This article was downloaded by:
On: *24 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK

Journal of Macromolecular Science, Part A
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597274

Amphiphilic Block Copolymer-Lipase Aggregate in Aqueous-Organic Media

Kensuke Naka[ab]; Tohru Nakamura[a]; Akira Ohki[a]; Shigeru Maeda[a]
[a] Department of Applied Chemistry & Chemical Engineering, Faculty of Engineering Kagoshima University Korimoto, Kagoshima, Japan [b] Department of Polymer Chemistry, Kyoto University, Kyoto, Japan

To cite this Article Naka, Kensuke , Nakamura, Tohru , Ohki, Akira and Maeda, Shigeru(1997) 'Amphiphilic Block Copolymer-Lipase Aggregate in Aqueous-Organic Media', Journal of Macromolecular Science, Part A, 34: 4, 587 — 595
To link to this Article: DOI: 10.1080/10601329708014986
URL: http://dx.doi.org/10.1080/10601329708014986

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

J.M.S.—PURE APPL. CHEM., A34(4), pp. 587–595 (1997)

AMPHIPHILIC BLOCK COPOLYMER-LIPASE AGGREGATE IN AQUEOUS-ORGANIC MEDIA

KENSUKE NAKA,*† TOHRU NAKAMURA, AKIRA OHKI, and SHIGERU MAEDA

Department of Applied Chemistry & Chemical Engineering
Faculty of Engineering
Kagoshima University
Korimoto, Kagoshima 890, Japan

Downloaded At: 14:26 24 January 2011

ABSTRACT

Light-scattering analysis indicated that the polymer aggregate of an amphiphilic block copolymer (**1b**) consisting of poly[(*N*-nonanoylimino)ethylene] and poly[(*N*-acetylimino)ethylene] in aqueous solution maintained its structure even at the addition of 80% methanol, whereas an aggregate structure of an amphiphilic block copolymer (**1a**) consisting of poly[(*N*-pentanoylimino)ethylene] and poly[(*N*-acetyliminio)ethylene] in aqueous solution was disrupted by the addition of 40% methanol. The polymer aggregate of **1b** also maintained its structure even with the addition of 60% acetonitrile. Lipase P was largely incorporated into the aggregate of **1b** in aqueous solution. Without the polymer aggregate of **1b**, activity was significantly decreased at the addition of 30% acetonitrile. However, in the presence of the polymer aggregate, more than 40% activity still remained even at an acetonitrile content of 30%.

†Present address: Department of Polymer Chemistry, Kyoto University, Yoshida, Sakyo-ku, Kyoto, 606-01, Japan.

Copyright © 1997 by Marcel Dekker, Inc.

INTRODUCTION

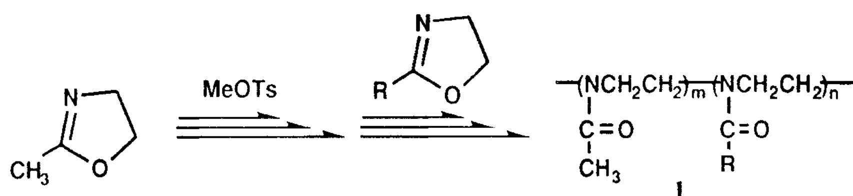
Polymer aggregate is a novel class of a self-organized macromolecular system for constructing functional fine particle. Many studies show that amphiphilic block copolymers form micelle-like polymer aggregates in water [1-9]. Hydrophobic bonding between the hydrophobic segments in these systems results in stabilization of compact structures, analogous to micelles. Some of these polymer systems undergo noncovalent interactions with enzymes which improve their activity and stability in aqueous and organic solvents [7-12]. These complexes are easily formed by mixing polymers and enzymes in aqueous buffer solutions. We recently showed that a nonionic amphiphilic block copolymer (**1a**) consisting of poly[(*N*-pentanoylimino)ethylene] (hydrophobic chain) and poly[(*N*-acethylimino)ethylene] (hydrophilic chain), which can easily be prepared since the polymerization proceeds in a living mechanism, forms a micelle-like aggregate in water and incorporates hydrophobic molecules or enzymes such as lipase and horseradish peroxidase (HRP) [7, 8]. From an organic synthesis point of view, it is important to study how to stabilize and use enzymes in organic solvents. Our polymer-enzyme aggregates showed enhanced activity compared with native enzymes in hydrophobic organic solvents such as chloroform and benzene. Although hydrophilic organic solvents tend to inactivate enzymes, there are many advantages in employing such organic solvents for enzymatic reactions. Thus, it is important to study how to improve the activity and stability of enzymes in water-miscible organic media. Poly[(*N*-acetylimino)ethylene] (hydrophilic chain) has high solubility not only in water but also in many hydrophilic organic solvents such as methanol and acetonitrile. Therefore, we assumed that the polymer aggregate could maintain its structure when an insoluble segment for such hydrophilic organic solvents was used as a hydrophobic block. Previously we used the block copolymer (**1a**) consisting of poly[(*N*-pentanoylimino)ethylene] which is a soluble segment for the usual polar organic solvents. For this paper we used a block copolymer (**1b**) contained poly[(*N*-nonanoylimino)ethylene] as a hydrophobic segment to stabilize the aggregate conformation in aqueous-organic media, prepared polymer-Lipase P aggregates and studied their activity in aqueous-organic media. In a series of these polymers the hydrophilicity and hydrophobicity are easily controlled by selecting their acyl group, i.e., 2-alkyl substituent of oxazoline monomers. Therefore, the micellar behaviors of these block copolymers in selective solvents can be controlled by their acyl group.

