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Design of Environment-sensitive Supramolecular Assemblies for Intracellular Drug Delivery: The Polymeric Micelles Responsive to Intracellular pH Change**

Younsoo Bae, Shigeto Fukushima, Atsushi Harada, and Kazunori Kataoka*

1. Synthesis of acid-sensitive amphiphilic block copolymer
2. Preparation of the micelles
3. Evaluation of pH sensitivity (RPLC)
4. Fluorescence quenching property
5. Confocal laser scanning microscopic (CLSM) observations
6. In vitro growth-inhibition assay

1. Synthesis of acid-sensitive amphiphilic block copolymer

1.1 Chemicals and devices

β-Benzyl L-aspartate was purchased from Sigma Chemical Co., Inc., USA. A bis-(trichloromethyl)carbonate (triphosgene), N-methylmorpholine (NMM), isobutyl chloroformate (IBCF), carbazic acid tert-butyl ester (CAt-BE) and hydrobromic acid/acetic acid solution (HBr/AcOH, 25g, 30% HBr, for peptide research) were purchased from Tokyo Kasei Organic Chemicals Co., Ltd., Japan. These chemicals were used without further purification. α-Methoxy-ω-amino poly(ethylene glycol) (CH_3O-PEG-$CH_2CH_2CH_2$-NH_2, PEG; MW=12,000) was kindly supplied by Nippon Oil & Fats Co., Ltd.. PEG was purified by ion-exchange gel column (CM-Sephadex C-50, Amersham Pharmacia Biotech.) and dehydrated by freeze-drying from benzene prior to use for block copolymer synthesis. Tetrahydrofuran(THF), n-hexane, N,N-dimethylformamide(DMF), dimethyl acetamide(DMAc), methanol(MeOH), trifluoro acetic acid(TFA), acetonitrile(CH_3CN), dimethyl sulfoxide(DMSO) and diethyl ether were purchased from Wako Pure Chemical Industries, Co., Ltd., Japan. THF, n-hexane and DMF were twice distilled. Adriamycin hydrochloride(ADR-HCl) was a generous gift from Nippon Kayaku Co., Ltd., Japan, and its purity was checked by RPLC. Sephadex LH-20 gel was purchased from Amersham Pharmacia Biotech Co., Ltd., Sweden.

1.2 Polymerization of Poly(ethylene glycol)-*block*-poly(β-Benzyl L-aspartate):PEG-PBLA

PEG-PBLA was synthesized as follows: Ring-opening polymerization of β-benzyl-L-aspartate N-carboxy-anhydride (BLA-NCA) was initiated from the terminal primary amino group of PEG under an argon atmosphere in distilled DMF. Prepared PEG-PBLA was precipitated from diethyl ether followed by

freeze-drying from benzene. PEG-PBLA was narrowly distributed in molecular weights(P.D.I.=1.108), which was confirmed by GPC (TSK-gel G3000PWXL and TSK-gel G4000PWXL columns, DMF with 10 mM LiCl; 0.8 ml/min flow rate, RI detection at 40 °C). The composition of PEG-PBLA was determined by ^{1}H-NMR measurements in DMSO-d_6 at 80 °C (Figure 1S A) from the peak ratios of the methylene protons of PEG (-OCH_2CH_2-:δ3.5ppm) and the phenyl protons of γ-benzyl groups of PBLA (-$CH_2\boldsymbol{C_6H_5}$:δ7.3ppm). Table 1S shows the composition of the products.

1.3 Synthesis of Poly(ethylene glycol)-*block*-poly(aspartic acid hydrazide *tert*-butoxycarbonyl): PEG-p(Asp-Hyd-BOC)

The benzyl groups of PEG-PBLA (1g) were removed by acid hydrolysis using HBr/AcOH. Hydrazide groups were attached to the end of aspartic acid side chains of the block copolymer via acid anhydride reaction. NMM(1.01g, 10mmol) was added to [PEG-p(α-Asp)](500mg) dissolved in DMAc(30ml), followed by dropwise addition of IBCF(1.36g, 10mmol) under an argon atmosphere at 4 °C. After 5min, CAt-BE(2g, 15mmol) in DMAc(10ml) was added. The mixed solution was allowed to react for 30 min at 4 °C and 2 more hours at room temperature. Clear light-brown solution of PEG-p(Asp-Hyd-BOC) was obtained by filtering off the white sediment of the byproduct between NMM and IBCF. The product was precipitated from diethyl ether and dialyzed in DMSO with molecular weight cut off (MWCO) 1,000 membranes for further purification. After substituting DMSO with distilled water, freeze-drying was carried out. PEG-p(Asp-Hyd-BOC) was synthesized from the obtained PEG-PBLA and shown to contain 28 units(75.68 mol%) of hydrazide groups at the side chain of the p(Asp) block from the ratios of the methylene protons of PEG (-OCH_2CH_2-:δ3.5ppm) and the methyl protons of BOC groups [-$COOC(CH_3)_3$:δ1.4ppm] by ^{1}H-NMR measurements in DMSO-d_6

