Polymer Journal, Vol. 27, No. 11, pp 1071—1078 (1995)

Aggregates of Amphiphilic Block Copolymers Derived from Poly[(*N*-acylimino)ethylene]s and Their Complexes with Lipase in Water

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(Received February 20, 1995)

ABSTRACT: A series of amphiphilic block copolymers (**1**) containing poly[(*N*-acetylimino)-ethylene] as a hydrophilic block was prepared and solution properties were studied by gel-filtration method, dynamic light scattering, ^{1}H NMR, and viscosity measurement. Except for polymers containing poly[(*N*-propionylimino)ethylene] as a hydrophobic segment, all polymers formed micelle-like polymer aggregates. The proportion of the aggregate fraction increased with hydophobicity of the polymer. Regardless of the structures of the hydrophobic segments, the particle sizes of the polymer aggregates fractionated by gel filtration chromatography were *ca.* 150 nm by dynamic light scattering. Interactions of Lipase P with the resulting polymer aggregates in water were studied by the gel-filtration method. We found all aggregates to strongly incorporate Lipase P, and enhance the hydrolysis of Lipase P. The maximum amount of Lipase P incorporated to the polymer aggregate was 4000.

KEY WORDS Polymer Aggregate / Amphiphilic Block Copolymer / Poly[(*N*-acylimino)ethylene] / Gel-Filtration / Enzyme / Lipase / Polymer–Enzyme Hybrid / Complex /

Many studies show that block copolymers of hydrophobic segments and hydrophilic segments form spherical micelle-like aggregates in water.[1-10] These water-soluble polymer aggregates are of interest due to their host properties for various materials. In aqueous solution, ionic or nonionic polymer surfactant forms intramolecular micelles under appropriate conditions. Hydrophobic bonding between the hydrophobic segments in these systems results in stabilization of compact structures, analogous to micelles. Typical examples are block copolymers of polystyrene and poly(ethylene oxide).[1,5,6] Their micelle-like properties and binding abilities for small molecules have been extensively investigated.[2,3,5,10]

The polymer aggregate may serve as a host not only for various small organic molecules but for large biopolymers such as proteins. However, there are few studies on interactions between polymer aggregates and proteins.[9-11] We reported that an amphiphilic block copolymer consisting of poly[(*N*-acetylimino)-ethylene] and poly[(*N*-pentanoylimino)ethylene] formed micelle-like aggregate in water and incorporated enzymes such as horseradish peroxidase (HRP)[9] or Lipases[10] as well as small hydrophobic organic molecules.

These conjugates may lead to a novel polymer–enzyme hybrid system. The ester hydrolysis activity of lipase increased about 30% in the presence of the aggregate in water.[10] Our polymer–enzyme aggregate can function not only in aqueous but also in organic solvents. The resulting polymer HRP aggregate showed significantly enhanced activity compared with

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native HRP in chloroform and benzene.[9] We also applied these polymer-enzyme aggregate to the easiest preparation of biosensors.[12] The aggregate easily encapsulates enzymes only by mixing the block copolymer and enzymes in water, and the polymer–enzyme aggregate was sandwiched between two porous polymeric membranes to provide an enzyme membrane.

In this paper, we synthesized a series of amphiphilic block copolymers (**1**) containing poly[(*N*-acetylimino)ethylene] as hydrophilic block and several hydrophobic poly[(*N*-acylimino)ethylene] segments and studied the solution properties in water. Aggregate formation was studied by gel-filtration chromatography, dynamic light scattering, ^{1}H NMR, and viscosity measurement. The interactions of Lipase P with the resulting polymer aggregates in water were investigated by gel-filtration analysis and hydrolysis activity. We also estimated the maximum amount of Lipase incorporated into a polymer aggregate.

