On−Off Control of Enzymatic Activity Synchronizing with Reversible Formation of Supramolecular Assembly from Enzyme and Charged Block Copolymers

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The supramolecular assembly has recently received considerable attention from various fields of science and technology.[1,2] This includes polymeric micelles formed from block copolymers in selective solvents. Many studies on polymeric micelles have been extensively carried out so far by several research groups from fundamental aspects to specified application.[3−8] The latter includes drug delivery systems,[9−12] separation technology,[13,14] optoelectronic devices,[15] and surface modification,[16,17] in which the core−shell architecture of the micelles with a size of several tens of nanometers plays a substantial role.

Recently, we have reported a new concept for the formation of polymeric micelles in aqueous medium: The formation of polyion complex (PIC) micelle through electrostatic interaction between a pair of oppositely charged block copolymers with poly(ethylene glycol) (PEG) segments.[18,19] PIC micelles are highly water soluble and narrowly distributed even under an electrically neutralized mixing ratio. High water solubility of PIC micelles is apparently due to the steric stabilization effect of PEG corona surrounding the segregated PIC core. PIC micelles were also formed by mixing an oppositely charged pair of block copolymers with a variety of polyelectrolytes including vinyl polymers, poly(amino acid)s, oligonucleotide, and enzyme.[20−24]

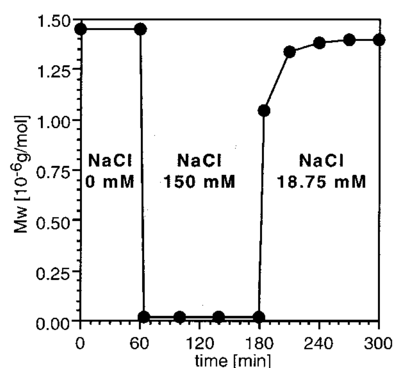

Figure 1. Reversible formation and dissociation of PIC micelles through a change in NaCl concentration: 0−60 min, 0 mM of NaCl; 60−180 min (shaded area), 150 mM of NaCl; 180−300 min, 18.75 mM of NaCl; temperature, 25.0 ± 0.1 °C.

From the standpoint of extending the micelle application in pharmaceutical and biological fields, the enzyme-loaded PIC micelles are of a particular interest, because they may have potential utilities as nanometric-scaled biochemical devices for therapy, diagnosis, and production of specialty chemicals. In a series of previous papers,[22,25] we reported in detail the physicochemical characterization of PIC micelles formed by electrostatic interaction between chicken egg white lysozyme, a cationic enzyme, and poly(ethylene glycol)-poly(α,β-aspartic acid) block copolymer [PEG-P(Asp)]. PIC micelle behaves as a nanocompartment of intact lysozymes, which are segregated from an outer environment by the PEG corona. It is of further interest to explore the activity of lysozyme entrapped in PIC micelles from the standpoint of utilizing these lysozyme-loaded PIC micelles as functional materials. Here, we report the on−off regulation of a lytic activity of lysozyme against *Micrococcus luteus* cells synchronized with the dissociation and formation of PIC micelle structure responding to a salt concentration (ionic strength) of the milieu.

The stability of PIC micelles prepared from the mixing of lysozyme (Sigma, St. Louis, MO) and PEG-P(Asp) (PEG Mw = 12000 g/mol, P(Asp) units = 15) at stoichiometric mixing ratio in sodium phosphate buffer (PBS; 10 mM; pH 7.4; $Na_2HPO_4 \cdot 12H_2O$ 2.865 g/L, $NaH_2PO_4 \cdot 2H_2O$ 0.312 g/L) was evaluated by a light scattering technique (DLS-700; Otsuka Electronics Co., Ltd., Osaka, Japan) based on the Debye equation as follows:

$$KC/\Delta R(0) = 1/\mathrm{Mw} + 2A_2C \qquad (1)$$

where C is the concentration of the polymer, $\Delta R(0)$ is the difference between the Rayleigh ratio at the detection angle of zero of the solution and that of the solvent, Mw is the weight average molecular weight, A_2 is the second virial coefficient, and $K = (4\pi^2 n^2 (\mathrm{d}n/\mathrm{d}c)^2)/(N_A\lambda^4)$ (N_A is Avogadro's number). Here, the second term of the right-hand side can be negligible, compared to the very large change in the first term (1/Mw) with the dissociation of macromolecular assembly on the order of 10^6 g/mol. Consequently, the Debye equation approximates

$$KC/\Delta R(0) \approx 1/\mathrm{Mw} \qquad (2)$$

The ionic strength of the milieu was changed in a stepwise manner by the sequential addition of PBS with or without NaCl, and the time course of a change in the scattering intensity [$\Delta R(0)$] was monitored. The monitored $\Delta R(0)$ was converted to the Mw using

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Table 1. Average Diameter and Polydispersity Index for Original PIC Micelles and Re-Formed PIC Micelles[a]

	av diameter [nm]	polydispersity index
original micelle	55.8	0.09
re-formed micelle	56.1	0.10

[a] These values were determined by cumulant analysis of DLS.

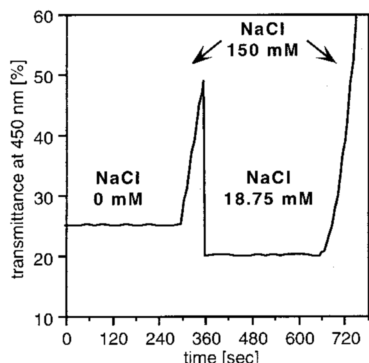


Figure 2. Change in lytic activity of lysozyme with the reversible micellization synchronizing with a variation in NaCl concentration: 0−300 s, 0 mM of NaCl; 300−360 and 660, sec (shaded area), 150 mM of NaCl; 360−660 s, 18.75 mM of NaCl; substrate, *Micrococcus luteus* cells; temperature, 25.0 °C.

