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Horseradish Peroxidase Immobilized in Macroporous Hydrogel for Acrylamide Polymerization

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ABSTRACT: Thermosensitive hydrogel made up of poly(*N*-isopropylacrylamide) (PNIPA)-chitosan semi-interpenetrating network (semi-IPN) with ultrarapid responding rate was synthesized. Horseradish peroxidase (HRP) was then immobilized on this hydrogel that acted as an enzyme-carrier by glutaraldehyde bridge. Polymerization of acrylamide was initiated by a redox system (hydrogen peroxide/acetylacetone (Acac)) and was catalyzed by the immobilized enzyme at room temperature. The attention was focused on the properties of the carrier-enzyme systems. The hydrogel was proofed to be macroporous by environmental scanning electron microscope images. Swelling properties of the hydrogel such as swelling ratio and deswelling–reswelling kinetics were measured. The properties of the immobilized enzyme such as enzyme activity, storage stability, and thermostability were also studied. The immobilized enzyme could be used repeatedly. Gel permeation chromatography measurement of the resulted polyacrylamide (PAAm) showed that the molecular weight reduced as the repeated times of the immobilized enzyme catalysis increased. In conclusion, the macroporous hydrogel would be a suitable enzyme carrier for practical applications in future. © 2008 Wiley Periodicals, Inc. J Polym Sci Part A: Polym Chem 46: 2222–2232, 2008

Keywords: enzymatic polymerization; enzyme immobilization; enzymes; hydrogels; macroporous polymers; polyacrylamide; thermosensitive hydrogel

INTRODUCTION

Recently, much interest has been taken in enzymatic polymerization, which denotes an *in vitro* polymerization via nonbiosynthetic pathways catalyzed by an isolated enzyme, as a new methodology of polymer syntheses.[1] Hydrolases (glycosidases and lipase) and oxidoreductases (horseradish peroxidase (HRP), soybean peroxidase (SBP), and laccase) have been proven to be efficient catalysts of polymer synthesis. HRP is a well-known enzyme that catalyzes the oxidation of several organic substances (such as phenols, anilines, ...) by hydrogen peroxide (H_2O_2) and a few hydroperoxides.[2] The generally accepted mechanism is that free radicals are produced during the catalysis.[3] The HRP-mediated polymerization of vinyl monomers was first reported by Derango et al. with acrylic monomers such as acrylamide (AAm) and hydroxyethyl methacrylate (HEMA).[4] β-Diketone was not involved and the authors assumed the oxidized form of the enzyme to be the initiator. Lalot and coworkers investigated the polymerization of AAm initiated by a redox system using β-diketones as reducing reactants and catalyzed by HRP.[5–9] Kobayashi and coworkers also studied enzyme-mediated vinyl polymerization in

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the presence of 2,4-pentanedione (acetylacetone, Acac).[10,11] Moreover, HRP was used to catalyze the initiation of other vinyl polymerization (styrene, derivatives of styrene, acrylates, and methyl methacrylate) in water-miscible cosolvents.[12,13]

For the purpose of reducing product cost, it is important to recover and reuse the expensive enzyme. Many methods have been applied for the immobilization of HRP over the years, such as covalent coupling,[14] adsorption,[15] crosslinking with glutaraldehyde,[16,17] encapsulation, and inclusion.[18,19] Immobilized HRP was widely used in applications, for instance, biosensor for hydrogen peroxide determination,[16,18,20] electrochemical immunoassay,[19] polyaniline synthesis,[17] and treatment of phenolic wastewater.[14,15] However, there were only a few reports upon the immobilized HRP that was utilized for enzymatic polymerization. There are several difficulties in enzymatic polymerization using immobilized enzyme. Most of the existing enzyme-carriers are nonporous and the specific surface areas are not so high, as a result, catalysis between the substrates and the enzymes is restrained. The viscosities of product solutions are usually high, leading up to hard recovery of immobilized enzyme. To get over these problems, macroporous poly(*N*-isopropylacrylamide) (PNIPA) hydrogel are proposed.

PNIPA hydrogel is well-known as an intelligent temperature-sensitive material and has been studied extensively. The phase-transition temperature (PTT) of PNIPA hydrogel is around 32 °C. In water, PNIPA hydrogel is in a highly swollen state below the PTT while the gel is in a shrunken state above the PTT, since the hydrophobic nature of isopropyl groups in the PNIPA plays a dominant role in excluding the water from the gel.[21,22] Some researchers used the PNIPA gels to immobilize enzymes lately. Harada et al. prepared a temperature-responsive gels to immobilize core-shell type bioconjugates physically, which were formed from the mixture of bovine pancreas trypsin and poly(ethylene glycol)-*block*-poly(α,β-aspartic acid).[23] Hamerska-Dudra used PNIPA hydrogel as thermosensitive carrier for invertase[24] and glucoamylase,[25] but low-molecular weight substrates and products are preferred. To synthesize porous hydrogel, several methods had been proposed. Akashi and coworkers[26–28] prepared porous PNIPA hydrogels in the presence of nanosized silica particles with subsequent acid treatment. Zhang et al. used PEG as a pore-forming agent during the polymerization.[29,30] In Chen et al.'s study, superporous hydrogels were synthesized by crosslinking polymerization of various vinyl monomers in the presence of gas bubbles formed by the chemical reaction of acid and $NaHCO_3$.[31] Recently, a novel method of fabricating macroporous PNIPA hydrogel was found in our laboratory.[32] Since then, PNIPA-chitosan semi-interpenetrating network (semi-IPN) was synthesized as enzyme carrier on which HRP was immobilized by crosslinking with glutaraldehyde, according to our schedule.