EXPERIMENTAL

Materials

2-Propyl-, 2-butyl-, and 2-octyl-2-oxazoline were prepared as described in the literature.[13] Commercially available 2-methyl- and 2-phenyl-2-oxazoline were dried over KOH and purified by distillation under nitrogen atmosphere. Amphiphilic block copolymers (**1**) were prepared by two-stage block copolymerization between 2-methyl-2-oxazoline and 2nd oxazoline according to the literature (Scheme 1).[14]

Lipase P was provided by Nagase & Co., Ltd. (Osaka Japan).

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. A sample was applied and eluted from the column in equilibration buffer. Fractions (2 ml) were collected at a flow rate of 20 ml h^{-1}.

^{1}H NMR and UV spectra were recorded on JOEL JNM-GSX400 and HITACHI Model U-2000 spectrophotometers, respectively. Dynamic light scattering measurements were carried out by a COULTER N4SD submicrometer particle analyzer.

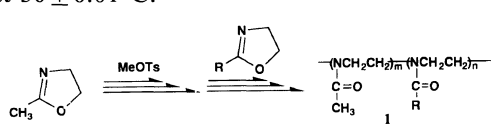
Viscosity was measured by an Ostwald viscometer at 25°C. Measurements were made in the concentration range 24—3 mg ml^{-1}. Plots of η_{sp}/c and polymer concentration were extrapolated to infinite dilution to obtain intrinsic viscosity. All solutions were filtered through membrane filters with a nominal pore size of 1.2 μm before measurement.

Preparation of Polymer Aggregate

Amphiphilic block copolymers (**1**) containing poly[(*N*-acetylimino)ethylene] as hydrophilic block were prepared by two-stage block copolymerization between 2-methyl-2-oxazoline and 2nd oxazoline (Scheme 1).[14] Table I lists the obtained block copolymers for this study. All block copolymers except the most hydrophobic polymer, **1d-2**, were soluble in water. Polymer sample 0.2 g was dissolved in 10 ml 0.05 M phosphate buffer (pH 7.0) at room temperature and stirred overnight. **1d-2** was first dissolved in a 1/1 mass percent mixture of ethanol-water and stirred at room temperature overnight. The polymer solution was dialyzed against distilled water 5 times, using a dialysis tube.

Enzyme Assay

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



Scheme 1.

RESULTS AND DISCUSSION

Solution Properties of Amphiphilic Block Copolymer Aggregate

Each block polymer (**1**) dissolved in 0.05 M phosphate buffer (pH 7.0) was eluted from Sephacryl *S*-500 HR column at room temperature (Figure 1). All samples showed peaks at fraction numbers 52—56, corresponding to molecularly dissolved chains of **1**, because a homopolymer of poly[(*N*-acetylimino)ethylene] ($M_n = 5000$) was eluted at the same loca-

