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Switching by Pulse Electric Field of the Elevated Enzymatic Reaction in the Core of Polyion Complex Micelles

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Enzymes have attracted an appreciable amount of interest in the biomedical and bioengineering fields as therapeutic compounds, sensor devices, and biocatalysts, because they regulate a variety of biochemical reactions with high specificity under mild conditions. For these application of enzymes, the increase in their stability under operating conditions as well as the proper control of their reactivity are important subjects, facilitating a study on enzyme modification, such as the chemical conjugation of smart polymers[1] and the inclusion into functionality containers.[1,2] As for the nanometric-scaled containers (nanocontainers) of enzymes, we have been focusing on the core−shell type polyion complex (PIC) micelles made from block ionomers.[3] PIC micelles easily incorporate enzymes in the core through an electrostatic interaction in aqueous medium.[4] The egg white lysozyme, a cationic enzyme, was successfully entrapped into the core of narrowly distributed PIC micelles with an average diameter of ca. 50 nm by simple mixing with a polyanion, poly(ethylene glycol)-*block*-poly(α,*β*-aspartic acid) [PEG-P(Asp)]. A unique finding, which we would like to report here, is the remarkable elevation of the lysozyme activity through the inclusion into the PIC micelles, thus achieving for the first time the drastic switching of enzymatic reactivity synchronizing with the application of a pulse electric field.

The enzymatic activity of lysozyme in the core of PIC micelles[5] was evaluated by a colorimetric assay using a series of *p*-nitrophenyl-*N*-acetyl-*β*-chitooligosides (NAGs) with varying chain lengths, $(NAG)_2$, $(NAG)_3$, $(NAG)_4$, and $(NAG)_5$, as substrates. The released amount of the reaction product, *p*-nitrophenol, was determined from the change in the absorbance at 400 nm. The enzymatic activity of the micelle-incorporated lysozyme against each substrate is shown in Figure 1 as the relative value to the activity of the native lysozyme. The micelle-incorporated lysozyme revealed higher activities for all of the substrates, demonstrating that the enzymatic reactivity was elevated through the incorporation into the core of the micelles. Notably, the elevation became more significant with a decrease in the length of the NAGs, and almost a 100 times increase in the activity toward $(NAG)_2$ was unprecedentedly observed. We previously explained the enhanced effect of enzymatic reaction toward $(NAG)_5$ by the substrate concentration on the micellar phase.[4c,d] Although this might also be a case for $(NAG)_4$, more than 1 order of magnitude higher reactivity of $(NAG)_2$ and $(NAG)_3$ is difficult to explain only by the simple concentration effect.

The lysozyme has six sugar binding sites named A, B, C, D, E, and F aligned in the active binding cleft.[6] Site C acts as a major interacting domain of the NAG to fix it into the cleft. A susceptible glyosidic bond of the NAG then lies between sites D and E, where the catalytic residues of Glu35 and Asp52 locate to promote the hydrolysis. However, $(NAG)_2$ and $(NAG)_3$ are too short to only occupy the sites of B−C and A−B−C, respectively, with a low probability of settling in the region between sites D and E. Eventually, they are hardly hydrolyzed by the native lysozyme.[6] The remarkable elevated reactivity shown in Figure 1 against $(NAG)_2$ and $(NAG)_3$ by lysozyme in the micelles suggests that there should be a substantial change in the binding mode between lysozyme and the substrate through the former inclusion into the PIC micelles. To get insight into the mechanism, the enzymatic reaction constants, the Michaelis constant (K_m) and maximum velocity (V_{max}), of both the micelle-incorporated and the native lysozymes were determined for a series of NAGs from the Lineweaver−Burk plots (Table 1). There was no significant difference in V_{max} for all of the samples regardless of the native or micelle-incorporated lysozyme. On the other hand, $(NAG)_2$ and $(NAG)_3$ showed a remarkable decrease in K_m as compared to $(NAG)_4$ and $(NAG)_5$ through the lysozyme incorporation into the micelles, indicating a crucial increase in the affinity of $(NAG)_2$ and $(NAG)_3$ toward the micelle-incorporated form of the lysozyme. Eventually, the lysozyme in the core of the PIC micelles apparently revealed an equivalent binding affinity to all of the substrates irrespective of their chain length. Presumably, the binding specificity of $(NAG)_2$ and $(NAG)_3$ to the catalytic site D in the binding cleft of the lysozyme may drastically increase in the core of the PIC micelles.

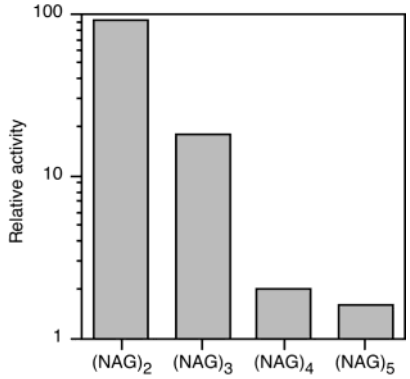

Figure 1. Relative activity of lysozyme in the core of the PIC micelles versus *p*-nitrophenyl-*N*-acetyl-*β*-chitooligosides (NAGs) (lysozyme concentration = 0.14 *μ*mol/L; PEG-P(Asp) concentration = 2.2 mg/mL; concentration of $(NAG)_2$, $(NAG)_3$, $(NAG)_4$, and $(NAG)_5$ = 500, 250, 50, and 50 *μ*mol/L; 25 °C).