The aim of this article is to study a novel type of macroporous thermosensitive hydrogel as an enzyme carrier. There is a high-specific surface area of this kind of hydrogel because of its macroporous property, which is significant for materials to be utilized as a catalyst support. Moreover, substrates as well as high-molecular weight products can easily transfer in this macroporous polymer matrix. Rapid responding property of the hydrogel synthesized by our group might be another advantage to be an enzyme carrier. After enzyme immobilization, the hydrogel is first heated in hot water to reach a shrunken state. On cooling the shrunken hydrogel in the substrate solution, the hydrogel expands rapidly and works like a pump that sucks up the substrates into its macropores, and then, enzyme catalysis takes place. When catalytic reaction finishes, the hydrogel is heated to shrink again, to squeeze the products out of the hydrogel. Following the earlier approach, catalysis and transfer could be enhanced, and on another hand, separation between products and the catalyst could be facile. Polymerization of AAm catalyzed by immobilized HRP enzyme, as a model enzyme, was studied in this article. Investigations in enzyme activity, storage stability, thermostability, and repetitive catalysis stability were carried on. The immobilized enzyme could be utilized for at least four times.

EXPERIMENTAL

Materials

N-isopropylacrylamide (NIPA, 99%, Acros Organics, NJ) was used after further recrystallization from *n*-hexane. *N*,*N*′-Methylenebisacrylamide (BIS, ≥98%, Fluka Chemika), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED, 99%,

Table 1. Feed Compositions of the Hydrogels

	Gel Samples						
	NG0	NG15	MG0	MG5	MG10	MG15	MG20
NIPA (mg)	150	150	150	150	150	150	150
BIS (mg)	5	5	5	5	5	5	5
Chitosan (mg)	0	150	0	50	100	150	200
4 wt % APS (μL)	100	100	100	100	100	100	100
TEMED (μL)	10	10	10	10	10	10	10
DDBAB (mg)	0	0	30	30	30	30	30
Water (mL)	1.5	1.5	1.5	1.5	1.5	1.5	1.5

Acros Organics), HRP (250 U/mg, Shanghai Huamei Bioengineering, China), chitosan ($M_w <$ 10,000, Jinan Haidebei Marline Bioengineering, China), Acac (CP, Sinopharm Chemical Reagent, China), glutaraldehyde (25% v/v, Sinopharm Chemical Reagent), ammonium persulfate (APS, $\geq$98%, Yixing Second Chemical Reagent Factory, China), dodecyl dimethyl benzyl ammonium bromide (DDBAB,95%, Shanghai Jingwei Chemical, China), guaiacol (AR, Tianjin Reagent Factory, China), and hydrogen peroxide (AR, 30% v/v, Shanghai Yuanda Peroxide, China) were used as received. Phosphate buffer solution (PBS pH: 7.0) was prepared by mixing 0.05-mol/L dihydrogenphosphate and monohydrogenphosphate in a ratio of 1:2.

Synthesis of PNIPA-Chitosan Semi-IPN Hydrogel

The process of synthesizing macroporous PNIPA hydrogel was introduced in our previous work.[32] Here, chitosan was involved to obtain PNIPA-chitosan semi-IPN hydrogel. Briefly, NIPA, BIS, chitosan, and DDBAB were dissolved in deionized water in glass vessel. The solution was cooled in a refrigerator at a temperature of -8 °C for 20 min, in which the solution was not frozen, and then polymerization took place after APS and TEMED were added. The polymerization was carried out at -8 °C for 12 h during which the whole reaction system was frozen. After the reaction, the resulted gel was cut into disks (20 mm in diameter and 5 mm in thickness). The samples were immersed in excessive deionized water at ambient temperature for 48 h, and the water was refreshed every several hours to remove the unreacted materials. Macroporous gels prepared at various amount of chitosan were labeled as MG0, MG5, MG10, MG15, and MG20. Normal gels which were synthesized at the absence (NG0) or at the presence (NG15) of chitosan were prepared at room temperature, in the absence of DDBAB. The feed compositions of the monomers and other reactants are summarized in Table 1.

Preparation of Immobilized Enzyme

Sakuragawa et al.[16] recommended a procedure of HRP immobilization on chitosan carrier. In our study, the enzyme was immobilized on chitosan involved in PNIPA-chitosan semi-IPN hydrogel by crosslinking with glutaraldehyde. After heating in hot water bath (45 °C) for 2 min, excessive water was wiped off from the surface of the gel samples ($\sim$1.5 g of each at swollen state) with wet filter paper. The samples were then placed into 50-mL beakers respectively, and 25-mL 25% glutaraldehyde solution was added into every beaker. The reaction took place for 2 h at room temperature (25 °C) under magnetic stir, and then the gels were rinsed repeatedly with distilled water to remove excess glutaraldehyde. Afterward, the gel samples were heated again in hot water bath (45 °C) for 2 min, excessive water was wiped off in the same way. Then 10-mL freshly prepared HRP solutions (1 mg/mL) were added to the activated semi-IPN gel samples, respectively. After 1 h reaction at room temperature under magnetic stir, the gel samples were rinsed with distilled water to remove the free HRP that was not immobilized on the carrier. The solution obtained during washing was analyzed for residual HRP, and the rinse was carried out until no HRP was found in the solution. The obtained hydrogels with the immobilized HRP were then used directly for AAm polymerization as well as other measurements. Gel samples after enzyme immobilization were renamed by suffixing "-A". For instance, NG0 was renamed NG0-A, which underwent the process 2.3.