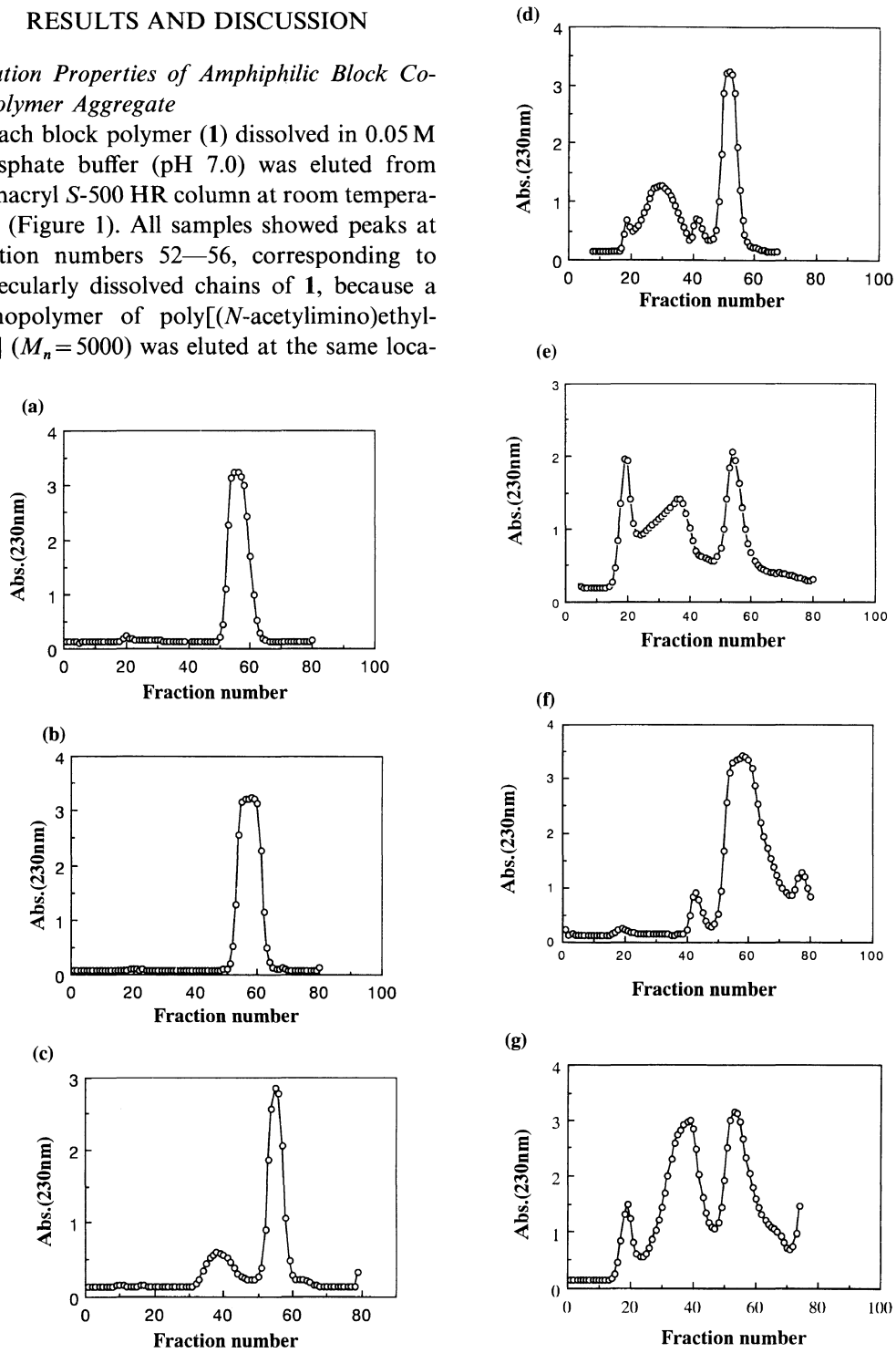


Figure 1. (a)~(g)

(Figure 1. (h), (i) and the figure caption: see page 1074)

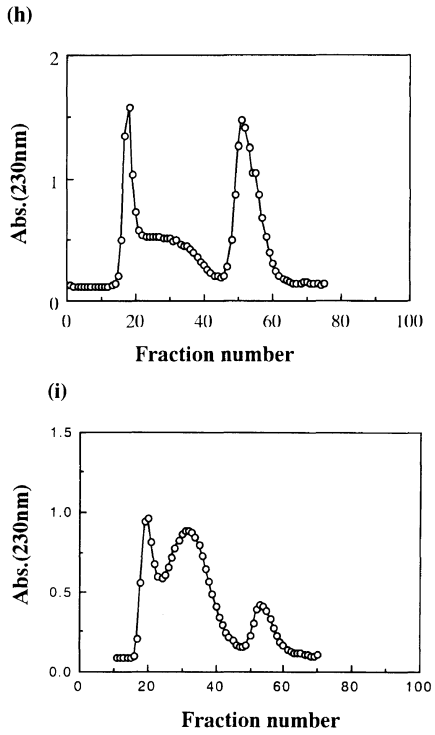


Figure 1. (h) and (i)

(Figure 1. (a)~(g): see page 1073)

