# Preparation of Photoresponsive Polymeric Adsorbent Containing Amphiphilic Polymer with Azobenzene Moiety and Its Application for Cell Adhesion Chromatography

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## Synopsis

In order to develop a new type column chromatography, a polymeric adsorbent was prepared by grafting a photoresponsive polymer containing an azobenzene moiety on the surface of controlled pore glass beads. The polarity of the surface of the polymeric adsorbent was increased by UV irradiation because of *trans–cis* isomerization of azobenzene moiety. Adhesion chromatography of erythrocytes was carried out using the photoresponsive polymeric adsorbent. The erythrocytes were adhered to the adsorbent in the dark and separated from the adsorbent by UV irradiation. This behavior appears to be caused by the photoresponse of the polarity change on the surface of the polymeric adsorbent. The morphology of the erythrocytes which adhered on the surface of the photoresponsive polymeric adsorbent remained unchanged both in the dark and after UV irradiation. The amount of erythrocytes which adhered to the adsorbent increased with increasing the molecular weight of the grafted polymer and the hydrophobicity of the surface.

## INTRODUCTION

A column chromatography technique using polymeric adsorbents with biospecific affinity has been reported recently for the separation and purification of biosubstances such as proteins or blood cells.[1] This is a powerful method for the continuous separation of biosubstances. Generally, these polymeric adsorbents are prepared by the introduction of the ligands with biospecific affinity into hydrophilic polymeric carriers.

Hofstee et al. have indicated that some biosubstances can be separated by the hydrophobic interaction between an adsorbent and an adsorbate.[2] In this case, hydrophobic alkyl or aryl groups are introduced into hydrophilic carrier as ligands. The hydrophobic interaction is nonspecific; however, many substances ranging from low-molecular-weight compound (amino acids, hormones) to molecular assembly (blood cells) can be separated by this method.

When the adsorbed substances are desorbed from the polymeric adsorbent, the interaction between them is weakened by the addition of an eluent in order to change in the polarity of the moving phase. However, if the polarity of the fixed phase can be changed by an external physical signal, it is considered that not only the desorption of the adsorbed substances will be achieved without denaturation by the eluent, but also more continuous separation is possible.

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We have already reported the preparation of photoresponsive polymeric adsorbents containing an azobenzene group or a spiropyran compound in their side chain and the photoregulation of the adsorption–desorption steps for antibiotics,[3,4] proteins,[5,6] and erythrocytes.[7] In this paper, we will report the development of a new type of photoresponsive polymeric adsorbent which can regulate adhesion–dehesion of blood cells. The polymeric adsorbent was synthesized by the grafting of an amphiphilic polymer, poly(2-hydroxyethyl methacrylate) (PHEMA), onto the surface of controlled pore glass (CPG), followed by the introduction of azobenzene moiety in the side chain of PHEMA.

Compared with hydrophilic polymeric carriers, CPG is more rigid and stronger and its flow properties are good. Furthermore, the interaction between adsorbent and adsorbate will occur more effectively in grafted polymer on the surface of CPG than on hydrophilic polymeric carriers.

Column chromatography of erythrocytes was investigated using this new type photoresponsive polymeric adsorbent. Moreover, the influence of the property of the polymer grafted on the surface of CPG on adhesion-dehesion behavior of erythrocytes by light irradiation was studied.

## EXPERIMENTAL

### Materials

2-Hydroxyethyl methacrylate was distilled under reduced pressure in nitrogen atmosphere and the fraction of bp 65.8°C/3 mm Hg was used. $\alpha,\alpha'$-Azobisisobutyronitrile (AIBN) and *N,N*-dimethylformamide (DMF) were purified by the conventional methods. 2-Aminoethanethiol (AESH), 3-aminopropyl triethoxysilane, and glutaraldehyde were pure grade commercial reagent and used without further purification. Controlled Pore Glass was purchased from Electronucleonics Laboratories Ltd., Fairfield, Conn. *p*-Phenylazobenzoyl chloride (PABC) was synthesized by the method of Coleman et al.[8]

ANAL. Calcd for $C_{13}H_9ON_2Cl$: C, 63.82%; H, 3.71%; N, 11.40%. Found: C, 63.85%; H, 3.38%; N, 11.40%.

