is incorporated in the micelle core and a change in fluorescence reflects the, release of the ADR from the micelles. Thus, the pH sensitive structural change of the micelles can be detected through the change in fluorescence.

Observations using confocal laser scanning microscopy (CLSM) reveal the intracellular localization of micelles that were incubated with human small cell lung cancer cell SBC-3. As shown in Figure 3, a time-dependent fluorescence change in intensity was observed over 24 h. After 1 h exposure, an increase in fluorescence intensity was observed for SBC-3 incubated with ADR (Figure 3a), but no such increase was detected with the micelles (Figure 3c). On the other hand, a considerable fluorescence change was observed in the cells exposed to the micelles after 24 h incubation (Figure 3d), which clearly demonstrates intracellular distribution of the micelles and the released ADR. Compared with Figure 3b which shows that ADR is only accumulated in cell nuclei, Figure 3d indicates that the localized fluorescence is dot-shaped within the cytoplasm suggesting the presence of the micelles trapped in the endocytic vesicles. In general, it is very difficult to distinguish between the fluorescence material ADR and its polymer conjugates in solution because both exhibit intense fluorescence. However, the micelles solve this problem because of their characteristic fluorescence quenching effects.

As a system releasing bioactive molecules, the micelles are required to maintain the ability of the loaded ADR to suppresses cell growth by binding with DNA strands in the cell nucleus. Figure 4 shows the growth-inhibition effects of

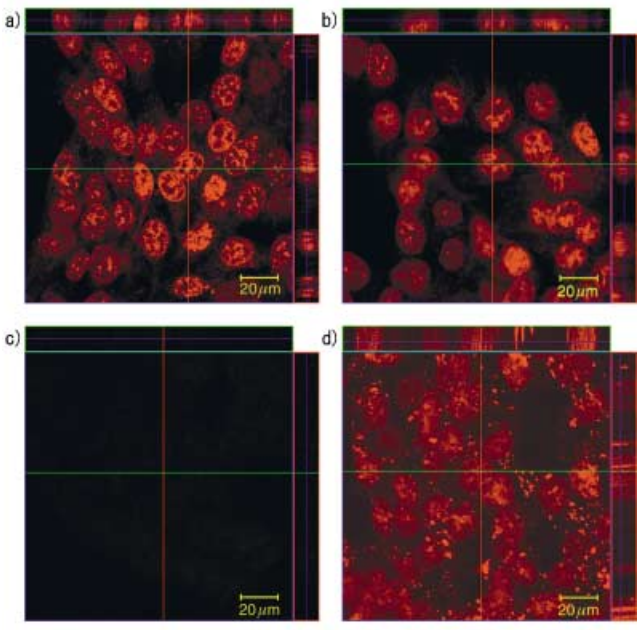

Figure 3. CLSM images of the SBC-3 cells incubated with ADR and the micelles (10 $\mu g\,mL^{-1}$). In contrast to free ADR, the fluorescence of the ADR in the micelles is only detected when they are activated. A series of optical sections was stacked (Z-stacked) by moving the focal plane of the instrument step-by-step through the depth of the cell. The Z-stacked images clearly reveal that the micelles are localized within the cytoplasm with a dotlike shape, assumed to be micelles in acidic lysosomal compartments, while most of the ADR released from the micelles is in the cell nucleus. a) free ADR after 1 h exposure, b) free ADR after 24 h incubation, c) micelles after 1 h exposure, d) micelles after 24 h incubation.

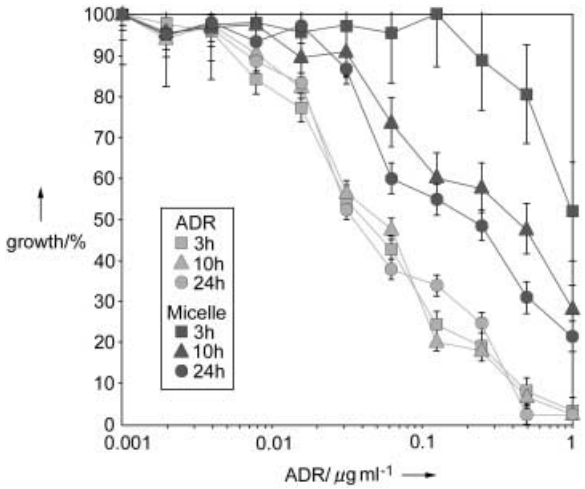

Figure 4. Growth inhibition assay results on human small lung cancer cells SBC-3 with different ADR concentrations and exposure times. As time elapses, the curve indicating the inhibition effect of the ADR-containing micelles approached that of free ADR.

the micelles on SBC-3 cells. The results obtained with the micelles gradually approach those of free ADR, which demonstrates that the ADR released from the micelles is pharmaceutically active. Therefore, we conclude that ADR accumulates in the cell nuclei after release from the micelles localized within the cytoplasm.

In summary, we have shown the intracellular localization of pH-sensitive polymeric micelles whose functions are controlled by live cells. As a multifunctional biomolecular device, the micelles undergo dynamic changes in structure and/or function in response to environmental stimuli (pH value). Furthermore, the ADR released from the micelles fluorescences which allows its localization within the living cells to be detected. CLSM reveals that the micelles are trapped in lysosomes where they are programmed to function by responding to low pH, and the released ADR

accumulates in the cell nuclei and effectively suppresses the synchronizing cell viability of cancer cells. These results suggest that highly controlled functional biomolecular devices have become available.

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