Morphology Observations of the Gels

Morphologies of gel NG0-A, NG15-A, MG15 (swollen state of the forenamed), and MG15-A (both swollen and shrunken state) were studied by environmental scanning electron microscope (ESEM) observation. The wet gel samples were observed directly without further treatment. The ESEM morphology was studied using ESEM (XL Series-30, Philips, Netherlands).

Measurement of Swelling Ratio of the Gels

The gel samples were measured gravimetrically after excessive water was wiped off with wet filter paper in the temperature range from 15 to 45 °C. Before the measurement, the gel samples were immersed in deionized water for at least 24 h at each given measurement temperature. The swelling ratio (SR) is defined as W_s/W_d, where W_s is the weight of water in the swollen sample at a given temperature and W_d is the weight of the sample at dry state.

Measurement of Deswelling–Reswelling Kinetics of the Gels

The equilibrated gel samples at a temperature of 15 °C were quickly transferred into hot deionized water of 45 °C, and then the deswelling kinetics were measured gravimetrically after removing excessive water from the surface of samples with wet filter paper. The reswelling kinetics of the shrunk samples that immersed in the hot water of 45 °C for at least 24 h were determined gravimetrically at 15 °C.

The deswelling and reswelling kinetics were defined as temporal weight changes for the samples. The change of weight was converted to the normalized swelling degree (SD), which indicated the volume changes of the samples between equilibrium swollen (100%) and equilibrium shrunken (0%) states. The SD is defined as $100 \times (W_t - W_{15})/(W_{45} - W_{15})$, where W_t is the weight of sample at a given time, W_{15} and W_{45} are the weights of samples that reached equilibrium at 15 and 45 °C, respectively.

Special attention should be paid to the normalization of SD. Most of the researchers prefer to use WR ($100\times (W_t - W_d)/W_s$), since it indicates the ratio of water containing in the gels directly. However, if the gel has a high SR in the shrunken state above PTT, WR is hard to get a normalized result. Kaneko et al.[33] used SD to describe the swelling and deswelling kinetics because some of the gels had a high SR in the shrunken state, and so did we. The parameter SD does not directly indicate the proportion of water in the gel but indicates the swollen state of the gel between equilibrium swollen (100%) and equilibrium shrunken (0%) states.

Polymerization of Acrylamide

After several measurements (procedures 2.5. and 2.6.), the gel MG15 was considered to be the most suitable enzyme carrier among all the hydrogels we synthesized. So MG15-A was chosen to be the catalyst of AAm polymerization. AAm (100 mg), 10 μL of 0.012 mol/L H_2O_2, and 10-μL Acac were dissolved in 1.5 mL-deionized water in a glass vessel. After the solution was degassed for 10 min, 0.3-g shrunken gel MG15-A ($\sim$1.5 g at swollen state, at room temperature) was added into the solution. The gel sucked up the solution into its macroporous matrix, and polymerization took place. After 6 h, the reaction mixture was heated in water bath (45 °C) for 5 min so that the product solution would be squeezed out of the gel, while the gel shrinks. After the gel was filtered off, the solution was then added dropwise to excess of ethanol to precipitate polyacrylamide (PAAm). The gel was immersed in excessive deionized water at room temperature for 24 h, and the water was refreshed every several hours to remove the residual PAAm. The precipitated PAAm was filtered off, washed with ethanol and dried under vacuum. The recovered gel MG15-A was reused according to procedure 2.7.

For free enzyme, 25 μL of 1 mg/mL HRP solution was used instead of 0.3-g shrunken gel MG15-A.

Measurement of Molecular Weight of PAAm

The molecular weight and its distribution of resulted PAAm were determined by gel permeation chromatography (GPC, Waters 515, America) at 40 °C, with two columns (Ultrahydrogel^{TM} 2000 and Ultrahydrogel^{TM} 250) connected in series and a refractive index (RI) detector (Waters 2410), using 0.1 M $NaNO_3$ solution as the eluent at flow rate of 0.5 mL/min.

Measurement of the Activity of Immobilized Enzyme

The activity of the immobilized enzyme was measured by the method of Pütter[34] with some

modifications as follows. This method had been recognized for the estimation of enzyme activity.[16] The immobilized enzyme (~0.05 g, wet weight) was placed in a 100-mL conical flask; 50 mL of PBS (pH: 7) and 1.0 mL of 0.02 mol/L guaiacol solution were added to the flask. After the suspension at 25 °C for 15 min, 0.6 mL of 0.012 mol/L H_2O_2 was added to the mixture and the whole solution was stirred continuously. At intervals of 1 min, 3-mL portion of the reaction mixture was placed in a quartz cell and the absorbance was measured at 436 nm. The solution then returned to the reaction mixture to maintain the volume. After the measurements had been performed, the immobilized enzyme was collected and dried in an oven at 120 °C for 6 h. The dry weight of the immobilized enzyme was measured for calculating the activity. The reaction rate was estimated from the difference in absorbance fluctuation per minute at 436 nm. For the blank test, the same procedure was performed with water instead of H_2O_2. The activity of the immobilized enzyme in units (U) was calculated by the following equation, because tetraguaiacol was not produced without H_2O_2 as shown.

$$4\text{guaiacol} + 4H_2O_2 \xrightarrow{\text{HRP}} \text{tetra-guaiacol} + 8H_2O$$

$$U = (\Delta\text{test} - \Delta\text{cont}) \times (1/25.5) \times 4 \times 51.6,$$

where Δtest is the absorbance fluctuation per minute, Δcont is the absorbance fluctuation in the blank test, 25.5: the molar absorptivity of tetraguaiacol, 4: the reaction product is tetraguaiacol, and 51.6: total volume of the solutions. The value of activity per unit weight of the immobilized enzyme can be calculated from the value of U and the dry weight of the immobilized enzyme used.