### Preparation of Photoresponsive Polymeric Adsorbent

#### *Activation of the Surface of CPG*

CPG was heated in 0.1*N* $HNO_3$ for 3 h, then washed with water and dried in an oven at 90°C. 5 g of this pretreated CPG was put into a 200 mL of toluene solution containing 25 mL of 3-aminopropyl triethoxysilane and refluxed for 3 h. After this reaction, CPG was filtered off and washed with methanol and dried *in vacuo*. The content of amino group determined by elemental analysis was $5.01 \times 10^{-4}$ mol/g CPG.

#### *Coupling Reaction of PHEMA on the Surface of CPG*

Amino semitelechelic PHEMA was synthesized using AIBN as initiator and AESH as chain transfer agent by a procedure reported previously.[9]

50-fold of glutaraldehyde, compared with amino group on the surface of the aminopropyl–CPG, was added and the mixture was shaken for 12 h at room temperature. The reaction mixture was filtered off and washed with water. A methanol solution containing excess of amino semitelechelic PHEMA was added to the aldehyde–CPG, and the mixture was shaken for 12 h at room temperature. The reaction mixture was filtered off and washed with methanol to exclude uncoupling PHEMA. Then, in order to convert the remained aldehyde group to hydroxy group, PHEMA-grafted CPG obtained was put into an aqueous solution of ethanolamine and reacted for 12 h. After the CPG was filtered off, the Shiff base bond produced by the reaction between aldehyde group and amino group was reduced with sodium borohydride.

*Introduction of Azobenzene Group into the Side Chain of Grafted PHEMA*

A desired amount of PABC and the PHEMA grafted CPG were reacted in pyridine for 12 h at room temperature. The reaction mixture was filtered off and washed with acetone, water, acetone, and then dried *in vacuo*.

## Adhesion Chromatography of Erythrocytes

Fresh blood of adult mongrel dog was collected using heparin as anticoagulant, centrifuged to remove serum and lecocytes as the supernatant, washed with a Hanks' buffer solution (HBS), and centrifuged again. The precipitated erythrocytes were then diluted with the HBS to give a suspension containing about $7 \times 10^5$ erythrocytes/mL.

The photoresponsive CPG (about 300 mg) was packed into a glass column (12 $\times$ 0.5 cm I.D.) and the conditioning was carried out efficiently with the HBS at 37°C. The erythrocytes suspension (500 $\mu$L) was injected into the column in the dark and eluted with the HBS flowed at a rate of 250 $\mu$L/min. When no effusion of erythrocytes from the column in the dark was found, UV irradiation was carried out by means of a 500-W ultra-high-pressure mercury lamp, Ushio UI-501C, and the wavelength was selected with a filter ($\lambda = 350 \pm 50$ nm). In order to obtain high irradiation efficiency, a reflect mirror was used. A 1 wt % ammonium oxalate solution was added to the 250 $\mu$L portions of the effluent from the column to effect hemolysis, and the concentration of erythrocytes was measured with absorbance at 413 nm using an electronic spectrophotometer, Shimadzu UV-240.

## Other Measurement

Advancing contact angle was measured by a contact angle goniometer, Erma G-1, at room temperature in air. A scanning electron micrograph was obtained as follows: the adsorbent in the column was taken out, and it was placed in a saline solution containing 1.25% glutaraldehyde in order to fix adhered erythrocytes. The adsorbent was then freeze-dried, followed by coating with gold. The coated material was observed by a scanning electron microscope (SEM), Hitachi-Akashi MSM-101.

TABLE I
Synthesis of Amino Semitelechelic PHEMA

| [S]/[M][a] | Time (h) | Conversion (%)[b] | MW[c] |
|---|---|---|---|
| 0.075 | 1.0 | 13.9 | 14,600 |
| 0.15 | 1.0 | 14.2 | 10,300 |
| 0.20 | 1.5 | 16.7 | 7400 |
| 0.40 | 1.5 | 18.1 | 5000 |

[a] [M] = 2.5 mol/L, S = chain transfer agent, AESH.
[b] [AIBN] = $5 \times 10^{-3}$ mol/L in DMF at 60°C.
[c] Determined by amino group analysis.