at 80 °C. As shown in Figure 1S B, a peak from BOC appeared, while the peaks from the γ-benzyl groups of PEG-PBLA disappeared.

1.4 Preparation of drug-bound Poly(ethylene glycol)-*block*-poly(aspartic acid hydrazone adriamycin): PEG-p(Asp-Hyd-ADR)

Synthesized [PEG-p(Asp-Hyd- BOC)](200 mg) was treated by TFA(10 ml) to remove protective BOC groups. The obtained [PEG-p(Asp-Hyd)](100 mg) and an excess amount of ADR-HCl (200 mg, MW=580) with respect to drug binding hydrazide residues were dissolved in 500 ml of methanol with TFA as an acid catalyst. The solution was stirred at room temperature for 24 h while being protected from light and concentrated to a volume of 50 ml under reduced pressure at 30 °C. After overnight reaction, a dark orange solution was further concentrated to 4 ml and applied to the open-column filled with Sephadex-LH20 gel for separating the PEG-p(Asp-Hyd-ADR) block copolymer from the unbound free ADR. The applied solution was separated into two fractions, and the one eluted first was collected. After evaporation of the methanol, the red wine color product was evaluated by RPLC to confirm the absence of unbound free ADR. PEG-p(Asp-Hyd-ADR) was prepared by attaching ADR to the hydrazide drug bonding residues of the block copolymer through an imine, called the Schiff base bond. The absence of unbound free ADR was confirmed by RPLC from the disappearance of a sharp peak corresponding to free ADR on the chart after Sephadex-LH 20 gel separation.

