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One-Step Immobilization and Purification of His-Tagged Enzyme Using Poly(2-acetamidoacrylic acid) Hydrogel

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Abstract: Ni^{2+}-Complexed poly(2-acetamidoacrylic acid) (PAAA) hydrogel support was developed for the one-step immobilization and purification of recombinant histidine-tagged glutamyl aminopeptidase (His-tagged GAP). Ni^{2+}-PAAA hydrogel was prepared from the polymerization of 2-acetamidoacrylic acid (AAA) and 2,2-[(1,4-dioxo-1,4-butanediyl) diamino] bis(2-propenoic acid) (DBDBPA) with potassium persulfate in dimethyl sulfoxide (DMSO), followed by Ni^{2+}complexation. His-tagged GAP was immobilized directly from the cell lysate onto the Ni^{2+}-PAAA hydrogel support and then purified. Catalytic activity of immobilized His-tagged GAP for the hydrolysis of alanyl-*para*-nitroanilide revealed 90% conversion after 30 min of incubation, indicating sustained catalytic activity. The hydrogel-immobilized enzyme also exhibited enhanced thermal stability of sustained 70% activity after 1 h incubation at 60 °C, while the free enzyme activity was reduced to 50% at the same condition. After four cycles of hydrogel regeneration, the immobilized enzyme lost only 20% of its initial activity. Ni^{2+}-PAAA hydrogel provided a new and convenient immobilization/purification system for His-tag enzymes through easy and simple procedures.

Keywords: poly(2-acetamidoacrylic acid) hydrogel, purification, immobilization, his-tagged enzyme.

Introduction

Immobilized enzymes are being used in diverse applications through many analytical devices, including industrial processes as catalysts, for example medical applications in therapy, water remediation, biosensors, and textile industry.[1] Enzyme immobilization provided many advantages over the free enzymes, such as their enhanced stability, reusability, and the simplification of enzyme recovery and purification processes.[2] The initial development of enzyme immobilization was reported in 1970s with enzyme engineering studies.[3] During the pioneering years, several general strategies for enzyme immobilization were proposed and developed. The methods of enzyme immobilization could be categorized into matrix entrapment, encapsulation, and support binding. Among them, immobilization to support seemed to possess high enzyme-loading capacity with good mechanical properties, and the flow through the support could transport the substrate and the product with least diffusion restrictions. Enzyme binding to support was performed through adsorption, ionic interactions, or covalent binding. Adsorption was the simplest immobilization method, but the weak interactions between the enzyme and the support could be easily disturbed by solvents and ionic strength, where the enzymes tended to leach out with aqueous solvents. Ionic or covalent immobilizations of enzyme revealed stronger binding affinity to the support than adsorption, which became advantageous to reduce the enzyme leach from the surface.[1-5] Synthetic resin, biopolymer and inorganic polymer, such as hydrogel, agarose, chitosan, silica, and magnetic beads, were used as supports for the enzyme immobilizations.[6-8] In addition, various natural and synthetic hydrogels were used for enzyme immobilization. Interestingly, the enzyme immobilization into the hydrogel matrix revealed the enhanced resistances to thermal inactivation, pH, denaturing agents, and organic solvents.[9-14] Hence, hydrogels were readily used as supporting materials for the immobilization or separation of proteins. The capacity of hydrogel could be determined from the degree of swelling, which in

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turn could be controlled either by the chemical potentials of the aqueous phase or by the crosslinking-density of the hydrogel. Hydrogel with ligands for high metal affinity was recently introduced as a medium for protein immobilization and purification. For example, utilizing the affinity binding between histidine residues and a metal-ion complex, histidine-tagged rhamnosidase was successfully immobilized on Ca^{2+}-alginate beads.[15] The binding interactions of bioparticles by ligands with high affinity for metals in carboxylmethyl-aspartate (TALON) and resins with an affinity for sepabeads were reported by Zheng *et al.*.[16] Thus far, use of agarose,[17,18] hydrogels,[8-14] and magnetic beads[19] increased gradually in many applications of solid support immobilization systems.

Glutamyl aminopeptidase (GAP) was used as a model enzyme. GAP belongs to the M42 family with substrate specificity toward acidic amino acids. Since GAP can cleave amyloid and angiotensin, its potential medical and industrial applications are being investigated. GAP, also known as peptidase A (PepA) from *Streptococcus pneumonia* (*S. pneumonia*), was reported to show the substrate specificity against Glu, Asp, and Ala. In this study, Ala-*para*-nitroanilide was introduced for the purpose of monitoring the enzymatic activity of PepA.