## RESULTS AND DISCUSSION

### Preparation of Photoresponsive Polymeric Adsorbents and Their Photoresponse

CPG beads are widely used for the matrix of affinity chromatography.[10] Many replacement methods of silanol group on the surface of CPG for other functional groups have been reported. In this paper, the amino group was introduced on the surface of CPG by reaction with 3-aminopropyl triethoxysilane according to the method reported by Weetall.[11] The content of amino group in the aminopropyl-CPG was $5.01 \times 10^{-4}$ mol/g CPG. Then the aldehyde group was introduced on the surface of CPG by reaction between the aminopropyl–CPG and excess of glutaraldehyde. The CPG turned red in this step.

Table I shows the results of the synthesis of amino semitelechelic PHEMA. The molecular weight was determined by an end group analysis. The molecular weight was controlled in the range approximately from 5000 to 15,000 by the regulation of the ratio of the concentration of the chain transfer agent and the monomer. The amino semitelechelic PHEMA was dissolved in methanol, and the coupling reaction to the aldehyde–CPG was carried out. Figure 1 shows IR spectra of PHEMA (MW 14600)-grafted CPG compared with nonsubstituted CPG. It can be seen from the figure that the peak assigned carbonyl group of

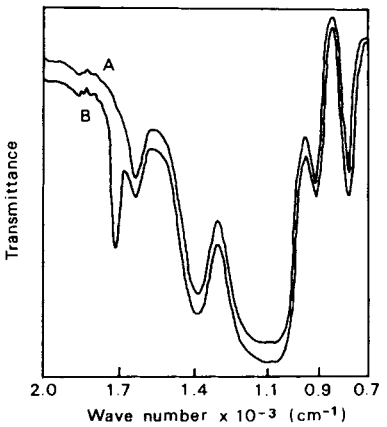


Fig. 1. IR spectra of nonsubstituted CPG (A) and PHEMA-grafted CPG (B).

TABLE II
Preparation of Photoresponsive Polymeric Adsorbent

| Code | MW of grafted PHEMA | Amount of grafted PHEMA (mol/g CPG) | [COCl]/[OH] | Content of azobenzene group (%) |
|---|---|---|---|---|
| CHA-1 | 14,600 | $7.12 \times 10^{-6}$ | 1.0 | 38.0 |
| CHA-2 | 14,600 | $7.12 \times 10^{-6}$ | 0.5 | 24.1 |
| CHA-3 | 14,600 | $7.12 \times 10^{-6}$ | 0.3 | 13.4 |
| CHA-4 | 14,600 | $7.12 \times 10^{-6}$ | 0.2 | 7.2 |
| CHA-5 | 10,300 | $6.88 \times 10^{-6}$ | 0.3 | 12.6 |
| CHA-6 | 7410 | $7.06 \times 10^{-6}$ | 0.3 | 11.8 |
| CHA-7 | 5000 | $6.52 \times 10^{-6}$ | 0.3 | 13.0 |
| CH-1 | 14,600 | $7.12 \times 10^{-6}$ | — | — |
| CH-2 | 10,300 | $6.88 \times 10^{-6}$ | — | — |
| CH-3 | 7410 | $7.06 \times 10^{-6}$ | — | — |
| CH-4 | 5000 | $6.52 \times 10^{-6}$ | — | — |

ester bond of PHEMA appeared at 1700 $cm^{-1}$. Moreover, the increase of carbon content was observed for PHEMA-grafted CPG from the elemental analysis. From these results, it is concluded that PHEMA was grafted on the surface of CPG.

An azobenzene group was introduced by the condensation reaction between PHEMA-grafted CPG and PABC in pyridine. After this step, the color of the CPG turned orange.

Table II shows the results of the preparation of the photoresponsive polymeric adsorbents. The amount of grafted PHEMA determined by the elemental analysis was not dependent on the molecular weight of PHEMA and approximately constant at $7 \times 10^{-6}$ mol/g CPG. The content of azobenzene group was regulated by the change of the ratio of the reaction groups, [COCl]/[OH]. When the ratio of the reaction group was 0.3, the content of azobenzene group was 12–13%, and it did not depend on the molecular weight of PHEMA.

