Aggregate of Amphiphilic Block Copolymer Derived from Poly[(*N*-acylimino)ethylene]s and Guest-Binding Properties for 8-Anilino-1-naphthalenesulfonic Acid, Pyrene, and Enzymes

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ABSTRACT: The aggregate of an amphiphilic block copolymer consisting of poly[(*N*-acetylimino)ethylene] and poly[(*N*-pentanoylimino)ethylene] was prepared and its host properties for hydrophobic molecules in aqueous solution were studied by gel-filtration and a fluorescent probe. The aggregate showed strong guest-binding ability for 8-anilino-1-naphthalenesulfonic acid (ANS) and pyrene compared with its monomeric form. The interactions of three enzymes, horseradish peroxidase (HRP), Lipase OF, and Lipase P, to the aggregate were investigated by ultrafiltration and gel filtration analyses. The hydrolysis activities of Lipase OF and Lipase P in aqueous solution increased about 30% in the presence of the aggregate. The polymer-lipase P aggregate showed remarkably enhanced activity in comparison with the native enzyme in water-saturated organic solvent.

KEY WORDS Polymer Aggregate / Amphiphilic Block Copolymer / Poly[(*N*-acylimino)ethylene] / Gel-filtration / Fluorescent Probe / Enzyme / Lipase / Polymer-Enzyme Hybrid / Organic Solvent /

Micelle-like polymer aggregates are a novel class of a self-organized macromolecular system for constructing functional fine particles. In aqueous solution, an ionic or nonionic polymer surfactant forms intramolecular micelles under appropriate conditions. Hydrophobic bonding between the hydrophobic side chains in these systems results in stabilization of compact structures, analogous to micelles. These organized assemblies act as hosts for various molecules. Typical examples are poly(ethylenimine) with hydrophobic side chains^{1–4} and acrylamide-styrene copolymers.⁵ Their micelle-like properties and binding abilities for small molecules have been extensively investigated. However, there are few studies on interactions between the polymer aggregate and biopolymers such as en-

zymes.^{6,7}

Recently, we found that an amphiphilic block copolymer (**1**) consisting of poly[(*N*-acetylimino)ethylene] and poly[(*N*-pentanoylimino)ethylene] formed micelle-like aggregate in water and it had capability of incorporating enzymes such as horseradish peroxidase (HRP).⁷ Apparent HRP activity was not hindered by incorporation into the polymer aggregate. Moreover, the polymer-HRP aggregate could be used in organic solvents. The resulting polymer-enzyme hybrid showed significantly enhanced activity compared with native HRP in chloroform and in benzene. We propose that this self-immobilized system may serve as a new polymer-enzyme hybrid for an amphiphilic biocatalyst.

In the present paper, we describe host properties of this aggregate system toward not only proteins but hydrophobic small organic molecules. A block copolymer of acrylamide–styrene is reported to solubilize up to 300 times more pyrene than the random copolymer.⁵ This confirms that the hydrophobic block is required for micellar-like aggregation and binding abilities for hydrophobic molecules. Here we used the amphiphilic block copolymer (**1**) consisting of poly[(*N*-acetylimino)ethylene] and poly[(*N*-pentanoylimino)ethylene] which can be prepared easily since the polymerization proceeds in a living mechanism. First we investigated interactions between the aggregate of **1** and fluorescent probes, ANS and pyrene, by gel filtration. The influence of the polymer aggregate on lipase activity in aqueous solution was studied. The aggregate enhanced hydrolytic activity. Furthermore, the lipase-polymer aggregate was found to act as an excellent biocatalyst in organic solvents compared with the native enzyme.

EXPERIMENTAL

Materials

An amphiphilic block copolymer (**1**) consisting of poly[(*N*-acetylimino)ethylene] (A block) and poly[(*N*-pentanoylimino)ethylene] (B block) was prepared by two-stage block copolymerization between 2-methyl-2-oxazoline and 2-butyl-2-oxazoline according to the literature.⁸ The number average molecular weight of **1** was estimated to be 4190 by vapor pressure osmometer measurement. The unit ratio of **1** was determined by ¹H NMR, and found to be 4 (A block): 1 (B block) in mole ratios.

Horseradish peroxidase (Type II) was obtained from Sigma Chemical Co. Lipase OF and lipase P were gifted from Nagase & Co., Ltd. (Osaka, Japan). 8-Anilino-1-naphthalene-sulfonic acid ammonium salt (ANS) and pyrene were purchased from Tokyo Kasei (Tokyo, Japan) and Nacalai Tesque (Kyoto, Japan), respectively.

Measurement

A Sephacryl S-500 HR column (2 × 85 cm) was equilibrated and eluted with 50 mM phosphate buffer (pH 7.0) at room temperature. The sample was applied to and eluted from the column in equilibration buffer. Fractions (2 ml) were collected at a flow rate of 20 ml h⁻¹.