Downloaded At: 14:26 24 January 2011

EXPERIMENTAL

Materials

2-Butyl- and 2-octyl-2-oxazoline were prepared as described in the literature [13]. Commercially available 2-methyl-2-oxazoline was dried over KOH and was purified by distillation under nitrogen atmosphere. Amphiphilic block copolymers (**1**) containing poly[(*N*-acetylimino)ethylene] as the hydrophilic block were prepared by two-stage block copolymerization between 2-methyl-2-oxazoline and a second oxazoline according to the literature (Scheme 1) [14]. Table 1 lists the block copolymers used for this study.



SCHEME 1.

Lipase P was a gift from Nagase & Co. (Osaka, Japan) and used without further purification.

Gel Filtration Chromatography

Gel filtration chromatography was performed with a Sephacryl S-500 HR column (2 × 85 cm). A sample was applied and eluted from the column with 0.05 M phosphate buffer (pH 7.0). Fractions (2 mL) were collected at a flow rate of 20 mL/h.

Downloaded At: 14:26 24 January 2011

Dynamic Light Scattering (DLS)

Solutions were passed through 1.2 μm filters. Dynamic light-scattering measurements were carried out at 90° scattering angle and at 25 ± 0.2°C using a COULTER N4SD submicrometer particle analyzer with a He-Ne laser as the light source. The concentrations of the samples were 0.2 mg/mL. Diffusion coefficients, D, were obtained from the average decay constants of the autocorrelation functions. The cumulants and CONTIN methods were used for data analysis of dynamic light-scattering results.

Preparation of Polymer Aggregates

To prepare an aqueous solution of a polymer aggregate, **1a**, 0.2 g was dissolved in 10 mL of 0.05 M phosphate buffer (pH 7.0) at room temperature and was stirred overnight. **1b** was first dissolved in ethanol/water (1/1, vol%) and then

TABLE 1. Properties of Block Copolymers of 2-Oxazolines and Their Aggregates

Sample	R	M_n[a]	DP[a] m	DP[a] n	Aggregate content,[b] % of total	Diameter,[c] nm
1a	n-C_4H_9	7140	54	20	67	140
1b	n-C_8H_{17}	6440	39	17	86	150

[a]Determination by ^{1}H-NMR spectra.
[b]Determined by gel filtration chromatography [9].
[c]Determined by dynamic light scattering.

stirred overnight at room temperature. The polymer solution was dialyzed against water 5 times using a dialysis tube.

Enzyme Assay

The ester hydrolysis activity 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*-nitrophenenoxide in UV spectroscopy at 400 nm. To 2 mL of enzyme solution in an aqueous-organic mixture in a cuvette, 50 μL of 0.11 M PNP acetonitrile solution was added at 30 $\pm$ 0.01 °C.

RESULTS AND DISCUSSION

Polymer Aggregates in Aqueous-Organic Media

Gel filtration chromatographies for aqueous solutions of **1a** and **1b** showed the existence of main high molecular weight portions and minor low molecular weight parts [9]. The second minor peak was seen at the same location as that due to a homopolymer of poly[(*N*-acetylimino)ethylene] (M_n = 5000) and was therefore assigned to molecularly dissolved chains or intramolecular micelles of **1**. The first main peak therefore corresponds to a polymer of high molecular weight and is attributed to a polymer aggregate. The proportions of the aggregates were estimated by the relative peak areas in the chromatograms, as listed in Table 1. A separation of the polymer aggregate from the second minor peak was performed by collecting the fraction corresponding to the polymer aggregate. The aggregate fraction was then reinjected into a Shephacryl S-500 column after one day incubation at room temperature. The presence of the low molecular weight part was hardly recognized from the gel filtration chart. The particle sizes of the polymer aggregate peak at a fraction of gel filtration chromatography were determined by dynamic light scattering (Table 1). Regardless of the structures of hydrophobic segment, cumulants analysis of the autocorrelation function showed that the average diameters of the aggregates were approximately 150 nm. The relaxation rate distributions were obtained by the CONTIN method, and the results were consistent with single particle behaviors.

