New Generation Polymeric Nanospheres for Catalase Immobilization

Sinan Akgöl,[1] Nevra Öztürk,[1] Adil Denizli[2]

[1]*Department of Chemistry, Faculty of Science and Arts, Adnan Menderes University, Aydin, Turkey*
[2]*Biochemistry Division, Department of Chemistry, Hacettepe University, Beytepe, Ankara, Turkey*

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ABSTRACT: Monosize poly(2-hydroxyethyl methacrylate-*co*-*N*-methacryloly-L-histidinemethylester) [mon-poly (HEMA-MAH)] nanospheres were prepared via surfactant-free emulsion polymerization method. L-Histidine groups of the mon-poly(HEMA-MAH) nanospheres were chelated with Fe^{3+} ions. Mon-poly(HEMA-MAH) nanospheres were characterized by Fourier transform infrared spectroscopy, proton NMR, and scanning electron microscopy. Particle size of the mon-poly(HEMA-MAH) nanospheres was measured by Zeta Sizer. Elemental analysis of MAH for nitrogen was estimated as 0.94 mmol/g polymer. The catalase immobilized onto the mon-poly(HEMA-MAH)–Fe^{3+} nanospheres resulted in increasing the enzyme stability with time. Optimum operational temperature for both immobilized preparations was the same, and the temperature profiles of the immobilized preparations were significantly broader. It was observed that enzyme could be repeatedly adsorbed and desorbed on the mon-poly(HEMA-MAH)–Fe^{3+} nanospheres without loss of adsorption capacity or enzymic activity. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 114: 962–970, 2009

Key words: catalase; nanospheres; adsorption; IMAC; amino acid ligand

INTRODUCTION

Immobilization confers additional stability to a variety of enzymes against several forms of denaturation. Enzymes have been immobilized on different shapes of supports (i.e., membranes, hollow fibers, or microbeads) either by adsorption, entrapment, or covalent binding. In the last decade, the application of nanometer materials has received increasingly great attention in the field of biotechnology and bioanalytical chemistry. Nanoparticles played an important role in the adsorption of biomolecules because of their large specific surface area and high surface energy.[1,2] Theoretical and experimental studies demonstrated that particle mobility, which is governed by particle size and solution viscosity, could impact the intrinsic activity of the particle-attached enzymes.[3,4] The enzyme supports are the most frequently obtained after chemical modification of natural and synthetic polymers such as chitin, chitosan, cellulose acetate, acrylic polymers, L-histidine-grafted poly(HEMA), poly(vinyl alcohol) (PVAL), or polyamide, and inorganic glass hollow fibers.[5–7]

Nonporous materials have a minimum diffusion limitation while enzyme loading per unit mass of support is usually low. On the other hand, porous materials can afford high enzyme loading, but suffer a much greater diffusional limitation of substrate. Reduction in the size of enzyme-carrier materials can generally improve the efficiency of adsorbed enzymes. In the case of surface attachment, smaller particles can provide a larger surface area for the attachment of enzymes, leading to higher enzyme loading per unit mass of particles.[8] In the case of enzyme adsorption into porous materials, much reduced mass-transfer resistance is expected for smaller porous particles owing to the shortened diffusional path of substrates when compared with large-sized porous materials. There have been extensive studies on the use of micrometer-sized particles for the adsorption of biomolecules.[9–11] Recently, a growing interest has been shown in using nanoparticles as carriers for enzyme adsorption.[12–17] The effective enzyme loading on nanoparticles could be achieved up to 10% weight because of a large surface area per unit mass of submicron particles and nanoparticles.[18]

IMAC is an effective analytical and preparative separation method for therapeutic proteins, peptides, nucleic acids, hormones, and enzymes.[19–23] The separation is based on the interaction of a Lewis acid (electron pair donor), i.e., a chelated metal ion, with an electron acceptor group on the surface of the protein.[24–26] Proteins are assumed to interact mainly through the imidazole group of histidine and, to a lesser extent, the indoyl group of tryptophan and the thiol group of cysteine. Cooperation between