Ultrafiltration analysis was performed by an ADVANTEC UK-200 membrane (M.W. Cut-off 200000). UV spectra were recorded on a Hitachi Model U-2000 spectrophotometer. The fluorescence spectra were obtained with a Hitachi Model F-3000.

Preparation of Polymer-Enzyme Aggregate

To a solution of 10 mg of enzyme in 10 ml of 0.05 M phosphate buffer was added 0.15 g of **1** with stirring at 25 ± 2°C. The mixture was stirred further at the same temperature overnight. Then, the free polymer was removed by dialysis against 100 ml of distilled water 5 times by ultrafiltration. After lyophilization, 36 mg of white powdery aggregate were obtained. The protein content of the aggregate was determined from the UV absorption at 260 nm.

Enzyme Assay

Ester hydrolysis in 0.05 M phosphate buffer (pH 7.0) was carried out by using *p*-nitrophenyl propionate (PNP). The consumption of PNP was determined by monitoring the production of *p*-nitrophenoxide in UV spectroscopy at 400 nm. To 2 ml of 0.05 M phosphate buffer (pH 7.0) in cuvette, 50 μl of 0.11 M PNP acetonitrile solution were added and 0.2 ml of enzyme solution in pH 7.0 phosphate buffer was added at 30 ± 0.01°C.

The ester synthetic activity in organic media was assayed by using *n*-caprylic acid and *n*-amyl alcohol. To 2 ml of an water saturated organic solution containing 450 μ mol of each substrate was added 1 mg of sample. The reaction mixture was incubated at $40 \pm 0.01^\circ\text{C}$. The conversion of the substrate was determined by ^1H NMR spectroscopy.

RESULTS AND DISCUSSION

Solution Properties of the Amphiphilic Block Copolymer Aggregate

Figure 1 shows an elution profile on gel filtration chromatography for the amphiphilic block copolymer (**1**) in phosphate buffer solution (pH 7.0). The existence of the high molecular weight portion and low molecular weight part is clearly shown. The second peak at fraction number 52–65 is seen at the same location as that due to a homopolymer of poly-[(*N*-acetylimino)ethylene] ($M_n = 5000$) which does not form the intermolecular aggregate, and is therefore assigned to molecularly dissolved chains of **1**. The high molecular weight part corresponds to the polymer aggregate. In the previous report,⁷ we showed that the diameter of the isolated aggregate was

distributed mainly from 16 nm to 133 nm with a mean value of *ca.* 22 nm by dynamic light scattering measurement. The average molecular weight of the aggregate was estimated as 3×10^6 on the assumptions that it is completely spherical and its specific gravity equals to 1.0.

The proportion of the aggregate to the total amount of **1** was estimated to be 20% on the basis of the gel filtration chart. The aggregate of **1** was prepared by stirring a 0.05 M phosphate buffer solution of **1** at pH 7.0. After overnight incubation, a white powdery product was obtained by dialyzation against distilled water 5 times by ultrafiltration (M.W. Cut-off 200000) and lyophilization. The average yield of the aggregate was 24%, which is in good agreement with the fraction on the gel filtration method described above. The unit ratio of the constituent block copolymer of the aggregate which was isolated by ultrafiltration was determined by ^1H NMR, and found to be 9 (A block):5 (B block). It is not difficult to consider that the higher proportion of the hydrophobic segment than that of original block copolymer was observed, since the molecular weight and unit ratio of the polymer have rather wide distribution. These results indicate that block copolymers containing higher proportion of the hydrophobic segment have stronger ability for the aggregation in aqueous solution.

Figure 1. Elution profile on gel filtration chromatography for **1** in 0.05 M phosphate buffer solution (pH 7.0). Sample (0.8 ml) containing 13 mg of **1** was applied to and eluted from a Sephacryl S-500 column as described in the text. The polymer was monitored by measuring absorbance at 230 nm.

Interaction between Polymer Aggregate and Fluorescent Probes

The fluorescence probe method was used to investigate the binding capability of the aggregate with organic small molecules. First, interaction between the polymer aggregate and ANS was studied in an aqueous solution. ANS in a 0.05 M phosphate buffer solution (pH 7.0) had weak fluorescence and the emission maximum was 515 nm. When ANS was added to an phosphate buffer solution of the aggregate eluted at fraction number 40 on gel filtration chromatography for **1** (Figure 1), the

Figure 2. Fluorescence intensity of ANS against the concentration of **1** in phosphate buffer solution. $[\text{ANS}] = 2.0 \times 10^{-5} \text{ M}$. Intensity was plotted at 465 nm of emission spectra ($E_x = 360 \text{ nm}$).

emission maximum shifted to 465 nm and fluorescence intensity drastically increased. On the other hand, in the presence of molecularly dissolved chains of **1** which was eluted at fraction number 56, the emission maximum shifted only 485 nm and the fluorescence intensity was only 20% that in the presence of the aggregate. In the presence of poly[(*N*-acetylrimino)ethylene], which does not form the aggregate, the emission maximum of ANS was 512 nm and fluorescence intensity was only 0.4% that of ANS in the presence of the aggregate. These data indicate that the polymer aggregate has strong binding property to ANS.