For free enzyme, 50-μL 0.02 mg/mL enzyme solution was used instead of the immobilized enzyme. The total volume of the solution was 51.65 instead of 51.6 in the above equation.

Relative Activity of Repetitive Immobilized Enzyme

The activity of the immobilized enzyme was measured for every repetitive procedure 2.7. The change of activity was converted to specific activity. Relative activity is defined as $(A_n/A_0) \times 100\%$, where A_0 is the activity of immobilized enzyme before catalysis of AAm polymerization, A_n is the activity after catalysis for the nth repeated time.

Storage Stability

Two grams of immobilized enzyme was stored in 50-mL pH 7 PBS at room temperature. The enzyme activity was measured every 3 days. The relative activity of freshly prepared enzyme was recognized as 100%. For free enzyme, 0.02 mg/mL enzyme solution in pH 7 PBS was measured instead.

Thermostability

Two grams of immobilized enzyme was stored in 50-mL pH 7 PBS at 70 °C water bath. The enzyme activity was measured every 15 min. The relative activity of the enzyme which was measured at the beginning of storage at 70 °C was recognized as 100%. For free enzyme, 0.02 mg/mL enzyme solution in pH 7 PBS was measured instead.

Degradation of Enzyme by H_2O_2

Two grams of immobilized enzyme was stored in 50-mL pH 7 PBS at room temperature. And then, 4-mL 0.012 mol/L H_2O_2 was added to the mixture. The enzyme activity was measured every 15 min. The relative activity of the enzyme which was measured at the beginning of H_2O_2 added was recognized as 100%. For free enzyme, 4-mL 0.012 mol/L H_2O_2 was added to 50-mL 0.02 mg/mL enzyme solution in pH 7 PBS before the measurement.

RESULTS AND DISCUSSION

Preparation of PNIPA-Chitosan Semi-IPN Hydrogel

All the MG gels (MG0, MG5, MG10, MG15, and MG20) were sponge like. A part of water absorbed by the hydrogel could be driven out of the gels, when a certain compressive stress was added directly toward the gels. For the NG gels (NG0 and NG15), the water in the gels could not be driven out even if the gels were brokenly compressed. The different properties implied that MG gels were macroporous and water could transfer in the MG gels more freely than in the NG gels.

For the preparation of macroporous hydrogel, DDBAB was used during the polymerization. The effect of DDBAB and the mechanism of

macroporous hydrogel formation were discussed in our previous article.[32] Briefly, a novel hydrophobic initiator dodecyl dimethyl benzyl ammonium persulfate (DDBAPS) was formed *in situ* as a product of the reaction of DDBAB and APS. To prevent DDBAPS from depositing out, the polymerization was carried out in frozen state. DDBAPS exited in the reaction medium in the form of agglomeration. In this heterogeneous initiation system, the monomer molecules around the DDBAPS agglomerations could be polymerized, but gelation hardly took place in the area which was at a certain distance from DDBAPS agglomerations, so, macroporous hydrogel could be formed because of the microphase separation of the reaction system. The real function of DDBAB was a reactant in the reaction of DDBAB and APS to get DDBAPS which could form the special reaction system of microphase separation.

Enzyme Immobilization

Chitosan as an immobilization carrier contains an amino group in its structure. Glutaraldehyde could react with the amino group to form a Schiff base. In the proposed method, a kind of low-molecular weight ($M_w < 10{,}000$) chitosan, which could be dissolved easily in neutral water solutions, was chosen. Firstly, chitosan was incorporated into the network of crosslinked PNIPA, forming a semi-IPN. And then, when glutaraldehyde was added, one aldehyde group of glutaraldehyde reacted with the amino group of chitosan. After HRP solution was added, the other aldehyde group of glutaraldehyde reacted with the amino group of HRP. In this way, HRP was immobilized in the thermosensitive carrier.

Figure 1 shows the activities of different immobilized HRP samples. For macroporous gels (MG0-A, MG5-A, MG10-A, MG15-A, and MG20-A), the activity increased with the content of chitosan. It indicated that more content of chitosan resulted in larger immobilization capacity of the gel. The activity of MG20-A was high up to nearly 70 U/g. Even if chitosan was not present, the activity of the immobilized enzyme (MG0-A) was slightly affected by direct adsorption of the enzyme onto the surface of the macroporous gel. The activity of NG15-A was only about 2 U/g. Although the contents of chitosan in the gels NG15-A and MG15-A were the same, the activity of MG15-A was nearly 30 times of the activity of NG15-A. The great difference between the enzyme activities was attributed to the different structures of these two kinds of gels. Gel MG15-A was macroporous with high-specific surface area, and gel NG15-A was a normal gel with low-specific surface area. So that much more HRP could be immobilized in MG15-A than that in NG15-A.