PHEMA was grafted on the surface of a glass plate by the same procedure described above. The advancing contact angle by pure water was measured on the surface of the glass plate at each intermediate state during the procedure in order to analyze the polarity change of the surface. The contact angle on the surface of the glass plate treated with 3-aminopropyl triethoxysilane was 68.1°, and the value was large compared with it on the surface of clean glass plate, which was 17°. On the surface of the glass plate grafted with PHEMA, the contact angle was 57.3°. We have already reported that the contact angle of PHEMA film casted from DMF solution was 56°.[12] Therefore, the decrease of the contact angle from 68.1° to 57.3° is considered to reflect the increase of the hydrophilicity by the grafting of PHEMA. When azobenzene group, about 10%, was introduced into the side chain of grafted PHEMA, the contact angle increased to 73.7° in the dark state. After UV irradiation for 10 min, the contact angle decreased to 66.4°. This change by UV irradiation was reversible. In other words, the hydrophobicity of the surface was increased by the introduction of azobenzene group and it was decreased by *trans–cis* photoisomerization of azobenzene group accompanying the increase of its polarity.[13] The polarity of the surface can be photocontrolled by the introduction of photoresponsive azobenzene group on the surface. Accordingly, it is considered that the photo-induced polarity change occur in azobenzene group attached to PHEMA grafted on CPG surface. The

use of the adsorbent having these properties may provide a system for the adsorption–desorption process that can be controlled by the regulation of the polarity of fixed phase with light irradiation.

## Column Chromatography of Erythrocytes on a Column Packed with Photoresponsive Polymeric Adsorbent

Figure 2 shows a result of the adhesion chromatography of erythrocytes conducted using 300 mg of CHA-3 as a packing material. Erythrocytes, approximately $3.5 \times 10^5$ in number, were injected into the column in the dark, and rinsed with the HBS. Unadhered erythrocytes were eluted from the column. Adhesion of about $2.56 \times 10^5$ was observed, and the percentage of the adhesion was 73.3%. It can be seen from the figure that when UV irradiation was carried out after effusion of erythrocytes could not be found in the dark, the absorbance of elution at 413 nm increased. This increase corresponded to elute of erythrocytes. There was no hemolysis of erythrocytes during the chromatographic operations. However, when the same experiment was carried out using CH-1, which does not contain the azobenzene group, no elution of erythrocytes was found after UV irradiation. From these results, it is concluded that the dehesion of adhered erythrocytes is induced by the photoresponse of azobenzene group introduced in the side chain of PHEMA grafted on the surface of CPG. Shaltiel et al. reported that the erythrocytes tend to adhere more extensively to a hydrophobic surface.[14] Hence, it may be concluded that the dehesion of the adhered erythrocytes is caused by the change in the hydrophobicity of the surface of CHA-3 induced by UV irradiation. The number of erythrocytes remained after UV irradiation was $1.9 \times 10^5$, and the efficiency of the dehesion by UV irradiation was 20.2%.

Figure 3 shows the SEM view of erythrocytes adhered on the surface of a series of CPG. On the surface of CPG [Fig. 3(A)], erythrocytes adhered separately to each other, the cell membrane deformed, and many projections could be observed. On the surface of CPG grafted with PHEMA [Fig. 3(B)], CH-1, erythrocytes adhered with little conformation change of cell membrane and holding almost their native morphology. In addition, on the surface of CHA-3 having azobenzene group, erythrocytes were adhered maintaining their native mor-

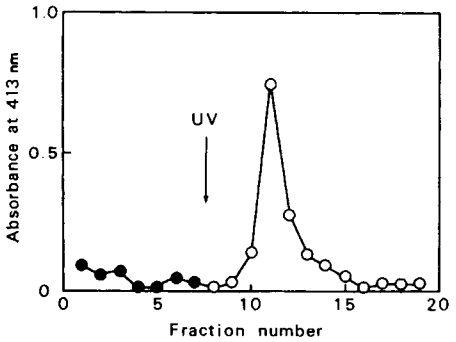


Fig. 2. Chromatography of erythrocytes on a column of CHA-3 at 37°C. Elution was done under UV irradiation, 250 $\mu$L portions of eluted solution were collected to monitor the absorbance at 413 nm: (●) in the dark; (○) under UV irradiation.