Figure 2 shows the fluorescence intensity of ANS against the concentration of the block copolymer in aqueous solution containing 0.1 M KCl at pH 7.0. A critical point was clearly observed at $2.6 \times 10^{-5} \text{ M}$ of the repeating unit. Linear relationships between intensity and polymer concentration were observed above and below this critical point and the slope above $2.6 \times 10^{-5} \text{ M}$ was larger than that below the critical concentration. These results clearly indicate that **1** forms the polymer aggregate above this critical concentration.

The interaction between pyrene and polymer aggregate was studied by a gel filtration analysis in a similar manner as above. The elution pattern showed that pyrene molecules

Figure 3. Elution profile on gel filtration chromatography for **1** in the presence of pyrene. 3 ml of phosphate buffer solution containing 54 mg of **1** and 13 mg of pyrene was stirred for 10 h at room temperature. After a precipitate was removed, the filtrate (0.8 ml) was applied to and eluted from a Sephacryl S-500 column as described in the text. The polymer was monitored by measuring at 230 nm, and pyrene was monitored by fluorescence intensity at 374 nm of emission spectra ($E_x = 310 \text{ nm}$).

were strongly incorporated into the polymer aggregate, and did not interact with the free polymer (Figure 3).

Interaction between Polymer Aggregate and Enzymes

The polymeric aggregate could work as a host not only for various small organic molecules but for large biopolymers such as proteins. Especially, a conjugate of enzyme and the polymer aggregate would lead to a novel polymer-enzyme hybrid system. In the previous report,⁷ we found that the aggregate incorporated enzymes such as HRP after mixing **1** and the enzyme in a phosphate buffer solution (pH 7.0), followed by dialyzation through a membrane (M.W. Cut-off 200000). HRP content was found to be 9.4% from UV spectroscopy. Here we examined the binding properties of the aggregate for proteins in detail. For this purpose, we used three enzymes, HRP, Lipase OF, and Lipase P, and investigated their interactions with the aggregate by ultrafiltration and gel filtration analysis.

HRP was incubated with the polymer ag-

Figure 4. Remaining HRP concentration after dialysis through the membrane (M.W. Cut-off 200000) several times in the presence of **1** (○), and poly[(*N*-acetylimino)ethylene] (△), and in the absence of the polymer (●). HRP was monitored at 403 nm.

gregate for 5 h in a phosphate buffer solution (pH 7.0) and the remaining protein concentration after dialysis through membrane was estimated by UV spectroscopy and this was repeated several times. The results were compared with those for HRP without the aggregate and with poly[(*N*-acetylimino)ethylene] and are shown in Figure 4. The enzyme interacted with the polymer aggregate, because there was marked tendency to resist passage through the membrane. Such tendency was hardly recognized with the homopolymer of poly[(*N*-acetylimino)ethylene]. HRP was mixed with **1** in a phosphate buffer solution (pH 7.0), and the mixture was applied to Sephacryl S-500 HR column (Figure 7). Free HRP was also recognized. The concentration of the incorporated HRP estimated from the gel filtration chart was 2 wt%. This value was similar to that of the isolated polymer-HRP aggregate obtained by ultrafiltration described previously.⁷

Lipase P was mixed with **1** in a phosphate buffer solution (pH 7.0), and the mixture was applied to a Sephacryl S-500 HR column. It was hard to estimate protein concentration by UV spectroscopy, since protein content was not so much. Thus Lipase P concentration was

Figure 5. Elution profile on gel filtration chromatography for **1** in the presence of lipase P. 2 ml of phosphate buffer solution containing 53 mg of **1** and 12 mg of lipase P were stirred for 4 h at room temperature. The mixture (0.8 ml) was applied to and eluted from a Sephacryl S-500 column as described in the text. The polymer (a) was monitored by measuring at 230 nm, and concentration of lipase P (b) was determined from the hydrolysis activity of PNP. The elution profile of lipase P is shown as (c).

determined from the hydrolysis activity of PNP. The results of gel filtration analysis are shown in Figure 5. We found Lipase P was largely incorporated into the polymer aggregate, and contamination of free lipase P was hardly recognized.