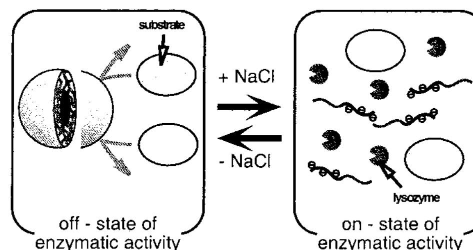
eq 2, and the results are shown in Figure 1. At the intial condition (0−60 min), the Mw of PIC micelles was monitored to be $\sim 1.5 \times 10^6$ g/mol, indicating that ~50 molecules of lysozyme were incorporated into PIC micelles.[25] Obviously, the Mw immediately decreased to 1.4×10^4 g/mol, which is very close to the molecular weights of lysozyme (1.43×10^4 g/mol) as well as that of the PEG-P(Asp) (1.46×10^4 g/mol), demonstrating the complete dissociation of the PIC micelles at 150 mM of NaCl concentration. With a decrease in NaCl concentration of the milieu to 18.75 mM by the addition of NaCl-free PBS at 180 min, the Mw increased again to the initial value at 300 min, indicating the re-formation of PIC micelles.

The reversible nature of a salt-sensitive micellization was also confirmed by the comparison of the average diameter and the polydispersity index of the re-formed PIC micelles at 300 min and the original PIC micelles prepared at the same concentration of NaCl (18.75 mM). As given in Table 1, both micelles have almost identical size and distribution, demonstrating the reversible formation of PIC micelles through a change in ionic strength.

The reversible micellization of lysozyme with PEG-P(Asp) responding to a change in ionic strength gave basis for the preparation of an intelligent bioreactor in which an enzymatic reaction is reversibly switched through the formation and dissociation of the enzyme-entrapped micelles. *Micrococcus luteus* cells (Seikagaku Kogyo Co., Tokyo, Japan) were selected as the substrate for lysozyme, and their lysis was monitored from an increase in the transmittance at 450 nm due to the rupture of the bacterial cell wall.[26,27]

Figure 2 shows the influence of ionic strength, that is, the concentration of NaCl, on a cell lysis by the lysozyme/PEG-P(Asp) system. No increase was observed in the transmittance in NaCl-free PBS, demonstrating the complete inhibition of a cell lysis. Note that lysozyme in the milieu was entrapped in the core of PIC micelles under this condition. PIC micelles have core−shell structure in which PEG segments surrounding the PIC core formed from lysozyme and the P(Asp) segments, and obviously, *Micrococcus luteus* cells cannot interact with lysozyme segregated in the core of PIC micelles due to the steric repulsion of the PEG corona. However, by increasing NaCl concentration to 150 mM at 300 s, we observed a continuous increase in the transmittance due to a cell lysis, indicating an immediate recovery of the enzymatic activity synchronized with the dissociation of PIC micelles. Furthermore, the specific activity of the lysozyme in the region of 300−360 s of incubation time was calculated from the initial slope of an increase in T%. It was determined to be 0.98 of native lysozyme, indicating that the entrapped lysozymes totally recovered their lytic activity after their release from the PIC micelle through a dissociation process. It is worth noticing that the lytic activity was totally again shut-off by decreasing NaCl concentration to 18.75 mM at 360 s via the addition of the stock suspension of Microccocus luteus cells in NaCl-free PBS. The transmittance remained constant for an additional 300 s (incubation time: 360−660 s). This complete inhibition of a lytic activity is certainly due to the re-compartmentalization of lysozyme into the core of the re-formed PIC micelles. It should be noted that the change in the transmittance from 25 (starting point) to 50 T% (inhibiting point) approximately corresponds to the change in the cell concentration from 8 to 2.6 mg/mL (~67.5% cell lysis), indicating that PEG-P(Asp) formed PIC micelles with the lysozyme even in coexistence with a substantial amount of the cell debris of the lytic cells. It was also confirmed as a control that an osmotic shock with a change in NaCl concentration did not induce such change in T% as shown in Figure 2. As can be seen from a total recovery of a cell lysis reaction by the re-addition of NaCl at 660 s, the on−off regulation of the enzymatic reaction can be repeated without any loss of lysozyme activity by a simple change in NaCl concentration.

Scheme 1. Schematic Model of On−Off Control of Enzymatic Activity through the Reversible Formation of PIC Micelles



In conclusion, this study demonstrates the on−off control of enzymatic activity synchronized with the reversible micellization of lysozyme with PEG-P(Asp) through a change in ionic strength (NaCl concentration) as shown in Scheme 1. Lysozyme entrapped in the core of PIC micelles showed no enzymatic activity against *Micrococcus luteus* cells (off-state in Scheme 1), because the PEG corona effectively inhibits the cells to interact with lysozyme in the core. However, an increase in ionic strength results in the dissociation of PIC micelles allowing the lysozyme expose in the milieu to show native lytic activity against *Micrococcus luteus* cells (on-state in Scheme 1). It is noteworthy that the association of lysozyme and PEG-P(Asp) by a decrease in ionic strength is totally reversible even in the presence of the substrate cells, and the enzymatic activity of lysozyme was completely inhibited through the re-formation of PIC micelles. This synchronized switching of the enzymatic activity with the micellization is a good example of an intelligent bioreactor that might be useful in the diverse area of medicine and biotechnology.

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Supporting Information Available: The detailed experimental procedure on the evaluation of on−off regulation of enzymatic activity synchronized with reversible formation of PIC micelles. This material is available free of charge via the Internet at http://pubs.acs.org.

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