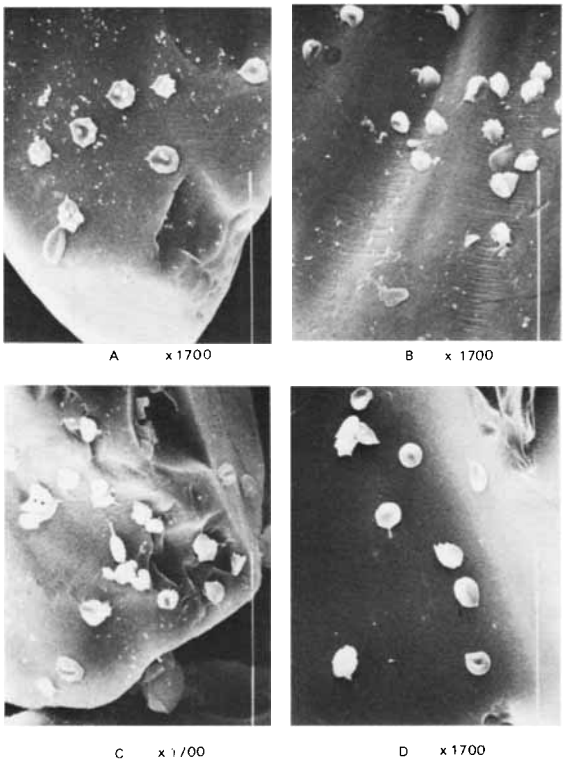


Fig. 3. Scanning electron micrographs of erythrocytes adhered on the surface of a series of CPG: (A) nonsubstituted CPG; (B) PHEMA-grafted CPG (CH-1); (C) photoresponsive CPG having azobenzene group (CHA-3) in the dark; (D) CHA-3 after UV irradiation.

phology, also, on the surface of CH-1, both in the dark state [Fig. 3(C)] and after UV irradiation [Fig. 3(D)]. That is to say, it is considered that the morphology change of adhered erythrocytes is not induced by the introduction of azobenzene group or UV irradiation. These results lead to the conclusion that the adhesion–dehesion process of erythrocytes to the surface of the polymeric adsorbent in the system without an eluent is possible by the photocontrol of the surface polarity using the photoresponsive polymer.

## Adhesion Behavior of Erythrocytes to Photoresponsive Polymeric Adsorbent

Figure 4 shows the relation between the adhesion amount of erythrocytes on the surface of CPG grafted with PHEMA and the molecular weight of grafted PHEMA. Azobenzene content was almost equal at 12–13%. The adhesion amount increased with increasing the molecular weight of PHEMA and saturated in the range of about $1.2 \times 10^4$–$1.5 \times 10^4$, regardless of the introduction of azobenzene group and UV irradiation. For two materials grafted with the same molecular weight of PHEMA, the adhesion amount of erythrocytes was increased by the introduction of azobenzene group. Moreover, a decrease in the amount of erythrocytes adhered to the adsorbent was observed after UV irradiation in all adsorbents containing azobenzene group.

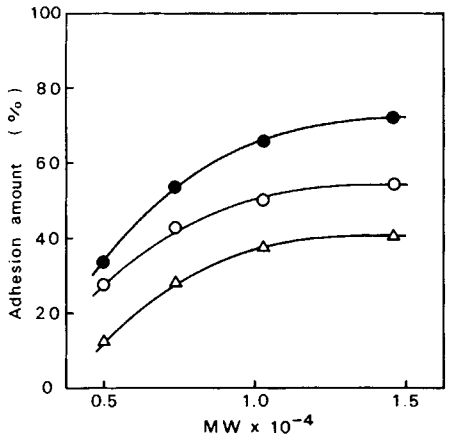


Fig. 4. Relationship between the adhesion amount of erythrocytes and the molecular weight of PHEMA grafted on the surface of the CPG: (△) PHEMA-grafted CPG, photoresponsive CPG having azobenzene group; (●) in the dark; (○) after UV irradiation.

It is well known that PHEMA swells in water. When PHEMA was grafted onto CPG, and the column was filled with the HBS, the PHEMA chain hydrated and expanded into the space surrounding CPG. The expansion seems to increase with increasing of the molecular weight of PHEMA. The increase of the adhesion amount of erythrocytes with increasing the molecular weight of grafted PHEMA could be attributed to the expansion of the space where the erythrocytes can interact with the PHEMA chains or to the increase of the hydrophobicity on the surface of adsorbent.

The difference in the amount of erythrocytes adhered between in the dark and after UV irradiation represents the dehesion amount of erythrocytes from the polymeric adsorbent by UV irradiation. Apparently, the value of the dehesion amount increases with increasing the molecular weight of grafted PHEMA.