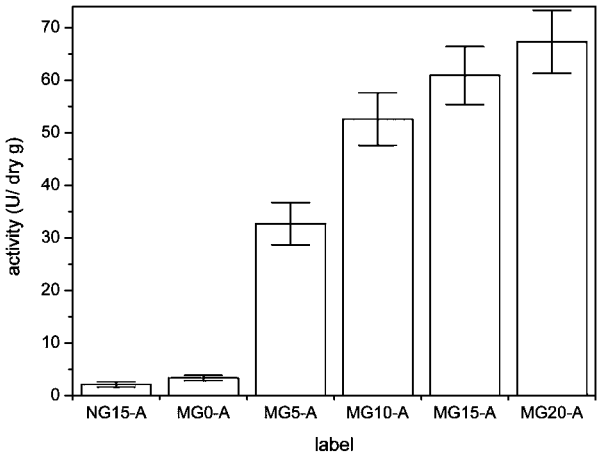

Figure 1. Activities of immobilized enzyme.

Morphology of the Gels

The ESEM micrographs of wet gels are shown in Figure 2. The gels NG0-A, NG15-A, MG15, and MG15-A were observed after reaching their equilibrium swollen state at room temperature. Furthermore, the gel MG15-A was observed after reaching its equilibrium shrunken state in hot water bath (45 °C). The matrixes of NG0-A and NG15-A were compact with few pores. These two normal gels were synthesized without DDBAB. For gel MG15, which was synthesized in the present of DDBAB, pores with the size of about 100 μm in diameter can be found. The reason for formation of macropores had been discussed before.[32] The gel MG15-A is also macroporous, but the pore size of gel MG15-A is smaller than the pore size of gel MG15. It indicates that enzyme immobilization process would decrease the pore size. Figure 2(e) shows the morphology of shrunken MG15-A. Pores still existed, which implied that macromolecules could transfer easily even in the shrunken gel.

Swelling Ratio

The equilibrium SRs of the NG and MG gels before and after enzyme immobilization are shown in Figures 3 and 4, respectively. The PTT are around 32 °C for all the gels. The SRs of all

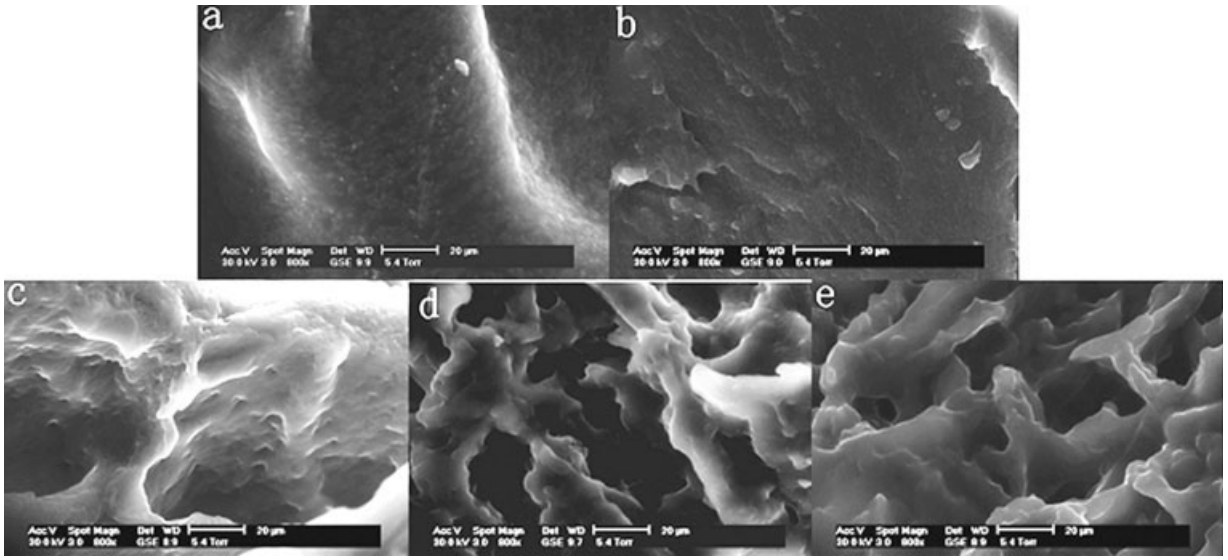

Figure 2. Micrographs of wet hydrogels: (a) swollen gel NG0-A; (b) swollen gel NG15-A; (c) swollen gel MG15; (d) swollen gel MG15-A; and (e) shrunken gel MG15-A.

the MG gels (MG0, MG5, MG10, MG15, and MG20) before enzyme immobilization are almost the same. An inapparent decrease of SRs with the content of chitosan can be observed. The SRs of the MG gels are obviously higher than that of the NG gels. The SR of NG15 which contains chitosan is higher than that of NG0. Property of high SR of MG gels is owed to their macroporous structure. For the NG gels, incorporation of chitosan into PNIPA gel networks increases the hydrophilicity of the systems and results in increased SRs.[35]

For the MG gels, the macropores cannot be occupied entirely by the hydrophobic crosslinked polymer chain at shrunken state above PTT, and the pores [about 20 μm in diameter according to Fig. 2(e)] which can hold a part of water still exist. For this reason, the SRs of MG gels are much higher than those of the NG gels at shrunken state.

