Environment-Sensitive Stabilization of Core−Shell Structured Polyion Complex Micelle by Reversible Cross-Linking of the Core through Disulfide Bond

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The controlled association of diblock copolymers has attracted much attention in the fundamental and applied fields of polymer science.[1,2] In most cases, a block copolymer forms polymeric micelles or other supramolecular structures due to the difference in the solubility between the two blocks in a selective solvent.[3,4] Recently, Kabanov et al. and our group have reported a new class of polymeric micelles, the polyion complex (PIC) micelle, in which the driving force of the association is the electrostatic interaction between a pair of oppositely charged polymers.[5,6] The PIC micelle formed from poly(ethylene glycol)-*block*-poly(L-lysine) (PEG-P(Lys)), a cationic block copolymer, and anionic homopolymers of natural and synthetic origin is a spherical particle with a diameter of several tens of nanometers.[7,8] Because of the size and the characteristic core−shell structure, these PIC micelles have potential use as a carrier of oligonucleotides and plasmid DNA for human gene therapy.[9]

The stability of the micelles and the micellization behavior are influenced by various factors such as concentration, temperature, and chemical structure of the polymer. PIC micelles as well as micelles composed of amphiphilic block copolymers dissociate upon dilution when the concentration reaches the critical association concentration. In addition, the salt concentration is a key parameter for the dissociation of the PIC micelle since the Coulombic interactions between charged segments are screened by the added salt. Several groups have reported the stabilization of the polymeric micelle by cross-linking of the core[10] or the shell.[11] In these cases, the cross-linkage fixed the structure of the micelle and permanently suppressed the dissociation. For application in drug delivery systems, however, the micelle must dissociate to release entrapped drugs at the targeted site. To this end, the cross-linking by reversible bonds is a promising method if the bond is cleaved in response to physical or chemical stimuli given in the environment at the site of the drug action.

In this contribution, we report the synthesis and the characterization of the PIC micelle with the core cross-linked by the disulfide bond (Figure 1). The core of PIC micelle composed of PEG-P(Lys) and poly(α,β-aspartic acid)(P(Asp)), a model for physiologically active polyelectrolytes including an oligonucleotide, was cross-linked by the oxidation of thiols introduced in the side chains of lysine units of PEG-P(Lys). The stability under a high salt concentration and the dissociation behavior after the addition of a reducing reagent were examined by light scattering measurements. The advantage of the PIC micelles with a disulfide cross-linkage in the field of drug delivery is that the cleavage of the disulfide bond would occur within the cell because the intracellular compartment has a stronger reducing environment than the extracellular fluid.[12] This ability of the micelle with a disulfide cross-linkage might allow the controlled release of the entrapped compounds such as oligonucleotides selectively inside the cells.

Thiolation of Lysine Units of PEG-P(Lys). PEG-P(Lys) and P(Asp) were synthesized as previously described.[5] The molecular weight of the poly(ethylene glycol) (PEG) used in this study was 5000. The polymerization degree of the poly(L-lysine) block of PEG-P(Lys) and P(Asp) was determined to be 22 and 15 from the ^{1}H NMR spectra, respectively. Partial substitution of the amino groups of the polylysine block with pyridyldithiopropionyl (PDP) groups was achieved using *N*-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP),[13] which is a widely used heterobifunctional cross-linking reagent. The substitution degree (SD) of the PDP groups in the side chains was determined by ^{1}H NMR in D_2O and UV spectroscopy. From the ^{1}H NMR measurement, the SD value was determined to be 6.8 groups per one polymer chain from the peak intensity ratio of the pyridyl protons (C_5H_4N; 7.6 ppm) and the methylene protons of PEG(OCH_2CH_2; 3.5 ppm). In the UV measurement, SD was determined to be 6.7 from the absorbance of released 2-thiopyridone ($\lambda_{max} = 343$ nm, $\epsilon = 7.06 \times 10^3$)[14] after the addition of an excess of dithiothreitol (DTT), a reducing agent of disulfide, in 10 mM phosphate buffered saline (PBS, pH 7.4). The substitution degree determined by two different methods indicated ∼30% of lysine residues was modified.