Figure 1. Elution profile on gel filtration chromatography for **1a-1** (a), **1a-2** (b), **1b-1** (c), **1b-2** (d), **1b-3** (e), **1c-1** (f), **1c-2** (g), **1d-1** (h), and **1d-2** (i) in 0.05 M phosphate buffer solution (pH 7.0). Sample (0.8 ml) containing 13 mg of **1** was applied and eluted from the Sephacryl S-500 column as described in the text. The polymer was monitored by measuring absorbance at 230 nm.

tion. When block copolymers are placed in a selective solvent, *i.e.*, a good solvent for one block but a nonsolvent for the other, colloidal particles or polymeric micelles are formed due to association of the insoluble segment.[7] In our amphiphilic block copolymer system, hydrophobic interaction between water-insoluble segment may be the main driving force to form aggregates. Except **1a-1** and **-2**, the peak at the lower elution volume was seen and corresponded to polymers of higher molecular weight. Elution profiles for **1a-1** and **-2** indicated that Poly[(*N*-propyonylimino)ethylene] does not have enough hydrophobicity to form polymer aggregates.

The proportion of the aggregates was estimated by relative peak areas in the chromatograms, as listed in Table I. The proportion of aggregate fraction varied considerably as a function of the block copolymer composition. The proportion of aggregate increased with the length of the hydrophobic segment, *e.g.*, for the series **1b-1**, **-2**, and **-3**, the aggregate content increased from 24% to 67%. The proportion of the aggregate also increased with the hydrophobicity of 2nd oxazoline. The most hydrophobic block copolymer **1d-2** showed the highest aggregate content.

The following experiment was performed to determine whether the presence of a molecu-

Table I. Properties of block copolymers of 2-oxazolines and their aggregates

Sample	*R*	M_n[a]	*DP*[a]		Aggregate content[b]	Diameter[c]
			m	*n*	% of total	nm
1a-1	*n*-C_3H_7	3630	32	8	0	no[d]
1a-2	*n*-C_3H_7	5070	33	20	0	no[d]
1b-1	*n*-C_4H_9	4190	36	9	24	—
1b-2	*n*-C_4H_9	4890	38	13	47	150
1b-3	*n*-C_4H_9	7140	54	20	67	140
1c-1	Ph	5200	49	7	7	—
1c-2	Ph	9280	71	22	58	170
1d-1	*n*-C_8H_{17}	4730	34	10	53	—
1d-2	*n*-C_8H_{17}	6440	39	17	86	150

[a]Determination by ^1H NMR spectra. [b]Determined by gel filtration chromatography. [c]Determined by dynamic light scattering. [d]No aggregate was formed.

larly dissolved chain peak is a manifestation of dynamic equilibrium between single chains and polymer aggregates. The polymer aggregate was separated from a molecularly dissolved chain peak by collecting the aggregate fraction in gel filtration chromatography. The aggregate fraction was then reinjected into the Sephacryl S-500 column after the aggregate fraction was incubated at room temperature for one day. Figure 2 shows the chromatogram of the **1d-2** sample after separated from a single chain peak. The area of the single chain peak in the original mixture corresponds to 14% of the total area of the peaks. The presence of molecularly dissolved single polymer chain peak is hardly recognized in Figure 2(b).

Particle sizes of the polymer aggregate peaks at fraction numbers **23—45** of gel filtration chromatography were determined by dynamic light scattering (Table I). As mentioned above, aggregate solutions contained only unimodal high molecular weight parts after separated by gel filtration. Regardless of the structures of hydrophobic segments, diameters of the aggregates were approximately 150 nm.

The structure of original **1b-3** sample was confirmed by ^{1}H NMR spectrum in $CDCl_3$, in which its characteristics peaks of the hydrophobic and hydrophilic segments are clearly identified (Figure 3a). When ^{1}H NMR of original **1b-3** sample was measured in D_2O, small, broad peaks were seen at 0.9, 1.3, 1.6, and 2.3 ppm which identified the protons of the pentyl group of the polymer (Figure 3b). The small, broad signals indicate restricted motion of these protons within the micellar core. The peak area of poly(ethyleneimine) moiety at 3.5 ppm was reduced to 70% of ideal peak signal, which indicated the poly[(*N*-

