Polyion Complex Micelles with Reactive Aldehyde Groups on Their Surface from Plasmid DNA and End-Functionalized Charged Block Copolymers

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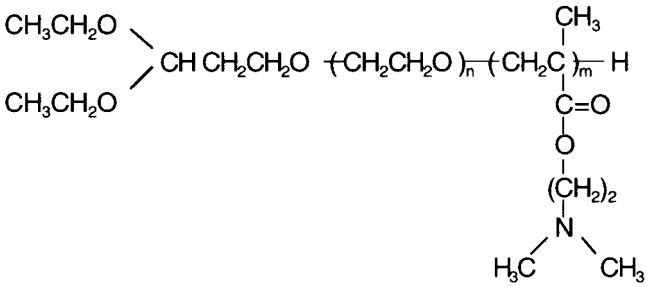
Polymer micelles formed in aqueous medium through self-assembly of block copolymers have recently received considerable attention in both the fundamental[1] and applied fields.[2] Most of the extensive studies done in these fields are related to polymer micelles from amphiphilic block copolymers composed of hydrophilic (or ionic) and hydrophobic segments assembled through the phase separation of hydrophobic segments from the aqueous medium. Recently, a novel group of polymer micelles in aqueous medium called polyion complex (PIC) micelles or block ionomer complexes was found by us[3] and Kabanov et al.[4] They are formed through electrostatic interaction of an oppositely charged pair of block copolymers with various polyelectrolytes of synthetic and natural origin. PIC micelles show unique physicochemical properties including an extremely narrow-sized distribution with core−shell architecture. Furthermore, an exclusive chain-length recognition was found to occur in the process of PIC micelle formation from a pair of charged block copolymers, indicating the process to be strictly driven through phase separation between the core- and shell-forming segments.[3c] PIC micelles were formed not only from synthetic block ionomers but also from an oppositely charged pair of block copolymers with natural polyelectrolytes including oligonucleotide, plasmid DNA, and enzyme.[5] These PIC micelles entrapping bioactive molecules have a promising utility in the field of drug delivery for targeting therapy of various diseases including cancer. Indeed, we have confirmed that the resistance of plasmid DNA against nuclease digestion remarkably increased by its incorporation into the core of the PIC micelles.[6]

From the standpoint of utilizing these PIC micelles in the field of drug delivery, the addition of ligand molecules on the tethered chain end of the shell-forming segment is of a particular importance to achieve their effective uptake into target cells through a receptor−ligand interaction. Furthermore, ligand-installed PIC micelles may have the potential utility as a starting component to construct a supramicellar assembly of two or three dimensions, which opens a new field in supramolecular chemistry. These interests in both the fundamental and applied fields led us to the present study to develop a novel way of constructing reactive PIC micelles with an aldehyde functionality on their outer layer. For this purpose, α-acetalpoly(ethylene glycol)-*block*-poly(2-(*N*,*N*-dimethylamino)ethyl methacrylate) (acetal-PEG−PAMA) was newly synthesized in this study on the basis of our previously established procedure of heterobifunctional poly(ethylene glycol) synthesis[7] as well as of anionic polymerization of (*N*,*N*-dimethylamino)ethyl methacrylate (AMA) initiated by a metal alkoxide.[8] It should be noted that the acetal end group of the block copolymer is readily transformed into a reactive aldehyde group by gentle acid treatment in aqueous medium.

Because of not only the low nucleophilic reactivity but also its aggregation characteristics, an alkoxide with an alkali metal countercation was believed to show no initiation ability to polymerize methacrylic ester monomers.[9] Recently, however, we have found that a methacrylic ester possessing a siloxy group at the *β*-position of the ester moiety can be polymerized by a simple alkoxide such as potassium ethoxide.[10] We then proposed the increased reactivity of the alkoxide initiator by the cheletion of the monomer molecule to the initiator. This polymerization system was expanded to other monomers possessing other cheleting moieties in the ester group.[11] Especially, in the case of a methacrylate possessing the 2-(*N*,*N*-dialkylamino)ethyl ester group, the polymerization proceeds without any side reaction even at 50 °C to form a linear polymer having a narrow molecular weight distribution (MWD).[8] Suitable cheletion of the monomer to the potassium alkoxide may prevent possible side reactions. Indeed, even the alkoxide of *ω*-hydroxypoly(ethylene glycol) was used as an initiator, and a linear block copolymer of PEG and PAMA was obtained without any side reaction.[12] Herein, we found that this polymerization reaction successfully proceeded even when the alkoxide of α-acetal-*ω*-hydroxy-PEG, prepared as reported,[7b] was used as an initiator to form α-acetal-PEG−PAMA as follows:



After potassium 3,3-diethoxypropanolate (PDP, 1 mmol) was prepared by the reaction between the corresponding alcohol and potassium naphthalene in THF (45 mL), 113.5 mmol of condensed ethylene oxide (EO; Saisan, Saitama, Japan) was added via a cooled syringe to the PDP solution. After the 2 day reaction of EO, 60 mmol of AMA (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added to the reaction mixture and stirred for a further 60 min at ambient temperature for the block copolymerization. The block copolymer was

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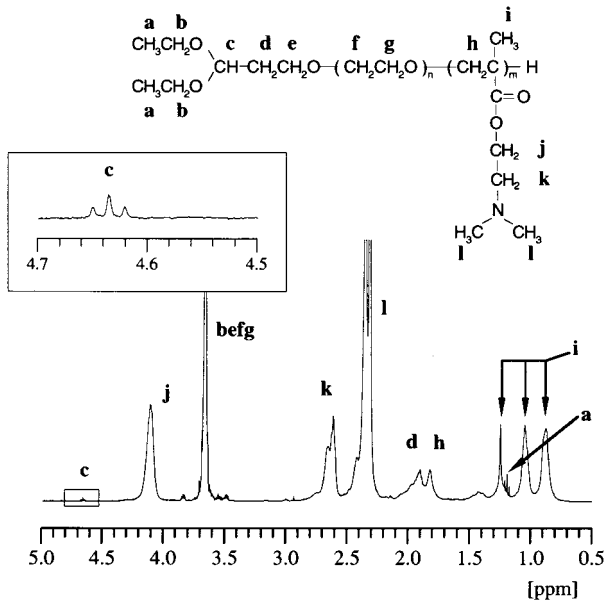

Figure 1. The 400 MHz ^{1}H NMR spectrum of α-acetal-PEG−PAMA block copolymer (solvent, $CDCl_3$).

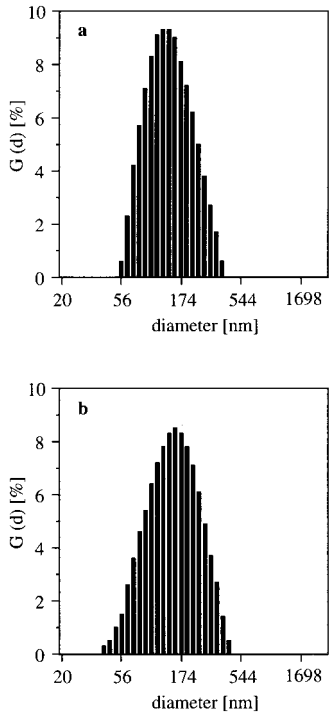

Figure 2. Comparison of the *z*-weighted size distribution before (a) and after (b) the acid treatment for PIC micelles (detection angle, 90°; temperature, 25.0 °C).

recovered by the precipitation into a large excess of 2-propanol. As we previously reported, PDP initiated polymerization of EO without any side reaction to form an α-acetal-PEG with a very narrow MWD (M_w/M_n = 1.05).[7b] The molecular weight of the prepolymer was estimated to be 5000 g/mol from the size exclusion chromatogram (SEC), which was in good agreement with the calculated data based on the feed amounts of the monomer versus initiator (5150 g/mol). By the addition of the AMA monomer to the polymerization system after the consumption of all the EO, a smooth block polymerization proceeded with only a small amount of the prepolymer remaining. Separation of the block copolymer from the unreacted PEG was accomplished by the batchwise treatment of an aqueous solution of the sample with an ion-exchange resin (SP-Sephadex C-25, Pharmacia Biotech, Tokyo, Japan) at pH 5. The block copolymer on the resin was desorbed in 0.2 M acetate buffer with 1.5 M NaCl (pH 5) and subjected to SEC analysis after a desalting by dialysis. It was confirmed from SEC that the PEG prepolymer was completely removed by this treatment, allowing to obtain the block copolymer with a narrow MWD (M_w/M_n = 1.41).

Figure 1 shows the ^{1}H NMR spectrum of the purified block copolymer in $CDCl_3$ (EX400, JEOL, Tokyo, Japan). Utilizing PEG, PAMA, and 3,3-diethoxypropanol as reference materials, the assignments were carried out and are listed in the figure. Along with both segments of PEG and PAMA, the end acetal signals (4.6 and 1.2 ppm) were observed. From the peak intensity ratio of α-methylene protons (4.1 ppm, $COOCH_2CH_2N(CH_3)_2$) of PAMA and ethylene protons of PEG (3.6 ppm, OCH_2CH_2), the polymerization degree and molecular weight of AMA were calculated to be 68 and 10 690 g/mol, which agreed well with the calculated value based on the feed molar amounts of AMA against PEG prepolymer (9580 g/mol).