Correspondence to: S. Akgöl (sinanakgol@yahoo.co.uk).

neighboring amino acid side chains and local conformations plays an important role in protein binding. Aromatic amino acids and the amino terminus of the peptides also have some contributions.[27] The low cost of metals and the reuse of adsorbents for hundreds of times without any detectable loss of metal-chelating properties are the attractive features of metal affinity separation.[28]

Catalase (EC 1.11.1.6) is a tetramer of four polypeptide chains, each over 500 amino acids long and regarded as one of the most common enzymes in plant and animal tissues. It contains four porphyrin heme (iron) groups, which allow the enzyme to react with the hydrogen peroxide and it has been used in industry for a long time.[28] Immobilized catalase has useful applications in various industrial fields such as food industry in the removal of hydrogen peroxide from food products after cold pasteurization, and in the analytical field, as a component of hydrogen peroxide and glucose-biosensor systems.[29–33]

In this study, a support matrix monosize poly(2-hydroxyethyl methacrylate-*co*-*N*-methacryloly-L-histidinemethylester) [mon-poly(HEMA-MAH)] nanospheres were prepared via surfactant-free emulsion polymerization method. Then, Fe(III) ions were incorporated into the histidine groups of the mon-poly(HEMA-MAH) nanospheres via metal chelation. Both monosize nanospheres were used for the immobilization of catalase.

MATERIALS AND METHODS

Materials

Bovine liver catalase (hydrogen peroxide oxidoreductase, E.C. 1.11.1.6), L-histidine methyl ester, and methacryloyl chloride were supplied by Sigma (St Louis, USA). EGDMA was obtained from Fluka A.G (Buchs, Switzerland), distilled under reduced pressure in the presence of hydoquinone inhibitor, and stored at 4°C until use. Poly(vinyl alcohol) (MW:100.000, 98% hydrolyzed) was supplied from Aldrich (St. Louis, USA). All other chemicals of reagent grade and purchased from Merck A.G. (Darmstadt, Germany). All water used in the experiments was purified using a Barnstead [(Dubuque, IA) ROpure LP®] reverse osmosis unit with a high-flow cellulose acetate membrane (Barnstead D2731). This membrane followed by a Barnstead 3804 NANOPURE® organic/colloid removal and ion-exchange packed-bed system.

Methods

Synthesis of MAH

For the synthesis of MAH, the following experimental procedure was applied; 5.0 g of L-histidine methylester and 1.82×10^{-3} mol of hydroquinone were dissolved in 100 mL of dichloromethane solution. This solution was cooled down to 0°C and 0.13 mol of triethylamine was added to the solution. Five milliliters of methacryloyl chloride was poured slowly into this solution, which was stirred magnetically at room temperature for 2 h. Hydroquinone and unreacted methacryloyl chloride were extracted with 10% NaOH solution. The aqueous phase was evaporated in a rotary evaporator. The residue (i.e., MAH) was crystallized in an ether–cyclohexane mixture and then dissolved in ethyl alcohol.

TABLE I
Polymerization Recipe and Conditions

Dispersion phase	Organic phase
50 mL of deionized water	0.01 mL MAH
0.0198 g KPS/45 mL water	0.6 mL HEMA
0.5 g PVAL/45 mL water (dissolved 1 h at 60°C in sonicator and taken 25 mL of it)	0.3 mL EGDMA
Polymerization conditions	
Reactor volume	100 mL
Stirring rate	150 rpm
Time	24 h
Temperature	70°C