Previously, we introduced the Ni^{2+}-PAAA hydrogel system for the purification of a His-tagged protein.[20] Ni^{2+}-PAAA hydrogel could be the solid support for simultaneous immobilization and purification of His-tagged enzymes. Here, we report for the utilization of the Ni^{2+}-poly(2-acetamidoacrylic acid) hydrogel support with reversible metal ion affinity for the one-step immobilization and purification of a recombinant histidine-tagged glutamyl aminopeptidase (His-tagged GAP), and investigated the characteristics of immobilized enzyme.

Experimental

Materials. 2-Acetamidoacrylic acid (AAA) (97%) and 2,2′-[(1,4-dioxo-1,4-butanediyl) diamino] bis(2-propenoic acid) (DBDBPA) (97%) were synthesized, as previously reported.[21] Imidazole (99.0%) and nickel(II) chloride hydrate (99.9%) were purchased from Aldrich. Potassium persulfate (99.0%) and dimethyl sulfoxide (DMSO, 99.0%) were purchased from Acros Organics and Junsei, respectively.

Synthesis of Poly(2-acetamidoacrylic acid) (PAAA) Hydrogel. AAA (500 mg; 4.00 mM), 2,2′-[(1,4-dioxo-1,4-butanediyl) diamino] bis(2-propenoic acid) (DBDBPA) (25.0 mg; 0.10 mM) and potassium persulfate (5.00 mg; 0.02 mM) were dissolved in DMSO (1.50 mL). The PAAA hydrogel was formed after heating the reaction mixture to 70 °C for 2 h, and the product was washed thoroughly with distilled water (Scheme I).

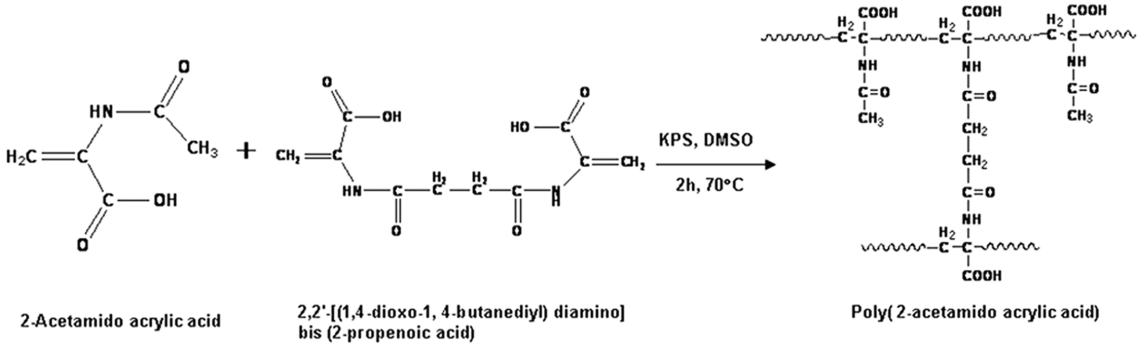
Swelling Ratio of PAAA Hydrogel. The PAAA hydrogel was fully swelled with distilled water over a seven-day period. The water on the surface of the hydrogel was absorbed with tissue paper, and the hydrogel was weighed. It was then vacuum-dried and weighed again. Three samples of each hydrogel were measured, the average value was calculated. The swelling ratio (Q_s) was determined from following formula:

$$Q_s = \frac{(W_s - W_d)}{W_d}$$

Where W_s is the weight of the swollen hydrogel, and W_d is the weight of the dry hydrogel.

Ni^{2+}-Complexed PAAA Hydrogel (Ni^{2+}-PAAA hydrogel). The PAAA hydrogel was immersed in nickel chloride ($NiCl_2$, 0.1 M) solution, and stirred continuously at room temperature for 24 h. The Ni-complexed hydrogel was washed with distilled water to remove the unbound or weakly bound Ni^{2+}. The amount of nickel in the Ni^{2+}-complexed PAAA hydrogel was determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES).