Light scattering is an appropriate technique to determine a disruption of the polymer aggregate in aqueous-organic media due to the fact that light-scattering intensity drops markedly in the absence of large particles. An aqueous solution of the polymer aggregate was obtained by ultrafiltration through a membrane (ADVANTEC UK-200, MW cut-off 200,000) to remove the single chain of **1b**. Gel filtration chromatography showed that contamination of the single chain of **1b** was hardly recognized. The polymer aggregate solution was diluted with water-methanol mixtures of different compositions and incubated for 1 day at room temperature. A plot of light-scattering intensity at 30 °C for the polymer aggregate solutions of **1a** and **1b** as a function of methanol content is shown in Fig. 1. A drop in the light-scattering intensity for **1a** was observed at a methanol content of more than 40%, which means that the aggregate structure of **1a** was disrupted by the addition of 40% methanol. However, a drop of the scattering intensity of the polymer aggregate solutions for **1b** was not observed even with the addition of 80% metha-

Downloaded At: 14:26 24 January 2011

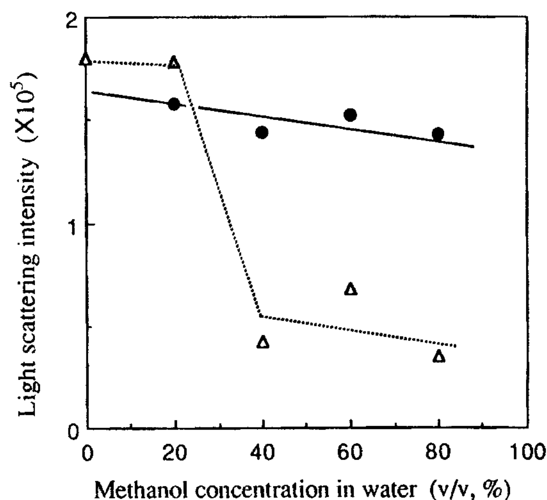


FIG. 1. Light-scattering intensity for the polymer aggregate solutions of **1a** (△) and **1b** (●) versus the concentration of methanol in water.

Downloaded At: 14:26 24 January 2011

nol. This result indicates that the polymer aggregate of **1b** maintains its structure at such a high concentration of methanol. Figure 2 shows a plot of the hydrodynamic diameter of the polymer aggregate of **1b**, which was obtained from cumulant analysis of the autocorrelation function, as a function of methanol content in aqueous solution. The result clearly shows that the hydrodynamic diameter of the aggregate was independent of methanol concentration. The polymer aggregate solution of **1b** was also diluted with water–acetonitrile mixtures and incubated for 1 day at room temperature. A plot of light-scattering intensity as a function of acetonitrile is

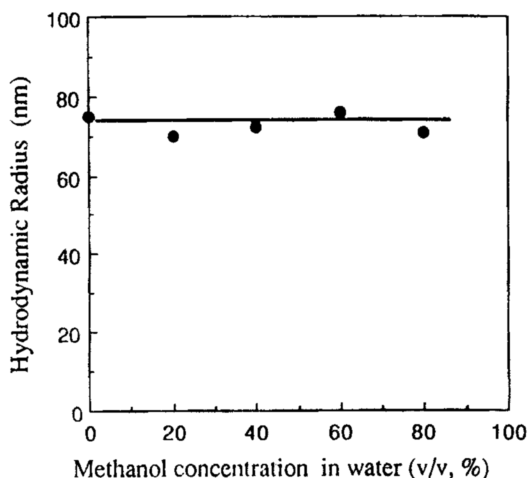


FIG. 2. Hydrodynamic radius for the polymer aggregate of **1b** versus the concentration of methanol in water.

shown in Fig. 3. A drop of scattering intensity was observed at an acetonitrile content at 80%. This result suggests that the polymer aggregate of **1b** maintains its structure even with the addition of 60% acetonitrile.