Contrasting Figures 3 and 4, the gels which contain chitosan (NG15, MG5, MG10, MG15, and MG20) have a significant decrease of SRs. The SR of MG20-A at 15 °C even reduces from

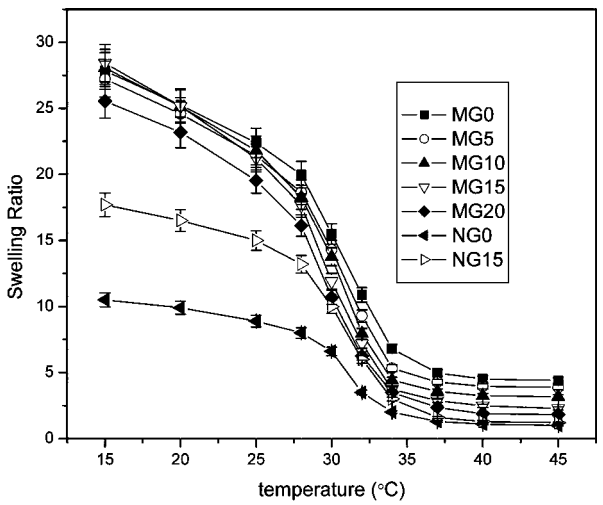

Figure 3. Equilibrium swelling ratio of gels before enzyme immobilization.

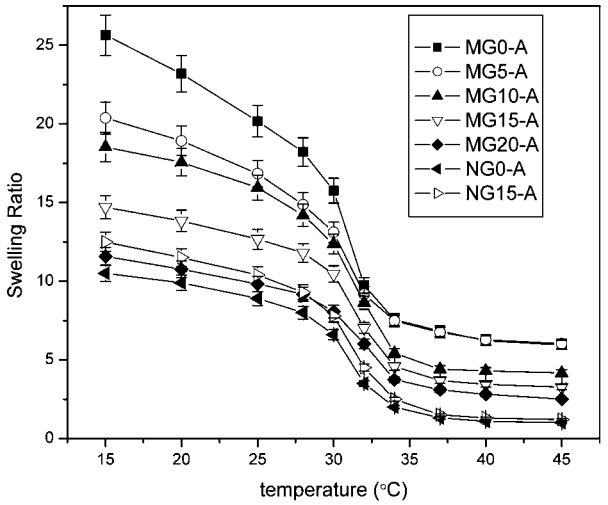

Figure 4. Equilibrium swelling ratio of gels after enzyme immobilization.

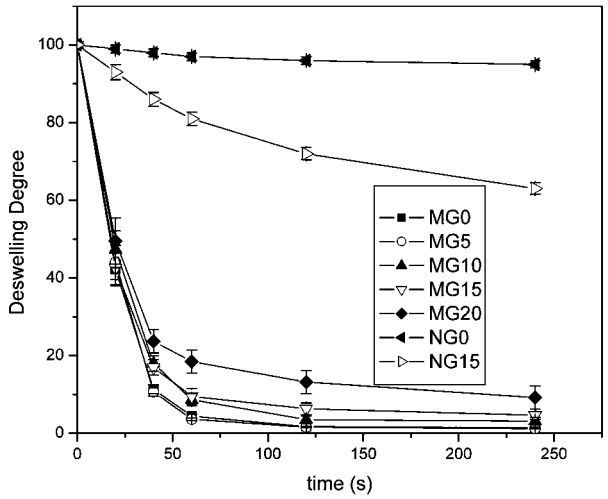

Figure 5. Deswelling kinetics of the gels before enzyme immobilization process at 45 °C from the equilibrium swollen state at 15 °C.

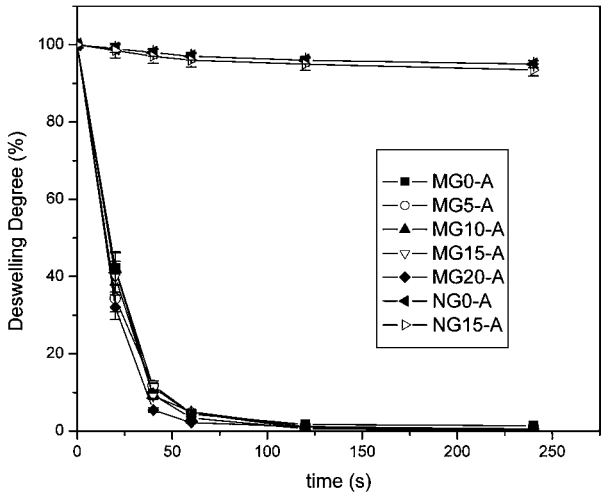

Figure 6. Deswelling kinetics of the gels after enzyme immobilization process at 45 °C from the equilibrium swollen state at 15 °C.

26 to 13 after immobilization process. However, SRs of the gels which do not contain chitosan (NG0 and MG0) are unaltered. It indicated that a part of the chitosan incorporated in the hydrogel matrix was crosslinked with glutaraldehyde, so that the hydrogel network was restricted, resulting in a decrease of SR.

Deswelling Kinetics

The deswelling kinetics of gels both before and after enzyme immobilization process that underwent a temperature jump from 15 (below PTT) to 45 °C (above PTT) are shown in Figures 5 and 6, respectively. The deswelling rate of gel NG0 was very slow and it could lose only 5% of water in 4 min. The deswelling rate of gel NG15 was faster then NG0, because the hydrophilic chitosan chains could act as water-releasing channels when deswelling and they enhance the shrinking rate.[35] The deswelling rates of all the MG gels were so fast that they almost reached their equilibrium shrunken states in just 2 min. The rapid deswelling property attributed to the macroporous structure of the gels. Water was squeezed out of the gels through the pores when deswelling. The hydrophilic chitosan chains in the MG gels absorbed a certain content of water which was hard to release through the macropores, so that deswelling rate of the gels that contained large amount of chitosan (MG15 and MG20) were a little slower than that of MG0. Contrasting Figures 5 and 6, changes of deswelling rate of the gels were not significant except the gel NG15. When enzyme immobilization process finished, a part of the chitosan chains were crosslinked with glutaraldehyde and lost the function of water-releasing, so that the deswelling rate of gel NG15-A was as slow as that of the gel NG0-A. For MG gels, macropores still existed after enzyme immobilization, so that the deswelling rates were still very fast.