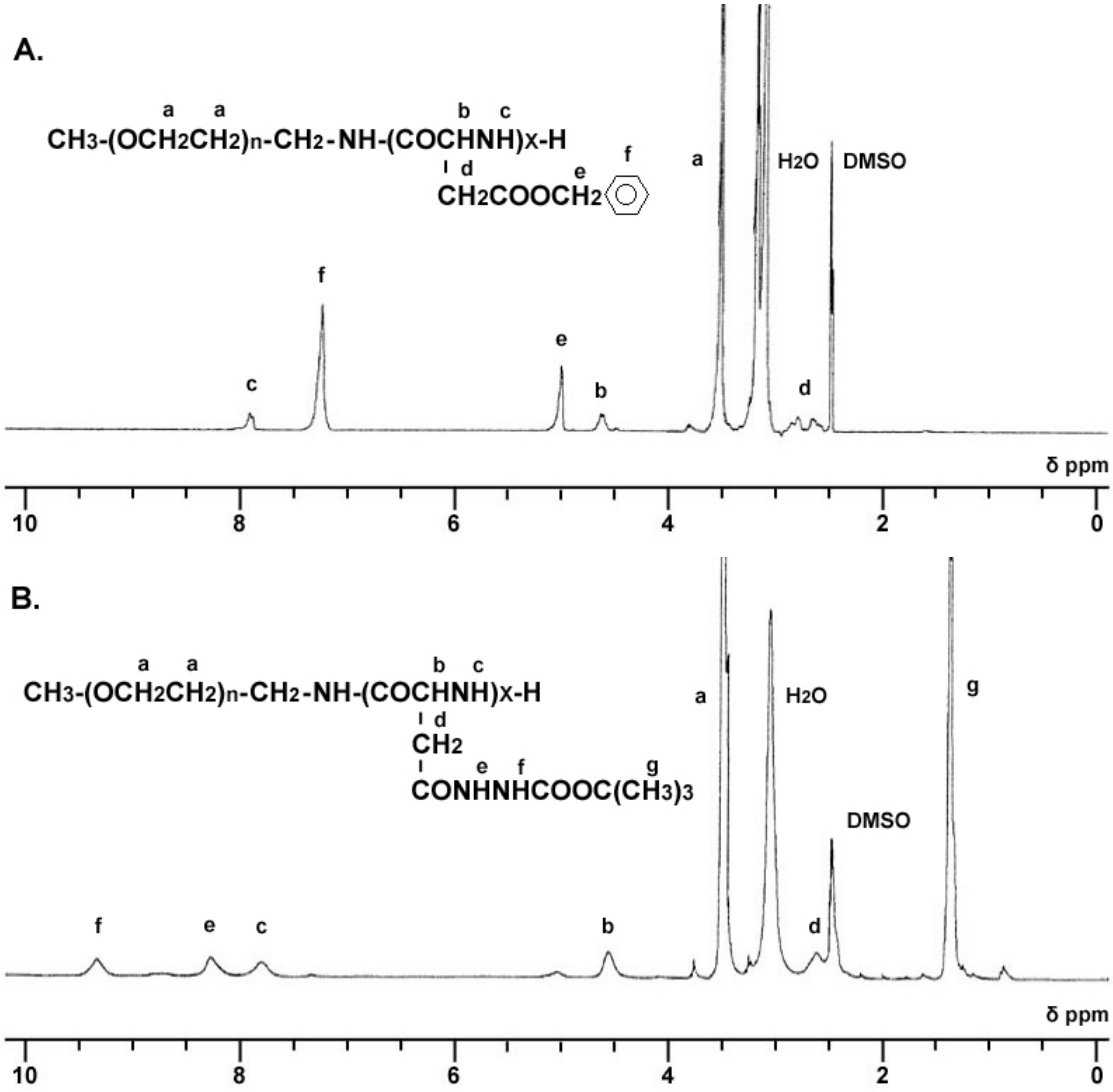

Figure 1S. ^{1}H-NMR spectra of PEG-PBLA(A) and PEG-p(Asp-Hyd-BOC) block copolymer(B) in DMSO-d_6 at 80 °C

Table 1S. Compositions of the block copolymers

Compounds	Composition [a]	Mol% [b]
PEG-PBLA	12-37	-
PEG-p(**Asp-Hyd**-BOC)	12-37-28	75.7
PEG-p(**Asp-Hyd-ADR**)	12-37-28-25 [c]	67.6 [c]

[a] Compositions are abbreviated X-Y, X-Y-Z and X-Y-Z-A. The letter X stands for molecular weight×10^{-3}, while Y, Z and A indicate the number of bold-faced units.
[b] The values are shown with respect to the number of aspartic acid residues of the block copolymer.
[c] The amount of ADR bound to copolymer was determined by RPLC.

2. Preparation of the micelles

The prepared PEG-p(Asp-Hyd-ADR) block copolymer (100 mg) was dissolved in DMAc (20 ml) and placed into the MWCO 12,000-14,000 membrane for dialysis against distilled water (pH 7.0) for 24 h at ambient temperature. The outer solution was exchanged at each 2, 6, 8, 12 and 24 h. As shown in Figure 2S, the hydrodynamic diameter of the micelles was determined by a dynamic light scattering (DLS) spectrometer (DLS-7000 Otsuka Electronics Co., Ltd.) equipped with a He/Ne ion laser (λ_0= 632.8 nm).

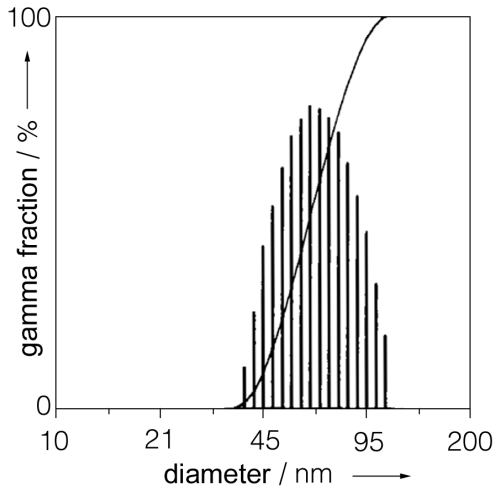

Figure 2S. Size distribution of the micelles from PEG-p(Asp-Hyd-ADR) block copolymers determined by DLS measurement.