Assessment of Lipase Activity in Aqueous-Organic Media

Lipase is a very important enzyme as a biocatalyst for organic synthesis in both aqueous and nonaqueous media. Therefore we chose to apply this enzyme for our system in aqueous-organic media. We had previously found that Lipase P was largely incorporated into polymer aggregates of both **1a** and **1b** after Lipase P was mixed with the polymer aggregates in a phosphate buffer solution (pH 7.0) for 3 hours, and that the hydrolysis reaction of *p*-nitrophenyl propionate (PNP) catalyzed by Lipase P in an aqueous solution increased more than 50% in the presence of both polymer aggregates [8, 9]. This high activity in the presence of the aggregate seems to be the reason for the increased local concentration of the substrate around the enzyme owing to its binding ability for hydrophobic molecules. Enhancement of lipase activity by interaction with the polymer is an alternative explanation [15, 16].

When a water-miscible organic solvent is used for an enzyme reaction, the water content can be a crucial factor for controlling enzymatic activity. Therefore we investigated the influence of a water-miscible organic solvent in an aqueous phosphate buffer solution on the hydrolysis activity of Lipase P. To stabilize the aggregate conformation in aqueous-organic media as described above, we used the polymer aggregate of **1b** for assessment of lipase activity in aqueous-organic media. Figure 4 shows the effect of acetonitrile content in mixed solvents of acetonitrile-aqueous phosphate buffer (0.05 M, pH 7.0) on the hydrolysis activities of Lipase P in the presence and in the absence of the polymer aggregate of **1b**. Under this condition, Lipase P was fully incorporated into the polymer aggregate. The high

Downloaded At: 14:26 24 January 2011

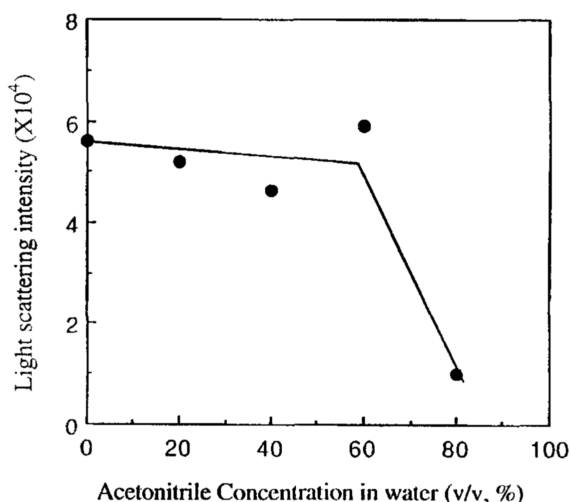


FIG. 3. Light-scattering intensity for the polymer aggregate of **1b** versus the concentration of acetonitrile in water.

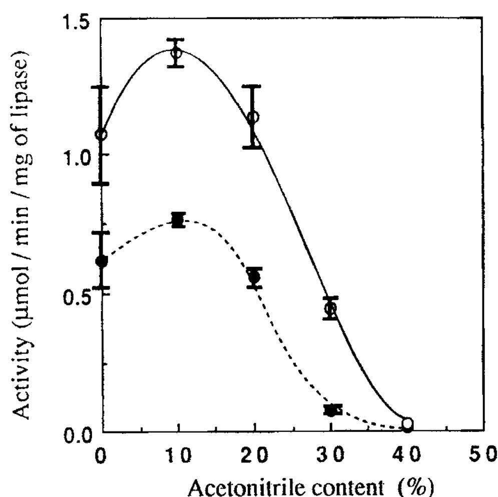


FIG. 4. Activity of Lipase P for the hydrolysis of PNP in acetonitrile-aqueous phosphate buffer (0.05 M, pH 7.0): (○) polymer-lipase aggregate; (●) native lipase.

Downloaded At: 14:26 24 January 2011

activity was still shown in the presence of the aggregate at 20% acetonitrile compared with that of native Lipase P in aqueous phosphate buffer solution (pH 7.0). Interestingly, a high activity value was obtained at 10% acetonitrile. Without the polymer aggregate, activity was significantly decreased with the addition of 30% acetonitrile. However, in the presence of the polymer aggregate, more than 40% activity still remained at an acetonitrile content of 30%. This result suggests that the deactivation of Lipase P is inhibited by the incorporation of Lipase P in the polymer aggregate. As mentioned above, light-scattering measurement indicated that the