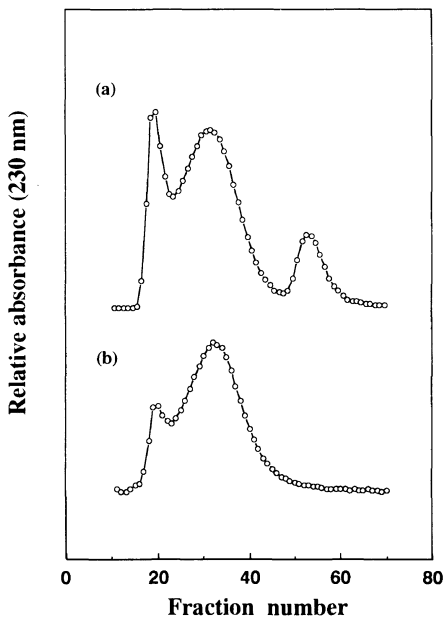


Figure 2. Isolation of the aggregate peak of **1d-2** by gel filtration chromatography. The elution profile for original **1d-2** (a), and reinjected sample after collecting fractions of 25—45 (b).

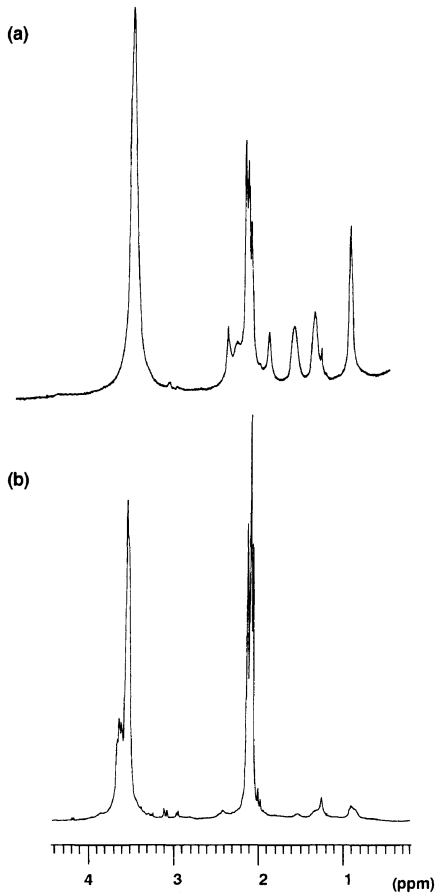


Figure 3. (a) ^{1}H NMR of original **1b-3** in $CDCl_3$. (b) ^{1}H NMR of original **1b-3** in D_2O.

pentanoylimino)ethylene] block to be a rigid structure. This suggests that the core of the polymer in aqueous solution is a very rigid structure and a hydrophilic segment is a relatively mobile shell. This behavior was also seen in the ^{1}H NMR spectra of the pheny groups of **1c-1** and **-2**.

To obtain the density of the polymer aggregate in aqueous solution, viscosity measurements were carried out on the polymer aggregate of **1b-3** which was separated from single chains by ultrafiltration (M_w cut-off 200000). The presence of molecularly dissolved single polymer chain peak was hardly recognized by gel filtration chart. We used the following relationship,[4] obtained from Einstein laws for a solution of sperical particles,

$$[\eta] = 2.5/\rho \qquad (1)$$

where $[\eta]$ is the intrinsic viscosity of the polymer aggregate, and ρ is the density of the polymer aggregate. The viscosity measurements performed on five copolymer concentrations give an intrinsic viscosity of 0.38 dl g^{-1}. We found the density of the polymer aggregate (ρ) of 0.07 g ml^{-1}. This indicates that the polymer aggregate must have a very loose structure in water, *i.e.*, less than 10% of the aggregate particles consisted of the block copolymer on the assumption that the polymer aggregate is completely spherical and its specific gravity is 1.0.