The obtained α-acetal-PEG−PAMA (PEG M_w = 5000 g/mol, DP of PAMA = 68) was mixed with pGL3-Luc plasmid DNA in 10 mM Tris-HCl buffer (pH 7.4) at an equal unit molar ratio of AMA to phosphate in plasmid DNA ([α-acetal-PEG−PAMA] = 30 mg/L, [plasmid DNA] = 42.3 mg/L). The mixture maintained its apparent transparency even after the long-term storage for more than 1 month. A dynamic light scattering (DLS) measurement was then carried out for this mixture at 25.0 °C using DLS-700 (Otsuka Electronics, Osaka, Japan). The histogram[13] and cumulant methods[14] were used in the DLS analysis. The cumulant analysis of the DLS data revealed the presence of PIC micelles having a diameter of 149.0 nm with a polydispersity index (μ_2/Γ^2) of 0.19. Also, the unimodal distribution of the PIC micelles was confirmed by the histogram analysis (Figure 2a).

The PIC micelles were then immersed in an acidic environment (pH 2.5) for 2 h to transform the acetal groups located on the surface of the PIC micelles into aldehyde groups. Comparing the size distribution before (Figure 2a) and after (Figure 2b) the acid treatment by DLS, it was obvious that the acid treatment induced no change in the micelle distribution. Also, no scission or denaturation of plasmid DNA took place in this process as confirmed by gel electrophoresis (Figure 3). The presence of an aldehyde group was monitored using 1,2-diamino-4,5-dimethoxybenzene dihydrochloride (DDB; DOUJIN Laboratories, Kumamoto, Japan), which generates a strong fluorescence at 402 nm through the reaction with an aldehyde group under acidic conditions.[15] In this experiment, 15 μL of DDB in Tris-HCl buffer (0.3 g/L, 10 equiv of DDB to acetal group) was added to 1.0 mL of the PIC micelle solution, and the change in the fluorescence spectra of DDB was monitored after the addition of 30 μL of 1.0 M HCl(aq). The fluorescence measurements (ex 338 nm, em 402 nm) were carried out at 25.0 °C using a FP-770F fluorescence spectrometer (JASCO, Tokyo, Japan). Figure 4 shows the time course of the change in the emission intensity (I_m) at 402 nm with the acid treatment. Obviously, I_m increased with time and saturated at about 90 min, demonstrating the DDB conjugation to aldehyde groups

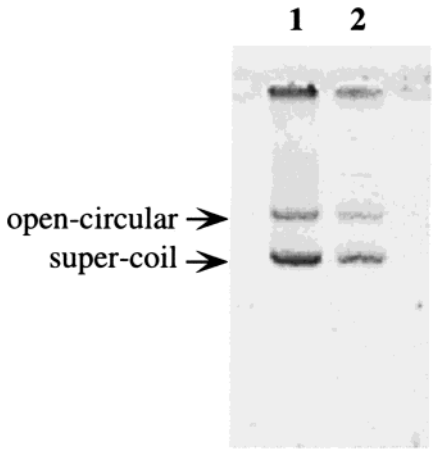

Figure 3. Electrophoretic migration of plasmid DNA in α-acetal-PEG−PAMA/plasmid DNA micelle (line 1; before acid treatment) and α-aldehyde-PEG−PAMA/plasmid DNA micelle (line 2; after acid treatment). (The excess amount of poly(vinyl sulfate) was added to the sample solution before electrophoresis analysis to release plasmid DNA from micelle. The samples were analyzed by electrophoresis under 20 mA current through 0.6% agarose gel using a Tris-HCl buffer. Gel was stained with ethidium bromide to visualize the DNA.)

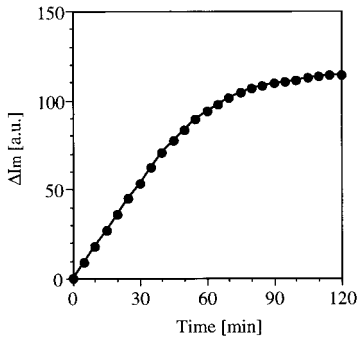

Figure 4. Change in the fluorescence intensity of α-aldehyde-PEG−PAMA/plasmid DNA micelle solution reacted with DDB (ex, 338 nm; em, 402 nm; DDB concentration, 4.48 mg/L; temperature, 25.0 °C).

at the tethered chain end of the PEG segments of the PIC micelle.

These reactive PIC micelles entrapping plasmid DNA may have potential utilities in the field of gene delivery as novel targetable carriers as well as a starting element to construct supramicellar architecture. Research in these directions is now underway in our laboratory, and the results will be reported elsewhere.

Supporting Information Available: The size exclusion chromatograms of α-acetal-PEG and α-acetal-PEG−PAMA before and after the purification by ion-exchange resin. This material is available free of charge via the Internet at jttp://pubs.acs.org.

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