Preparation of the PIC Micelles and Cross-Linking of the Core. PEG-P(Lys) and P(Asp) spontaneously associate in aqueous media through an electrostatic interaction at pH 7.4. At this pH, all of the amino groups of PEG-P(Lys) and the carboxyl groups of P(Asp) were assumed to be ionized based on the result of the titration for the nonsubstituted PEG-P(Lys) and P(Asp).[5] The number of positive charges on PEG-P(Lys) versus the number of negative charges on P(Asp) in the solution was set equal in this study. PEG-P(Lys) containing PDP groups was dissolved in 10 mM PBS (pH 7.4) and filtered through a 0.1-μm filter to remove any dust. A 3-fold excess of DTT was added to the polymer solution and stirred for 15 min to reduce the disulfide bond of PDP to a thiol. The P(Asp) solution was then added under a reductive environment to prevent formation of an intermolecular disulfide bond before the micellization of the block copolymers at ambient temperature. After that, DTT and released 2-thiopyridone were removed by dialysis against 1 L of PBS for 3 days. The micelle undergoes a cross-linking reaction by oxygen in air during the process of dialysis. After 3 days of aerial oxidization, almost no free thiol was observed using Ellman's method.[15] The stock micelle solution was optically transparent and precipitation was not observed as in the case of the original micelle[16] with the core−shell structure, in which the hydrophilic shell of PEG surrounds the core of the charge neutralized polyion complex.

Characterization of the PIC Micelles with the Cross-Linked Core. A dynamic light-scattering (DLS) study was carried out to elucidate the micelle structure at 25 °C (DLS-700 spectrophotometer, Otsuka Electronics Co., Ltd., Osaka, Japan). Details of the data analysis of the DLS measurements were reported

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(16) Original micelle (noncross-linked micelle) used as a control was prepared by simple mixing of solutions of the nonsubstituted PEG-P(Lys) and P(Asp) after filtration through a 0.1-μm filter.

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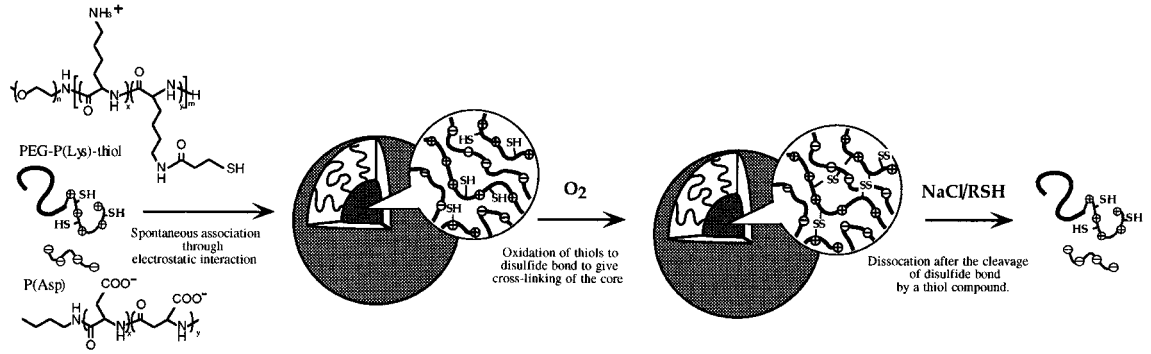

Figure 1. Schematic illustration of the environment-sensitive stabilization of the PIC micelle through the formation of a disulfide bond in the core.

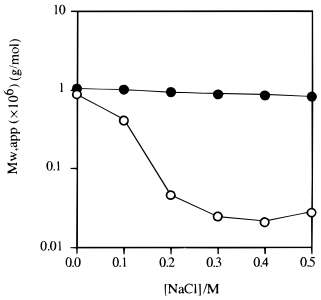

Figure 2. Change in the apparent molecular weight with the concentration of sodium chloride for: (○) the original (noncross-linked) micelle and (●) the cross-linked micelle (total concentration, 1 mg/mL; temperature, 25 °C; solvent, 10 mM PBS (pH 7.4)).