Complex Formation between Polymer Aggregate and Lipase

Among many enzymes, lipase is an important enzyme as a biocatalyst for organic synthesis. Therefore, we studied the host properties of polymer aggregates for Lipase P. First, Lipase P was mixed with each polymer in phosphate buffer solution (pH 7.0), and the mixture was applied to a Sephacryl S-500 HR column. Lipase P concentration in each fraction was determined from the hydrolysis activity of PNP. A typical elution profile on gel filtration chromatography for **1b-1** was shown in the previous paper.[10] We found all polymer aggregates obtained from this study to have strong capability of incorporating Lipase P, and contamination of free Lipase P was hardly recognized.

It was previously shown the hydrolysis activity of lipase P in the presence of the aggregate of **1b-1** increased about 30%.[10] To estimate the influence of the other polymer aggregates on lipase activity in an aqueous phosphate buffer solution (pH 7.0), hydrolysis activity for PNP was investigated. The solution containing the polymer aggregate and lipase was incubated for 3 h, and then used as an enzyme solution as described in the experimental section. Gel-filtration analysis revealed that all polymer aggregates completely incorporated Lipase P. The experimental results are given in Figure 4. All aggregate samples show activity enhancement. Especially, Lipase P with **1b-3** catalyzed the hydrolysis of PNP about three times faster than native Lipase P.

Figure 5 shows plots of hydrolysis activity of Lipase P *versus* incubation time with polymer aggregate of **1d-2** in a phosphate buffer solution (pH 7.0). The activity reached equilibrium after 3 h. It is assumed that the complexation between the aggregate and Lipase P was complete after 3 h incubation in the

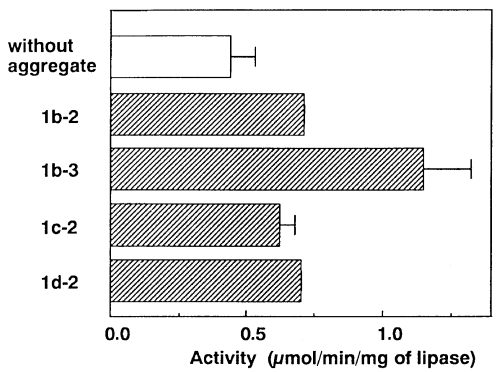


Figure 4. Hydrolysis activity of Lipase P in the presence of the polymer aggregate of **1b-2**, **-3**, **1c-2**, and **1d-2** in 0.05 M phosphate buffer solution (pH 7.0). Average data from six experiments except in the case **1b-2** and **1d-2**, of which data from a single experiment.

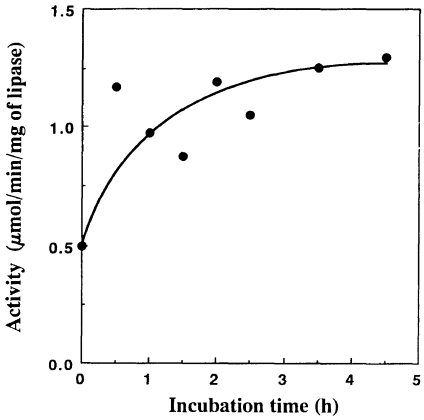


Figure 5. Plots of hydrolysis activity of Lipase P *versus* incubation time with polymer aggregate of **1d-2** in a phosphate buffer solution (pH 7.0). [Lipase P] = 0.2 g l^{-1}; [**1d-2**] = 7.0 × 10^{-2} g l^{-1}.

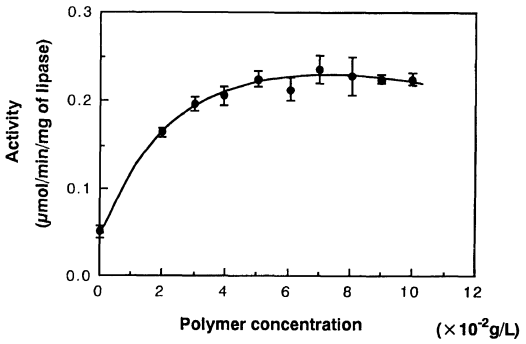


Figure 6. Plots of hydrolysis activity of Lipase P against the concentration of **1d-2** in a phosphate buffer solution. Activity was measured after the solutions containing the aggregate and Lipase P were incubated for 3 h. [Lipase P] = 0.2 g l^{-1}.

experimental conditions. Evidence for complete incorporation after 3 h incubation was also obtained by gel-filtration analysis as described before.