Reswelling Kinetics

Figure 7 shows the reswelling kinetics of the gels before enzyme immobilization underwent a temperature jump from the equilibrium shrunken

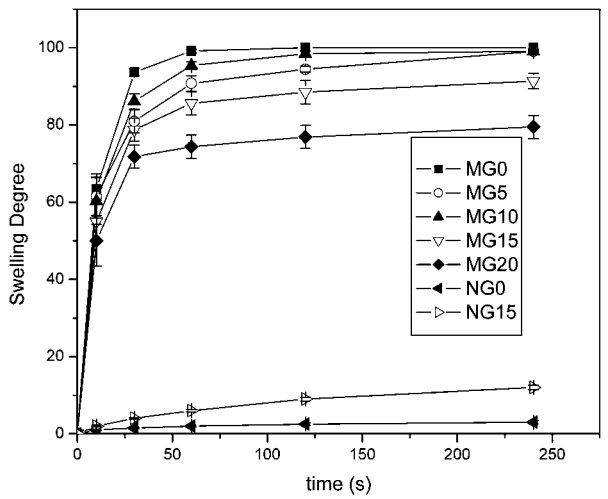

Figure 7. Reswelling kinetics of the gels before enzyme immobilization process at 15 °C from the equilibrium shrunken state at 45 °C.

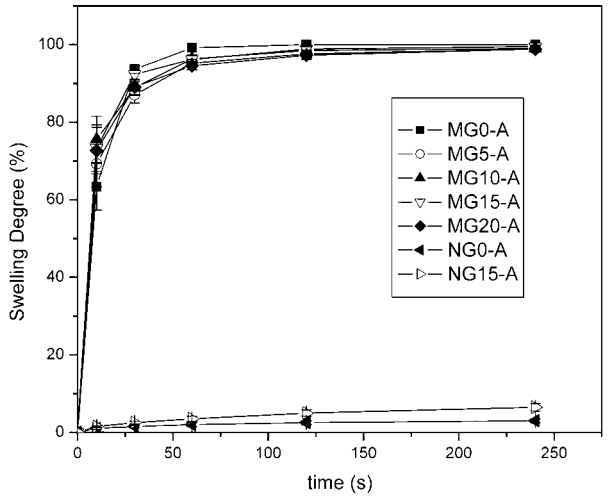

Figure 8. Reswelling kinetics of the gels after enzyme immobilization process at 15 °C from the equilibrium shrunken state at 45 °C.

state at 45 to 15 °C. Figure 8 shows the same process for the gels after enzyme immobilization. It can be seen from Figure 7 that gels MG0, MG5, and MG10 swell so fast that they reach the equilibrium swollen states in just 2 min. After enzyme immobilization process, the reswelling behaviors of all the MG gels were similar. However, it should be noticed that the SRs reduced obviously as the content of chitosan increased, according to Figure 4. Because of the incompletely closed porous structure at shrunken state, water molecules could easily diffuse into the interior of the gels. The surface pores enlarged when reswelling, permitting more water molecules to enter into the gels. So macroscopically, it seemed that the MG gels expanded rapidly when sucking up water just like the sponge did. It was difficult for water molecules to diffuse into the matrixes of NG gels because of their compact structure.

Polymerization of Acrylamide

The novel procedure of AAm polymerization, catalyzed by HRP immobilized on macroporous hydrogel, was carried out successfully. The responding rate of MG15-A was still rapid in resulted aqueous solution of PAAm. Table 2 shows the yields and molecular weights of the resulted PAAm, as well as the relative activities of the immobilized enzyme (gel MG15-A) after each reused time. It can be seen that the immobilized enzyme could be utilized repeatedly, however, the yield of PAAm and the activity of immobilized enzyme reduced obviously. The reduction was due to enzyme degradation by H_2O_2 as explained by Lalot and coworkers[8]. It was a pity that the immobilized HRP could not be used for more than four reused times because of this degradation.

Molecular weights of the resulted PAAm decreased with the reused time of the immobilized enzyme. When the immobilized enzyme (gel MG15-A) was used two times repeatedly, the molecular weights and distributions of PAAms were similar with that of PAAm catalyzed by free enzyme, indicating that the effect of H_2O_2 on enzyme degradation was not significant. The main reason for yield reduction was that a part of the resulted PAAm retained in the incomplete closed pores of the shrunken gel MG15-A. When the gel MG15-A was used for the third time, the yield, molecular weight, and enzyme activity decreased sharply. When the gel MG15-A was used for the fourth time, the activity of immobilized enzyme was very low, resulting in a low yield. The reason for reduction of the molecular weight was still unclear.

Stability of the Immobilized Enzyme

The results of storage stability of both free and immobilized enzyme are shown in Figure 9. The

Table 2. Results Summary of Polymerization of Acrylamide

Reused Time	Yield (%)	M_n ($\times 10^{-3}$ g/mol)	M_w ($\times 10^{-3}$ g/mol)	Polydispersity (DI)	Relative Activity After Polymerization (%)
1	78	174	466	2.68	79
2	70	137	446	3.26	62
3	42	40.6	229	5.64	43
4	23	12.7	14.9	1.17	31
Free enzyme	97	222	630	2.84	–[a]

[a] The activity of free enzyme could not be measured after polymerization of carylamide.