Preparation of the Cell Lysates Containing the Glutamyl Aminopeptidase (His-tagged GAP). PepA of *S. pneumonia R6* (GeneID: 15903724) gene was amplified by PCR from *S. pneumonia R6* genomic DNA and cloned into a pET-based vector with a tobacco etch virus (TEV) protease cleavable N-terminal hexa-histidine tag. Briefly, *E. coli* BL21 (DE3) cells (Novagen), harboring the recombinant plasmid (pVFT1S-PepA), were cultured in Luria Bertani medium at 37 °C to an OD600 value of 0.5. The recombinant protein was induced with 0.5 Mm isopropyl-*β*-*D*-thiogalactopyranoside for 4 h. The cell lysate, prepared by disrupting the



Scheme I. Synthesis of the poly(2-acetamidoacrylic acid) hydrogel.

harvested cell with sonicator, was centrifuged at 15,000 rpm for 20 min, and the supernatant was used for further purification with the metal affinity chromatography on a HiTrap™ chelating column (GE Healthcare) and a HiTrap™ Q column (GE Healthcare).

Purification and Immobilization of Enzyme with Ni^{2+}-PAAA Hydrogel (His-tagged GAP/Ni^{2+}-PAAA Hydrogel). The Ni^{2+}-PAAA hydrogel (0.10 g) was equilibrated with 10 mL of buffer A (50 mM Tris-HCl, 100 mM NaCl, 1 mM $ZnCl_2$, and pH 8.0) for 2 h, and adjusted to pH 8.0. The hydrogel was then added to the cell lysate solution and incubated for 2 h at 4 °C with constant shaking. The supernatant was removed from the hydrogel by centrifugation (3,000 rpm, for 5 min at 4 °C). The hydrogel was transferred to the column and washed with buffer B (3×4 mL) (20 mM Tris-HCl, 0.5 mM NaCl, 40 mM imidazole, and pH 8.0) to remove the non-bound His-tagged GAP. His-tagged GAP was eluted with 1 mL of buffer C (20 mM Tris-HCl, 0.5 mM NaCl, 250 mM imidazole, and pH 8.0). The Ni^{2+}-PAAA hydrogel (0.30 g) was equilibrated with 50 mL of buffer A for 2 h, and adjusted to pH 8.0. A 0.10 g of Ni^{2+}-PAAA hydrogel was then added to a 400 μL purified His-tagged GAP, and incubated for 2 h at 4 °C with constant shaking. After incubation, the hydrogel was transferred to the column and washed with buffer A to remove the excess His-tagged GAP.

Enzyme Activity Assays. The activity of the His-tagged GAP was determined by monitoring the hydrolysis of Ala from alanyl-*para*-nitroanilide (Ala-pNA) and the release of *para*-nitroaniline (p-NA) at 405 nm. The residual activity of the immobilized His-tagged GAP was determined by adding 2.50 μL of Ala-pNA (0.06 mM) into the reaction mixture of 0.30 g of His-tagged GAP (100 μg)/Ni^{2+}-PAAA hydrogel in 400 μL of buffer A, followed by incubation for 60 min at 37 °C. Increased absorbance of p-NA was detected at 405 nm from hydrolysis reaction by immobilized His-tagged GAP.

Thermal-Stability and Reusability Study of the Immobilized His-Tagged GAP. The free and hydrogel-immobilized His-tagged GAP samples were kept in buffer A at 30, 40, 50, and 60 °C for 60 min, with continuous shaking (100 rpm). The residual activity of each sample was determined by comparing with the respective non-incubated sample, which was assumed to maintain 100% activity. The hydrogel-immobilized His-tagged GAP was washed and recycled with buffer A, and its catalytic activity with enzyme substrate was determined at each repeat by comparing to the first cycle.

Results and Discussion

Purification and Immobilization of His-Tagged Enzyme. PAAA hydrogel was synthesized from 2-acetamidoacrylic acid (AAA) and 2,2′-[(1,4-dioxo-1,4-butanediyl) diamino] bis(2-propenoic acid) (DBDBPA), as in Figure 1(a). The inner structure of the PAAA hydrogel consisted of hydrogen bonds between the hydrogel matrix and water. This PAAA hydrogel could swell up and extend to more than 100 times of its dry weight. PAAA hydrogel were utilized in diverse biotechnological applications on an account of their water-absorbing capacity. The Ni^{2+} was complexed to the fully swollen hydrogel with distilled water (Figure 1(b)).