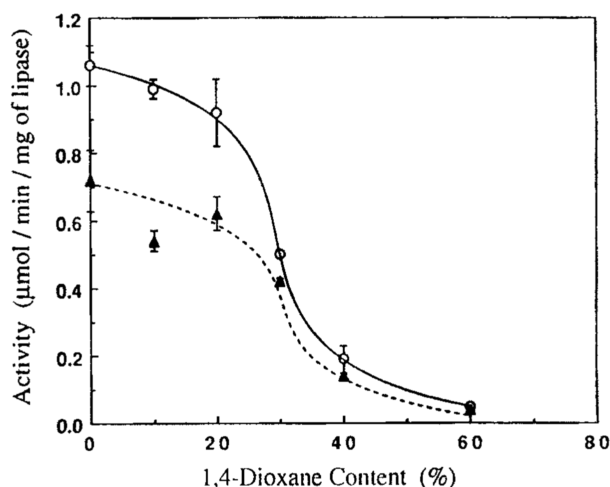


FIG. 5. Activity of Lipase P for the hydrolysis of PNP in 1,4-dioxane-aqueous phosphate buffer (0.05 M, pH 7.0): (○) polymer-lipase aggregate; (▲) native lipase.

polymer aggregate of **1b** is stable at an acetonitrile content of less than 60%. It seems that the polymer–enzyme aggregate maintains its organized structure in such an acetonitrile–water mixture.

Figure 5 shows the effect of 1,4-dioxane content in mixed solvents of dioxane–aqueous phosphate buffer (0.05 M, pH 7.0) on the hydrolysis activities of Lipase P in the presence and in the absence of the polymer aggregate of **1b**. The high activity was shown in the presence of the aggregate at 20% 1,4-dioxane compared with that of native Lipase P in aqueous phosphate buffer solution (pH 7.0). Although the activity decreased with the addition of 30% acetonitrile, a higher activity was still shown in the presence of the aggregate at 40% dioxane than of native Lipase P.

CONCLUSION

The present study clearly demonstrates one method to stabilize enzyme in aqueous–organic media. Light-scattering analysis indicates that the polymer aggregate of **1b** maintains its structure even with the addition of 60% acetonitrile or 80% methanol. The stability of the aggregate conformation in aqueous–organic media strongly depends on the hydrophobic segment of an amphiphilic block copolymer. This technique offers new possibilities for the design of effective biocatalysts in water-miscible organic solvents.

Downloaded At: 14:26 24 January 2011

ACKNOWLEDGMENT

This work was supported by Grant-in-Aid for Scientific Research 06780472 from the Ministry of Education, Science and Culture.

REFERENCES

[1] K. C. Dowling and J. K. Thomas, *Macromolecules, 23*, 1059 (1990).
[2] A. Desjardins and A. Eisenberg, *Ibid., 24*, 5779 (1991).
[3] M. Wilhelm, C.-L. Zhao, Y. Wang, R. Xu, and M. Winnik, *Ibid., 24*, 1033 (1991).
[4] R. Xu, M. A. Winnik, F. R. Hallett, G. Riess, and M. D. Croucher, *Ibid., 24*, 87 (1991).
[5] I. Astafieva, X. F. Zhong, and A. Eisenberg, *Ibid., 26*, 7339 (1993).
[6] G. Kwon, M. Naito, M. Yokoyama, T. Okano, Y. Sakurai, and K. Kataoka, *Langmuir, 9*, 945 (1993).
[7] K. Naka, A. Ohki, A., and S. Maeda, *Chem. Lett.*, p. 1303 (1991).
[8] K. Naka, Y. Kubo, A. Ohki, and S. Maeda, *Polym. J., 26*, 243 (1994).
[9] K. Naka, T. Nakamura, A. Ohki, and S. Maeda, *Ibid., 27*, 1071 (1995).
[10] K. Akiyoshi, K. Nagai, T. Nishikawa, and J. Sunamoto, *Chem. Lett.*, p. 1727 (1992).
[11] M. Otamiri, P. Adlercreutz, and B. Mattiasson, *Biocatalysis, 6*, 291 (1992).
[12] T. Nishikawa, K. Akiyoshi, and J. Sunamoto, *Macromolecules, 27*, 7654 (1994).

[13] H. Witte and W. Seeliger, *Justus Liebigs Ann. Chem.*, p. 996 (1974).
[14] S. Kobayashi, T. Igarashi, Y. Moriuchi, and T. Saegusa, *Macromolecules, 19*, 535 (1986).
[15] H. L. Brockman, J. H. Law, and F. J. Kezdy, *J. Biol. Chem., 248*, 4965 (1973).
[16] C. Chapus, M. Semeriva, C. Bovier-Lapierre, and P. Desnelle, *Biochemistry, 15*, 4980 (1976).

Received June 19, 1996
Revision received July 30, 1996

Downloaded At: 14:26 24 January 2011