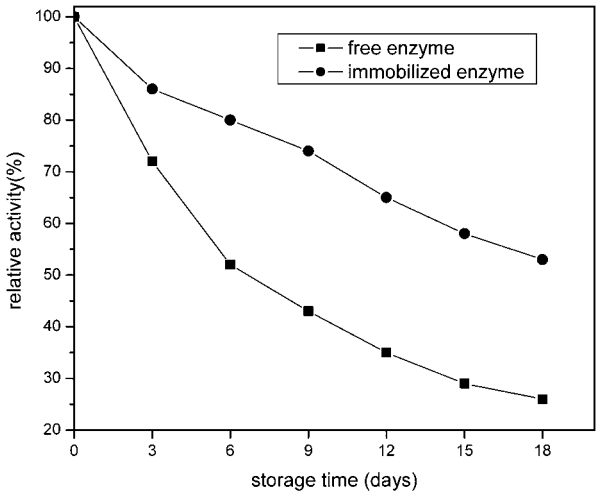

Figure 9. Storage stability of both free and immobilized enzyme.

activities of both free and immobilized enzyme reduced with storage time. Free enzyme retained less than 30% of the activity of freshly prepared enzyme solution after 18 days, while immobilized enzyme (gel MG15-A) retained about 55% of the activity that was measured as soon as the immobilized enzyme was synthesized. It seems that the immobilized enzyme was more stable when stored in water at room temperature than free enzyme.

Figure 10 shows the deactivation kinetics of both free and immobilized enzyme in 70 °C water bath. Free enzyme lost its activity rapidly. Only about 10% of the relative activity retained after storing at 70 °C for 50 min. However, high activity of immobilized enzyme retained and

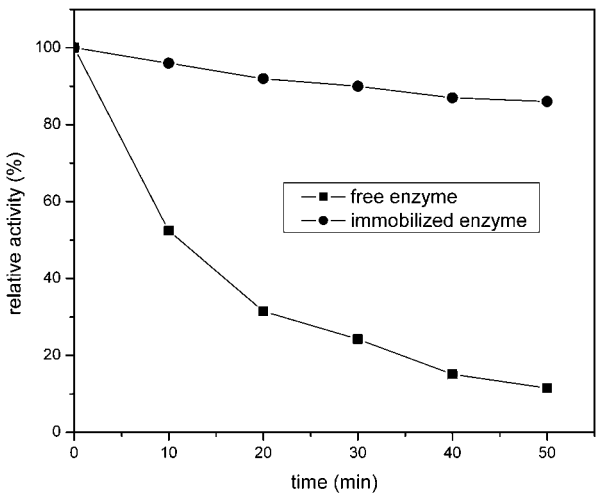

Figure 10. Thermostability of both free and immobilized enzyme.

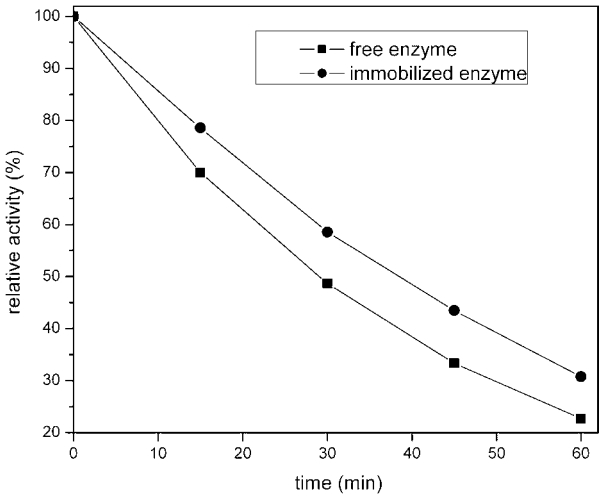

Figure 11. Enzyme degradation by H_2O_2.

high temperature (70 °C) had not so much effect on storage of immobilized enzyme. It indicated that the carrier for enzyme immobilization could protect the enzyme against the high temperature.

Figure 11 shows the enzyme degradation by H_2O_2. Both free enzyme and immobilized enzyme were deactivated rapidly when immersed in H_2O_2 solution of high concentration (about 10 times of the concentration used for initiation of AAm polymerization). Immobilization did not get over the enzyme degradation by H_2O_2 so that the reuse of immobilized enzyme was restricted.

CONCLUSIONS

A novel type of temperature-sensitive macroporous hydrogel which was made up of PNIPA-chitosan semi-IPN was successfully synthesized. HRP enzyme was immobilized on the hydrogel by glutaraldehyde bridge. Thermoresponding rate of the hydrogel was quite fast because of the macroporous property although a part of chitosan was crosslinked by glutaraldehyde. Macromolecules could transfer easily through the macropores of the hydrogel at both swollen and shrunken state. The content of HRP that immobilized on macroporous hydrogel was much larger than that immobilized on normal hydrogel. The immobilized enzyme could be used repeatedly for catalysis of polymerization of AAm for four times, but the yield and molecular weight of resulted PAAm reduced remarkably along with the reused times. The immobilized enzyme could be stored more stably than the free enzyme both at room temperature and at

70 °C, but could not get over the enzyme degradation by H_2O_2.

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