ICP-AES analysis provided the number of the Ni^{2+} ions per carboxylic acid group in the dry Ni^{2+}-PAAA hydrogel to be 0.18. His-tagged GAP was bound to the Ni^{2+}-PAAA hydrogel in an aqueous environment, as shown in Figure 1(c). In an attempt to purify recombinant aminopeptidase with Ni^{2+}-PAAA hydrogel, cell lysates were applied and the eluted fractions were analyzed by SDS-PAGE gel in Figure 2. The molecular mass of the purified His-tagged GAP band in Figure 2 was determined to be 43 kDa, approximately. His-tagged GAP was immobilized directly from the cell lysate onto the Ni^{2+}-PAAA hydrogel support and purified. This result confirmed that one-step for the immobilization and purification of histidine tag proteins could be achieved through specific interactions with Ni^{2+}-PAAA hydrogel.

Next, the hydrogel-immobilized aminopeptidase (His-tagged GAP/Ni^{2+}-PAAA) was used as a biocatalyst for the hydrolysis of alanyl-*para*-nitroanilide (Ala-pNA). The catalytic reaction of the immobilized His-tagged GAP was monitored through a spectrophotometric assay, based on the absorbance of hydrolyzed alanyl-*para*-nitroanilide (Ala-pNA) over time at 405 nm in Figure 3. 90% of conversion was achieved within 30 min, and the maximum was reached within 1 h for the hydrolysis of Ala-pNA.

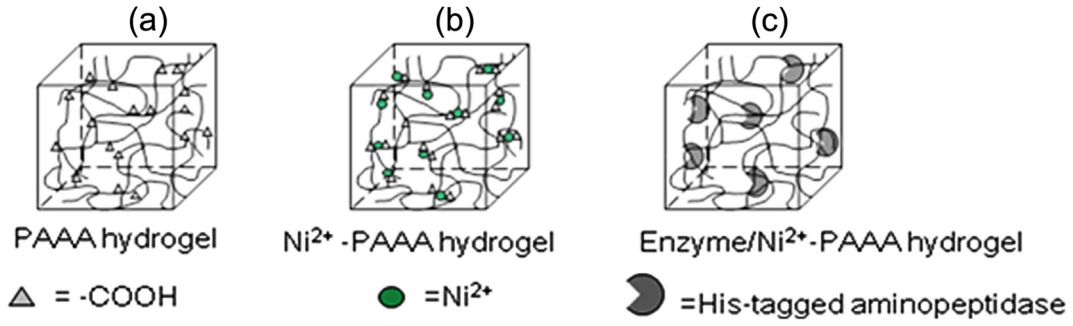


Figure 1. Schematic diagram of the PAAA hydrogel (a), Ni^{2+}-PAAA hydrogel (b), and His-tagged GAP/Ni^{2+}-PAAA hydrogel (c).

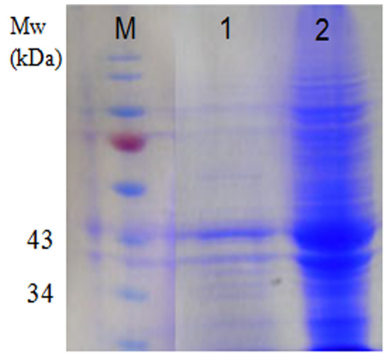


Figure 2. SDS-PAGE analysis of purified His-tagged GAP by the Ni^{2+}-PAAA hydrogel. Lane M: molecular weight markers; lane 1: purified His-tagged GAP from the Ni^{2+}-PAAA hydrogel with an elution buffer; lane 2: cell lysates.

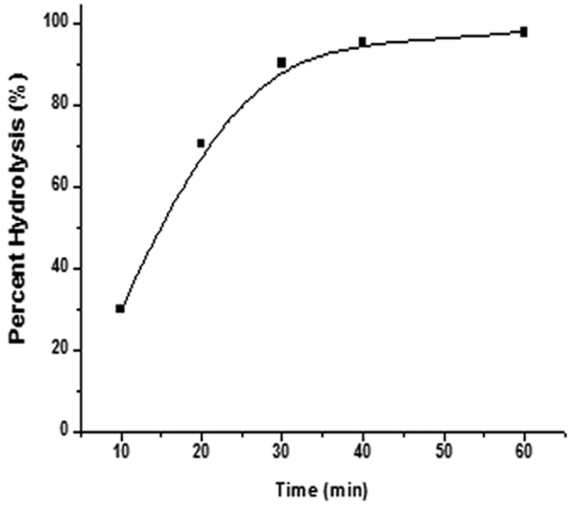


Figure 3. Detection of *para*-nitroanilide (pNA) from hydrolysis of alanyl-*para*-nitroanilide (Ala-pNA) by the hydrogel-immobilized His-tagged GAP as a function of time. The reaction was performed at 37 °C in 50 mM Tris-HCl (pH 8.0).