The aggregate of **1** with lipase P was easily prepared by mixing them in a 0.05 M phosphate buffer solution (pH 7.0). After overnight reaction, a white powdery product was obtained from dialysis by using ultrafiltration with a membrane (M.W. Cut-off 200000) and subsequent lyophilization. The gel filtration chart of the resulting product is shown in Figure 6. Obviously, the molecular weight of the main peak was much higher than those of lipase and the molecularly dissolved block copolymer. Although the isolated product contained a small amount of molecularly dissolved chains of **1**, the contamination of free lipase P was hardly recognized. The protein content in the product was estimated as 60 wt% on the basis of UV spectroscopy. This value was in fair agreement with the result of gel filtration. Figure 6 indicates that all lipase P should be incorporated into the polymer

Figure 6. Elution profile on gel filtration chromatography for the lipase P-polymer aggregate. A sample (0.8 ml) containing 4 mg of lipase-polymer aggregate was applied to and eluted from a Sephacryl S-500 column as described in the text. Concentration of the polymer (a) and lipase P (b) was monitored as Figure 5.

Figure 7. Elution profile on gel filtration chromatography for **1** in the presence of HRP. 3 ml of phosphate buffer solution containing 48 mg of **1** and 1 mg of HRP were stirred for 5 h at room temperature. The mixture (0.8 ml) was applied to and eluted from a Sephacryl S-500 column as described in the text. The polymer (a) was monitored by measuring absorbance at 230 nm, and the concentration of HRP (b) was determined at 403 nm.

aggregate, and the calculated lipase P content was 50 wt%.

Lipase OF was found to interact with the aggregate after ultrafiltration. However, the incorporation of Lipase OF was hardly recognized after gel filtration. These results suggest that the binding of the enzyme to the aggregate is highly dependent on the nature of the enzyme.

Table I. Enzymic activity of lipase P and lipase OF in aqueous media in the presence of the polymer aggregate

Enzyme	Activity/ $\mu\text{mol min}^{-1} \text{mg}^{-1}$ ^a	
	Without aggregate	With aggregate ^b
Lipase P	1.58 ± 0.10	2.08 ± 0.07
Lipase OF	4.67 ± 0.36	5.92 ± 0.64

^a Ester hydrolysis in 0.05 M phosphate buffer (pH 7.0) was carried out using *p*-nitrophenyl propionate (PNP) as described in the text. ^b 2 ml of phosphate buffer solution containing 2.2 mg of lipase P or 0.4 mg of lipase OF were added to 4 ml of the aggregate solution (0.5 mg ml^{-1}) and the mixture was stirred for 4 h at room temperature. 150 μl of this sample were used as the enzyme solution.

Influence of Polymer Aggregate on Lipase Activity in Aqueous Solution

We found that the apparent HRP activity was not reduced by the incorporation to the polymer aggregate.⁷ To estimate the influences of the aggregate on lipase activity in an aqueous phosphate buffer solution (pH 7.0), hydrolysis activities for PNP were investigated. The solution containing the polymer aggregate and lipase was incubated for 3 h, and used as an enzyme solution as described in the experimental section. The experimental results are given in Table I. It was surprising that both hydrolysis activities of lipase OF and lipase P in the presence of the aggregate increased about 30%. The reason for this high activity in the presence of the aggregate is not clear. Perhaps, under the present conditions, lipase should be incorporated to the polymer aggregate within 3 h, and the hydrophobic substrate also was mainly distributed in the aggregate. Therefore, the increased local concentration of the substrate around the enzyme enhanced the apparent activity. The enhancement of lipase activity by interaction with the polymer is an alternative explanation, since lipase exerts catalytic ability by its hydrophobic binding site, which is bound to a substrate to cause conformational change.^{9,10}

The hydrolysis activity of the isolated polymer-lipase P aggregate was also assayed by PNP in a 0.05 M phosphate buffer solution (pH 7.0). The activity of lipase P increased about 40% based on lipase content estimated from UV spectroscopy. This value agree very closely with that obtained in the above experiment.

Ester Synthetic Activity in Organic Solvent

Lipase is a very important enzyme as a biocatalyst for organic synthesis, since it catalyses ester synthesis, transesterification and peptide synthesis in non-aqueous media.¹¹⁻¹³ To compare the ester synthetic activity of the polymer-lipase P aggregate with that of the native lipase, esterification between *n*-amyl alcohol and *n*-caprylic acid in water-saturated *n*-hexane was investigated. The activities of free lipase P and the polymer-lipase P aggregate were 0.9 ± 0.2 and $2.4 \pm 0.6 \mu\text{mol h}^{-1} \text{mg}^{-1}$ of lipase, respectively. The aggregate showed remarkably enhanced activity, *i.e.*, more than 2 times that with the native enzyme in water-saturated *n*-hexane.

The polymer-HRP aggregate also showed enhanced activity compared with native HRP in water-saturated chloroform and benzene.⁷

These result shows that this new type of polymer-enzyme hybrid is an excellent biocatalyst in non-aqueous as well as aqueous media.

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