Figure 5 shows the relation between azobenzene content in PHEMA (MW 14,600) and the adhesion amount of erythrocytes. In the dark, the amount of erythrocytes which adhered was increased with increasing azobenzene content, up to the maximum amount of adhesion, which was found when azobenzene content was 12%. This result may be explained as follows: the hydrophobicity of the adsorbent and the adhesion amount increase with increasing azobenzene content. But, when the azobenzene content is above 12%, the hydrophobicity of the side chain of PHEMA grafted on CPG is so high that the hydrophobic interaction will occur as inter- or intramolecular, resulting in the shrinking of the PHEMA chain. Therefore, this interaction induces the narrowing of the space where PHEMA chain can interact with erythrocytes, and shorter length of PHEMA grafted on CPG results in decrease on the adhesion amount of erythrocytes. When UV irradiation was carried out, the decrease of the adhesion amount of erythrocytes could be found in all azobenzene contents. It is considered from these results that the adhesion amount of erythrocytes on the surface of GPC grafted with PHEMA, containing the photoresponsive azobenzene group, is influenced by both the molecular weight of grafted polymer, which dominants the interaction space between adsorbent and erythrocytes, and the hydrophobicity which contributes the force of interaction.

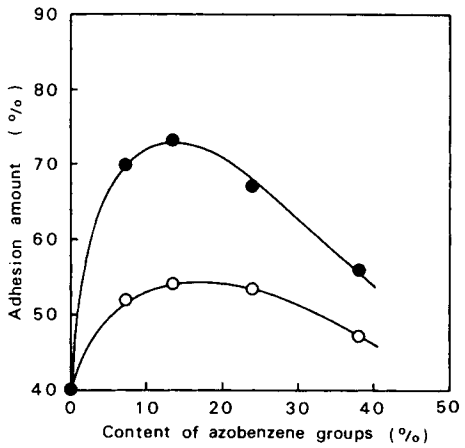


Fig. 5. Relationship between the adhesion amount of erythrocytes and the content of azobenzene groups in the polymer grafted on the surface of CPG: (●) in the dark; (○) after UV irradiation.

This new type photoresponsive polymeric adsorbent is expected to be useful for the separation of blood cells.

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## References

1. *Hydrophobic, Ion Exchange, and Affinity Methods*, R. Epton, Ed., Halsted, New York, 1979.

2. B. H. J. Hofstee and N. F. Otillio, *J. Chromatogr.*, **159,** 57 (1978).

3. N. Negishi, K. Ishihara, I. Shinohara, T. Okano, K. Kataoka, and Y. Sakurai, *Macromol. Chem. Rapid Commun.*, **2,** 95 (1981).

4. N. Negishi, T. Iida, K. Ishihara, and I. Shinohara, *Macromol. Chem. Rapid Commun.*, **2,** 617 (1981).

5. K. Ishihara, N. Negishi, and I. Shinohara, *J. Appl. Polym. Sci.*, **27,** 1897 (1982).

6. K. Ishihara, S. Kato, and I. Shinohara, *J. Appl. Polym. Sci.*, **27,** 4273 (1982).

7. K. Ishihara, S. Kato, I. Shinohara, T. Okano, K. Kataoka, and Y. Sakurai, *Kobunshi Ronbunshu*, **39,** 271 (1982).

8. G. H. Coleman, G. Nichols, C. M. McCloskey, and H. D. Anspon, *Organic Synthesis III*, Wiley, 1965, p. 713.

9. T. Okano, M. Katayama, and I. Shinohara, *J. Appl. Polym. Sci.*, **22,** 369 (1978).

10. *Affinity Chromatography*, I. Chibata, T. Tosa, and T. Matsuo, Eds., Kodansha, Tokyo, 1976.

11. H. H. Weetall, *Nature*, **232,** 473 (1971).

12. T. Okano, J. Aoyagi, and I. Shinohara, *Nippon Kagaku Kaishi*, **1976,** 161 (1976).

13. K. Ishihara, A. Okazaki, N. Negishi, I. Shinohara, T. Okano, K. Kataoka, and Y. Sakurai, *J. Appl. Polym. Sci.*, **27,** 239 (1982).

14. G. Halperin, and S. Shaltiel, *Biochem. Biophys. Res. Commun.*, **72,** 1497 (1976).

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