Effect of Temperature on the Catalytic Activity and Reusability. The thermal effect on the enzyme stability was quantitated by incubating the immobilized and free enzymes and by measuring their relative activities by varying temperatures ranging from 30 to 60 °C for 1 h incubation in 50 mM Tris-HCl buffer (100 mM NaCl, 1 mM $ZnCl_2$, and pH 8.0) (Figure 4). The activities of the free and immobilized His-tagged GAP revealed inverse correlations the temperature. Both the free and immobilized enzymes retained nearly 100% of their activities at temperature below 40 °C.

When the temperature was increased to 50 °C, the immobilized enzyme exhibited 95% activity, while the activity of free enzyme decreased to 84%. At 60 °C, the activity of the free enzyme was further decreased to 50%, whereas the activity of the immobilized enzyme decreased to 70%. Above results suggested the increased thermostability of enzyme significantly at high temperatures by the immobilization onto Ni^{2+}-PAAA hydrogel. The enhanced thermal stability of the enzyme upon immobilization could be explained through the aided stabilization of enzyme conformation by hydrogel network. It was reported that hydrogel could protect the conformational changes of enzyme from various environmental effects, such as heat, pH, and organic solvents.[22]

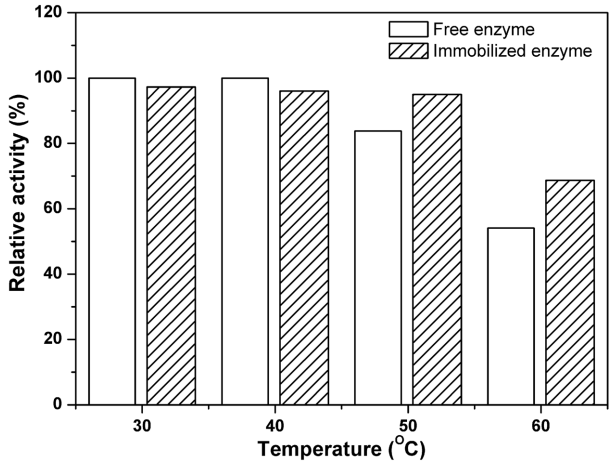


Figure 4. Thermal effects on the relative activities of free and immobilized enzymes. The releases of pNA by free and immobilized His-tagged GAP from Ala-pNA (0.06 mM) were monitored with the temperature range 30-60 °C in 50 mM Tris-HCl (pH 8.0).

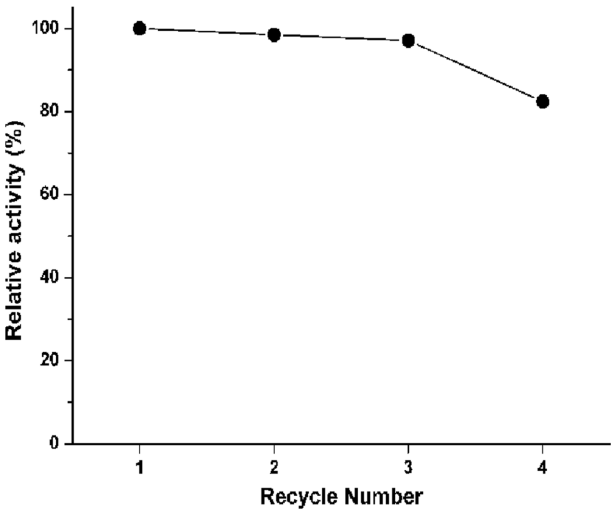


Figure 5. Sustained immobilization of His-tagged GAP on the regenerated Ni^{2+}-PAAA hydrogel. The hydrolysis of Ala-pNA by immobilized aminopeptidase was monitored at 37 °C for 1 h in 50 mM Tris-HCl (pH 8.0).