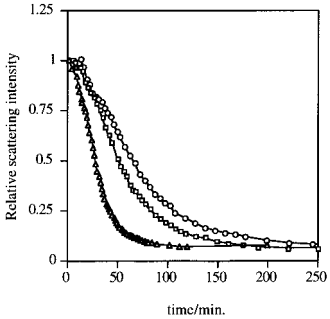

Figure 3. Change in the relative scattering light intensity after the addition of DTT ([DTT]: (○) 0.5 mM, (□) 1.0 mM, (△) 2.0 mM; total polymer concentration, 1.0 mg/mL; [NaCl], 0.3 M; temperature, 25 °C; solvent 10 mM PBS (pH 7.4)).

elsewhere.[8] The polydispersity index ($\mu_2/\bar{\Gamma}^2$), which is equivalent to the normalized *z*-average variance of the distribution of the diffusion coefficient, was determined to be as low as 0.08 by the cumulant method. The value indicated that the cross-linked micelle has a relatively narrow size distribution and a well-defined structure. The concentration dependency of the diffusion coefficient was essentially not observed in the range of the experiment (total polymer concentration = 0.5−4.0 mg/mL)(data not shown). From the diffusion coefficient of the micelle extrapolated to infinite dilution, the hydrodynamic radius (R_h) was calculated to be 16.1 nm. It should be noted that the radius determined by the cumulant method before the dialysis was 15.7 nm. The similar sizes of the micelle before and after cross-linking suggest that disulfide bond formation does not have significant effect on the structure of the micelle. No remarkable change in the size was observed for at least two weeks, indicating that the micelle has a high colloidal stability due to steric repulsion of the surrounding PEG corona.

To examine the effect of thiolation on the stability of the micelle, the dependence of the apparent molecular ($M_{w,app}$) weight on NaCl concentration was evaluated using a static light-scattering measurement with the Debye equation.[17] The $M_{w,app}$ vs NaCl concentration curves are shown in Figure 2. For the original micelle without cross-linking, a steep decrease in $M_{w,app}$ was observed when increasing the NaCl concentration since the polyion complex micelle dissociates under a high salt concentration due to the screening of the Coulombic force by the salt. Compared with the original micelle, the micelle formed between the thiolated PEG-P(Lys) and P(Asp) had a drastic improvement in stability. The decrease in $M_{w,app}$ of the core cross-linked micelle with NaCl concentration was fairy small and the cumulant diameters measured by DLS were in the range of 32.0−35.2 nm over the range of NaCl concentrations.

If the stabilization is caused by the formation of a disulfide bond, the dissociation of PIC micelle should occur when the cross-linkage of the disulfide bond is cleaved by a reducing reagent at a high salt concentration. The dissociation process after the addition of DTT at 0.3 M NaCl was monitored by the scattering intensity at the detection angle of 90°. Figure 3 shows the ratio of the scattering intensity at various incubation times to the initial value. A decrease in the scattering intensity corresponding to the dissociation was observed after the addition of the reducing agent, and the rate of dissociation was dependent on the DTT concentration. Two mM of DTT is enough to dissociate the micelle within 50 min at this condition. These results strongly suggest that the stabilization of the PIC micelle is attributed to the formation of a disulfide bond, and eventually, an entrapped substrate, in this case, poly(α,β-aspartic acid), can be released to the environment concurrently with the micelle dissociation. Note that glutathione, the most abundant reducing agent in most cells, has an intracellular concentration of approximately 3 mM,[18] while the concentration in blood is 300 times less than that and is in the range of 10 μM.[19] This significant difference in glutathione concentration between the extra- and intracellular environments gives a rationale for the intracellular delivery of charged compounds including that of DNA using the disulfide-stabilized PIC micelles with a tailored property to promptly dissociate under the physiological salt condition found inside cells. A further issue to be considered in this system is the transport of the micelle through cellular membranes. This is possibly overcome by introducing ligands on the surface of the micelle for the receptor-mediated endocytosis. A study in this direction is now in progress in our laboratory.

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Supporting Information Available: Detailed procedure of thiolation of lysine units of PEG-P(Lys) (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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