To estimate the maximum amount of lipase incorporated into the polymer aggregate, we studied the concentration of binding lipase against initial concentration of aggregate at constant lipase concentration. Since lipase incorporated into the polymer aggregate has higher activity than free lipase, we assumed activity increase indicates increased lipase binding to the aggregate. When polymer aggregate concentration is low, there is not enough capacity for incorporating all Lipase P. Therefore, free and binding Lipase P may coexist. At this stage increment of activity seems to directly correspond to increment of binding Lipase P. When the polymer aggregate is added enough to incorporate all enzyme, the activity enhancement becomes constant. We thus measured lipase activity against the concentration of the polymer aggregate of **1d-2** to estimate the saturation point of activity enhancement corresponding to the maximum amount of binding Lipase P to the polymer aggregate. Figure 6 shows plots of hydrolysis activity of Lipase P against concentration of the polymer aggregate. Activity was maximum at [Lipase]/[Aggregate] = 2.9, which indicates the maximum amount of binding Lipase P to the polymer aggregate is 4000.

The above assumption was further supported by gel filtration chromatography. Lipase P was completely incorporated into the polymer aggregate corresponded to the aggregate concentration beyond the saturation point (Figures 7b, c). Free lipase was recognized when excess lipase existed (Figure 7a). The maximum amount of binding Lipase P was 74 wt%. This is in good agreement with previous results that protein content in the isolated polymer aggregate was 60 wt% by UV spectroscopy and gel filtration analysis.[10]

Hydrolysis activity slightly decreased when polymer concentration increased beyond the saturation point. That activity enhancement may be due to increased local concentration of the substrate around enzyme. We already reported that the aggregate showed strong guest-binding ability for hydrophobic small molecules such as 8-anilino-1-naphthalenesulfonic acid and pyrene.[10] Fluorescence measurement indicated that these guest molecules interact immediately with the aggregate. The hydrophobic substrate should therefore be incorporated into the aggregate in which Lipase P already exists. When the polymer

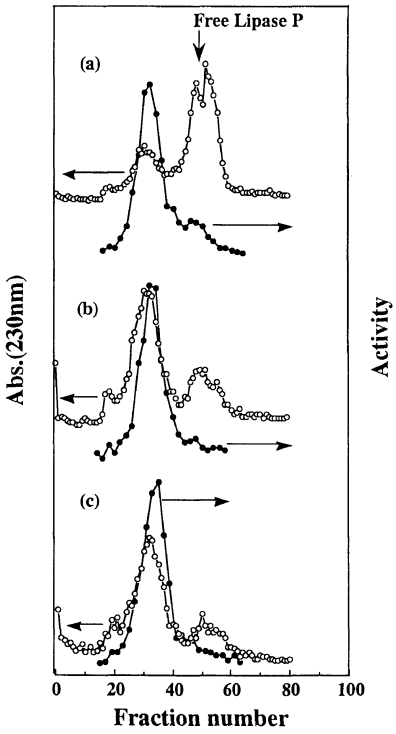


Figure 7. Elution profile on gel filtration chromatography for samples containing polymer aggregate of **1d-2** and Lipase P in a phosphate buffer solution (pH 7.0). Lipase P concentrations were $10\,g\,l^{-1}$ (a), $3.4\,g\,l^{-1}$ (b), and $2.0\,g\,l^{-1}$ (c), respectively. Aggregate concentration was $1.0\,g\,l^{-1}$. The conditions of (a), (b), and (c) correspond to the aggregate concentration below, equal, and beyond the saturation point in Figure 5, respectively.

aggregate increases beyond the saturation point, local concentration of enzyme in one polymer aggregate may decreased. For this reason, apparent hydrolysis activity decreases.

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