Reusability of the Ni^{2+}-PAAA hydrogel is one of the many advantages in the enzyme immobilization, which in turn would be commercially important in various biocatalyst applications. The enzyme must be very stable or become highly stabilized from the immobilization process. The changes in

enzyme activity were monitored after the regeneration of the Ni^{2+}-PAAA hydrogel to test its reusability in Figure 5. After four cycles of reuse of the Ni^{2+}-PAAA hydrogel at 37 ºC, the activity of the immobilized His-tagged GAP on the hydrogel retained relatively well; more than 80% were remained from the initial activity.

Conclusions

Ni^{2+}-PAAA hydrogel system revealed three-dimensional network structure with high capacity to retain large amounts of water. The soft and fluid environment of fully hydrated hydrogel can provide enzymes with near-physiological conditions, where the minimized denaturation and preserved activity of an immobilized enzyme could improve the thermal stability and its reusability. Ni^{2+}-PAAA hydrogel could provide a simple one-step procedure for the His-tag enzyme immobilization and purification at low cost, and its greater usage could be anticipated in both research and industrial applications.

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References

(1) D. A. R. Mahmoud and W. A. Helmy, *J. Appl. Sci. Res.*, **5**, 2466 (2009).
(2) D. Brady and J. Jordaan, *Biotechnol. Lett.*, **31**, 1639 (2009).
(3) H. H. Weetall, *Anal. Chem.*, **46**, 602 (1974).
(4) J. Krenkova and F. Svec, *J. Sep. Sci.*, **32**, 706 (2009).
(5) R. A. Sheldon, *Adv. Synth. Catal.*, **349**, 1289 (2007).
(6) E. Jang, S. Park, S. Park, Y. Lee, D. Kim, B. Kim, and W. Koh, *Polym. Adv. Technol.*, **21**, 476 (2010).
(7) P. Wang, *Curr. Opin. Biotechnol.*, **17**, 574 (2006).
(8) A. Kara, B. Osman, H. Yavuz, N. Besirli, and A. Denizli, *React. Funct. Polym.*, **62**, 61 (2005).
(9) N. A. Peppas, J. Z. Hilt, A. Khademhosseini, and R. Langer, *Adv. Mater.*, **18**, 1345 (2006).
(10) M. Basri, A. Harun, M. B. Ahmad, C. N. A. Razak, and A. B. Salleh, *J. Appl. Polym. Sci.*, **82**, 1404 (2001).
(11) G. S. Chauhan, S. Mahajan, K. M. Sddiqui, and R. Gupta, *J. Appl. Polym. Sci.*, **92**, 3135 (2004).
(12) S. S. Betigeri and S. H. Neau, *Biomaterials*, **23**, 3627 (2002).
(13) S. S. Kanwar, S. Pathak, H. K. Verma, S. Kumar, R. Gupta, S. S. Chimni, and G. S. Chauhan, *J. Appl. Polym. Sci.*, **100**, 4636 (2006).
(14) N. Pekel, B. Salih, and O. Guven, *J. Biomater. Sci. Polym. Ed.*, **16**, 253 (2005).
(15) M. Puri, A. Kaur, R. S. Singh, W. H. Schwarz, and A. Kaur, *Process Biochem.*, **45**, 451 (2010).
(16) H. Zheng, J. Chen, L. Su, Y. Zhao, Y. Yang, H. Zeng, G. Xu, S. Yang, and W. Jiang, *Enzyme Microb. Technol.*, **41**, 474 (2007).
(17) I. Ardao, M. D. Benaiges, G. Caminal, and G. Alvaro, *Enzyme Microb. Technol.*, **39**, 22 (2006).
(18) B. C. C. Pessela, R. Torres, M. Fuentes, C. Mateo, R. Munilla, A. Vian, A. V. Carrascosa, J. L. Garcia, J. M. Guisan, and R. F. Lafuente, *J. Chromatogr. A*, **1055**, 93 (2004).
(19) M. Sari, S. Akgol, M. Karatas, and A. Denizli, *Ind. Eng. Chem. Res.*, **45**, 3036 (2006).
(20) E. J. Ha, Y. Kim, S. A. An, Y. Kim, J. Lee, S. Lee, and H. Paik, *J. Chromatogr. B*, **876**, 8 (2008).
(21) H. Tanaka, T. Suzuka, K. Hada, and Y. Tezuka, *Polym. J.*, **32**, 391 (2000).
(22) J. Li, Y. Du, L. Sun, H. Liang, T. Feng, Y. Wei, and P. Yao, *J. Appl. Polym. Sci.*, **101**, 3743 (2006).