3. Evaluation of pH sensitivity

RPLC was performed to assess the pH sensitivity of the micelles, equipped with a μ-Bondasphere 5μC4-300A column (150×3.9 mm, Nihon Waters, Tokyo, Japan) using acetonitrile/water(with 1% AcOH) gradient as a mobile phase. The loading content of the drugs on each polymer chain was also calculated with RPLC by measuring the peak intensity of the released drugs. The micelles with 10 mg/ml concentration were incubated under various pH conditions from 7.4 to 3.0 [20 mM phosphate buffer (pH 7.4 - 6.0), 20 mM

acetate buffer (pH 5.8 - 3.0)] and their time and pH-dependent drug release profile was monitored. Every 30 minutes 10 μl of each sample was injected for 80 hr, and the amount of released ADR was measured from the peak intensity by a UV detector with 485 nm wavelength. (Figure 3S)

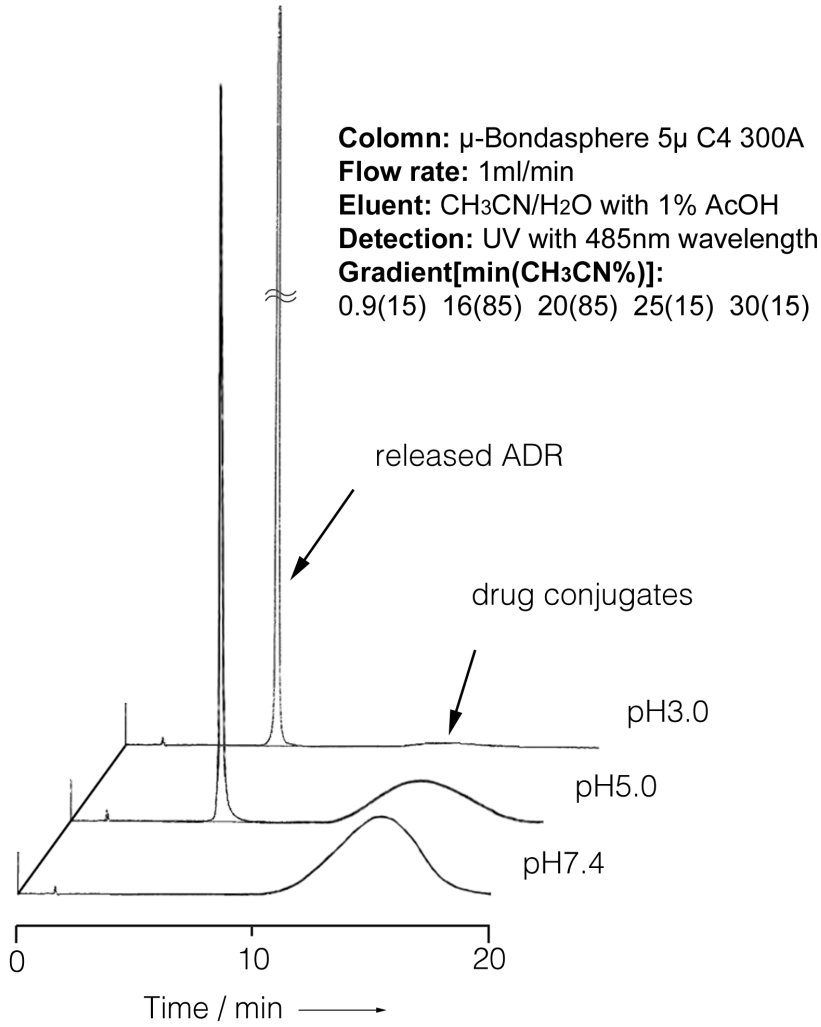

Figure 3S. The acid sensitivity of the micelles was evaluated by RPLC. As the pH decreased, the micelles released ADR which was stably bound to polymers at pH 7.4.

4. Fluorescence-quenching property

The change in the fluorescence intensity of intact ADR and the micelles was monitored by a spectrofluorometer (FP-777, JASCO, Japan) in cell culture medium, Dulbecco`s Modified Eagle`s Medium (DMEM) supplemented with 10%

fetal bovine serum for 24 h to confirm the fluorescence-quenching effect. Intact ADR and the micelle samples prepared with the ADR equivalent were excited with a wavelength of 485 nm, and the fluorescence at 590 nm was monitored. (Figure 4S)