This result of the elevated enzymatic reaction toward $(NAG)_2$ in the PIC micelles then led us to the idea of on−off regulation of the enzymatic reaction by an external electric field.[7] The rationale is that the structure of the PIC micelles should be sensitive to the electric field, because the main driving force of the PIC micelle formation is the electrostatic interaction. Figure 2 shows the

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Table 1. Enzymatic Reaction Constants of Micelle-Incorporated and Native Lysozyme

substrate	K_m [mol/L]	V_{max} [mol/s]
	micelles	
$(NAG)_2$	6.51×10^{-5}	1.74×10^{-8}
$(NAG)_3$	6.48×10^{-5}	2.81×10^{-8}
$(NAG)_4$	6.40×10^{-5}	4.97×10^{-8}
$(NAG)_5$[a]	6.37×10^{-5}	5.44×10^{-8}
	native	
$(NAG)_2$	1.61×10^{-2}	1.59×10^{-8}
$(NAG)_3$	3.27×10^{-3}	2.74×10^{-8}
$(NAG)_4$	2.58×10^{-4}	4.51×10^{-8}
$(NAG)_5$[a]	1.46×10^{-4}	4.96×10^{-8}

[a] Reference 4c.

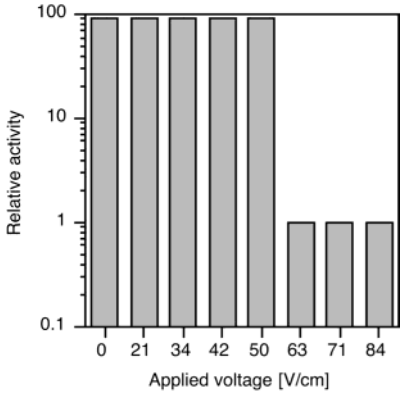

Figure 2. Change in the relative activity of micelle-incorporated lysozyme with the voltage of the applied electric field (lysozyme concentration, 0.14 μmol/L; PEG-P(Asp) concentration, 2.2 mg/mL; $(NAG)_2$ concentration, 500 μmol/L; 25 °C).

relationship between the applied voltages and relative activity of the micelle-incorporated lysozyme, where the relative activity was defined as the relative initial velocity against the native lysozyme without an electric field. Note that there was no influence of the electric field in the evaluated voltage range (0−90 V/cm) on the initial velocity of the enzymatic reaction of the native lysozyme. Obviously, the relative activity of the micelle-incorporated lysozyme discontinuously changed at the critical voltage between 50 and 63 V/cm. The elevated activity was completely shut-off and became unity at 63 V/cm.

On the basis of the result shown in Figure 2, a discrete switching of the enzymatic reaction by the alternating application of a pulse electric field across the critical voltage was expected. Indeed, Figure 3 shows such a switching of the enzymatic reaction of the micelle-incorporated lysozyme under the stepwise variation in the pulse electric field. A synchronized shut-off of the *p*-nitrophenol release is obvious from the figure. The reaction rates with and without the electric fields were 1.87×10^{-4} and 1.74×10^{-2} μmol/s, respectively, and the ratio of these reaction rates was calculated to be 93. This is in good agreement with the activity ratio of the micelle incorporated into the native lysozyme without the electric field, indicating that the elevated enzymatic reaction in the core of the PIC micelles is completely diminished by applying an electric field higher than a critical value. Notably, the *p*-nitrophenol release exhibits a very fast response without any delay time to the switching of the electric field, suggesting that the complete dissociation of the PIC micelles may not occur during this discrete switching of the enzymatic reaction. Presumably, the switching might be induced through the minute change in the local microenvironment in the core of the PIC micelles by the electric field over the critical voltage. The details of this structural change are now under investigation in our laboratory and will be reported elsewhere.[8]

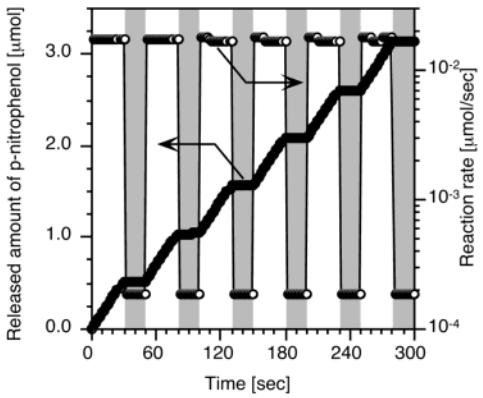

Figure 3. On−off control of the enzymatic reaction in the core of the micelles by a pulse electric field (●, released amount of *p*-nitrophenol; ○, reaction rate; pulsed voltage of 65−70 V/cm was applied in the shaded area; pulse width was 30 s with 20 s interval; lysozyme concentration, 0.14 μmol/L; PEG-P(Asp) concentration, 2.2 mg/mL; $(NAG)_2$ concentration, 500 μmol/L; 25 °C).

In conclusion, the remarkable enhanced effect of the enzymatic reaction and its on−off switching using a pulse electric field were confirmed for the lysozyme-incorporated nanocontainer of PIC micelles. These unique features of the PIC micelles are relevant for their use as smart nanoreactors in the diverse fields of medical and biological engineering.

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Supporting Information Available: The chemical structures of PEG-P(Asp) and NAGs, the synthesis and characterization of the block copolymer, and the experimental procedure of enzymatic activity evaluation (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(5) PIC micelles entrapping lysozyme in the core were prepared by mixing lysozyme and PEG-P(Asp) in sodium phosphate buffer (10 mM, pH 7.4) at the equal residual molar ratio of Asp in PEG-P(Asp) to Lys + Arg in lysozyme. The formed PIC micelles had 50.8 ± 10.1 nm of average diameter in cumulant analysis of dynamic light-scattering measurements.

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(7) A DC electric field was applied by soaking a pair of Pt wire electrodes in a quartz cell with the fixed distance of 1.0 cm.

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