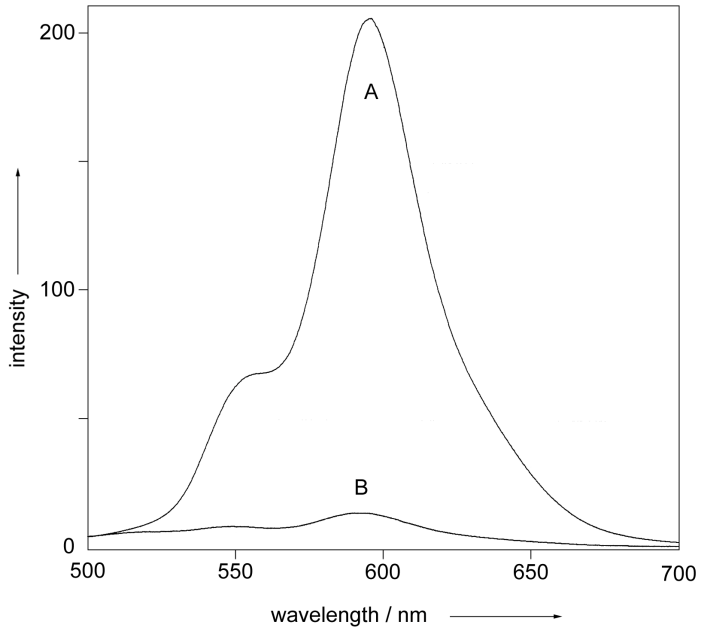

Figure 4S. The fluorescence-quenching effect of the micelles was confirmed bya UV/Vis spectrometer with 485 nm excitation. Fluorescence intensities between intact ADR (A) and the micelles (B) in the cell culture medium DMEM were considerably different in spite of the same 100 μg/ml of ADR equivalent concentrations ($ADR_{intact}/ADR_{micelle}$=1.02). The micelles selectively emitted fluorescence when they became active under acidic conditions.

5. Confocal laser scanning microscopic (CLSM) observations

A confocal laser scanning microscope (LSM 510, Carl Zeiss co., Ltd., Germany) was used for the observations on intracellular drug release of the micelles. The fluorescence images of the cells were observed with a 40X objective (Plan-neofluar, Carl Zeiss) at excitation wavelengths of 458 nm (Ar laser). For the experiments, a human small cell lung cancer cell line SBC-3 was obtained from the National Cancer Center Research Institute, Japan. The cells (1×10^4cell/dish) were cultured onto sterile 35mm glass-base dish (Iwaki Glass, Japan) in Dulbecco`s Modified Eagle`s Medium (DMEM, Sigma Chemical Co.,

Inc., USA) supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO_2 at 37 °C, followed by coincubating with ADR and the micelles while drug concentration in the medium was adjusted to 10μg/ml in terms of ADR equivalent. All images were acquired and processed with the accompanying software.

6. In vitro growth-inhibition assay

The tetrazolium dye assay, called MTT assay, was used to evaluate the growth-inhibitory effect of the micelles. MTT assay is a quantitative colorimetric assay for cell survival and proliferation, based on the ability of live cells to utilize a pale yellow substrate (a tetrazolium salt) and its subsequent modification into a dark blue formazan product. The tetrazolium is cleaved in mitochondria, so the reaction only occurs within living cells. The assay detects living, but not dead cells, and the signal generated is dependent upon the degree of activation of the cells. In 96-well culture plates, 100μl aliquots of exponentially growing cells (2×10^3/ml) were seeded and incubated for 24 h, followed by the addition of 20μl of ADR and micelle samples with various concentrations. After exposure to the samples for 3 h, 10 h and 24 h, the medium was discarded and the each cell was reincubated in fresh medium for another 24 h. After adding 20μl of MTT solution (5 mg/ml in PBS), the plates were incubated at 37 °C for 3 h. After centrifugation of the plates, the medium was aspirated, and 200μl of sodium dodecyl sulfate(SDS) solution was added to each well to dissolve the formazan. The optical density was measured at 570 nm using a Bio-RAD Microplate Reader 550. A well containing only DMEM with 10% FBS and MTT was used as control. For each drug concentration, eight wells were used. Percentage growth was calculated as (mean absorbance in 8 wells exposed

to samples – mean absorbance in 8 control wells)/(mean absorbance in 8 sample-free wells – mean absorbance in 8 control wells). The IC_{50} was defined as the ADR concentration required for 50% reduction in the optical density of each test. (Table 2S)

Table 2S: Growth-inhibitory effect of intact ADR and the micelles against the SBC-3 cell line (n=8)[a]

Sample	Exposure time (hour)	IC_{50} of samples [b] (μg/ml±SD)	Relative index [c]
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

[a] The letter "n" stands for the number of independent experiments.
[b] IC_{50} values of the micelles are calculated with the ADR equivalents.
[c] "Relative index" means the ratio between a control and the object for comparison.