Synthesis of mon-poly(HEMA-MAH) nanospheres

Mon-poly(HEMA-MAH) nanospheres were produced by surfactant-free emulsion polymerization. Table I shows a polymerization recipe and procedure in detail. In a typical polymerization procedure may be explained: the stabilizer, PVAL (0.5 g), was dissolved in 50 mL deionized water for the preparation of the continuous phase. Then, the monomer mixture (HEMA and MAH) was added to this dispersion phase, which was mixing in an ultrasonic bath for about half an hour. Before polymerization, initiator was added to the solution and nitrogen gas was blown through the medium for about 1–2 min to remove dissolved oxygen. Polymerization was carried out in a constant temperature shaking bath at 70°C, under nitrogen atmosphere for 24 h. After the polymerization, the nanospheres were cleaned by washing with methanol and water several times to remove the unreacted monomers. For this purpose, the nanospheres were precipitated and collected with the help of a centrifuge (Zentrifugen, Universal 32 R, Germany), which is at the rate of 18,000 rpm for 1 h and resuspended in methanol and water several times. After that mon-poly (HEMA-MAH) nanospheres were further washed with deionized water. Poly(HEMA) nanospheres were produced by same formulation without MAH comonomer.

FTIR spectra of the nanospheres and MAH monomer were obtained by using FTIR spectrophotometer

(Varian FTS 7000, USA). The dry sample monomer (about 0.1 g) was thoroughly mixed with KBr (0.1 g, IR Grade, Merck, Germany) and pressed into a tablet form, and the spectrum was then recorded.

The proton NMR spectrum of MAH monomer was taken in $CDCl_3$ on a Jeol GX-400 (Peabody, USA) instrument. The residual nondeuterated solvent ($CHCl_3$) served as an internal reference. Chemical shifts are reported in ppm (δ) downfield relative to $CHCl_3$.

The surface morphology of the monosize nanospheres was examined using scanning electron microscope (SEM). The samples were initially dried in air at 25°C for 7 days before being analyzed. A fragment of the dried bead was mounted on a SEM sample mount and was sputtered coated for 2 min. The sample was then mounted in a SEM (Phillips, XL-30S FEG, Germany). The surface of the sample was then scanned at the desired magnification to study the morphology of the nanospheres.

The particle size, size distribution, and surface charge were determined by Zeta Sizer (Malvern Instruments, Model 3000 HSA, England).

To evaluate the degree of MAH incorporation, the synthesized mon-poly(HEMA-MAH) nanospheres were subjected to elemental analysis using a Leco Elemental Analyzer (Model CHNS-932).

The degree of MAH incorporation in the synthesized mon-poly(HEMA-MAH) nanospheres was determined by elemental analyzer (Leco, CHNS-932, USA).

The surface area of mon-poly(HEMA-MAH) nanospheres was calculated using the following expression[31]:

$$N = 6 \times 10^{10}\ S/\pi\rho_s d^3. \quad (1)$$

Here, N is the number of nanospheres per milliliter, S is the % solids, ρ_s is the density of bulk polymer (g/mL), and d is the diameter (nm). The number of nanosphere in mL suspension was determined by utilizing from mass-volume graph of nanospheres. From all these data, specific surface area of mon-poly(HEMA-MAH) nanospheres was calculated by multiplying N and surface area of 1 nanosphere.

Incorporation of Fe^{3+} ions

Chelates of Fe^{3+} ions with mon-poly(HEMA-MAH) nanospheres were prepared as follows: 1.0 g of the monosize nanospheres was mixed with 50 mL of aqueous solutions containing 30 ppm Fe^{3+} ions, at constant pH of 5.0 (adjusted with HCl and NaOH), which was the optimum pH for Fe^{3+} chelate formation at room temperature. A 1000 ppm atomic absorption standard solution (containing 10% HNO_3) was used as the source of Fe^{3+} ions. The flask was stirred magnetically at 100 rpm for 1 h (sufficient to reach equilibrium). The concentration of the Fe^{3+} ions in the resulting solution was determined with a graphite furnace atomic absorption spectrometer (AA800, Perkin-Elmer, Bodenseewerk, Germany). The Fe^{3+} chelation step is depicted in Figure 1. The amount of adsorbed Fe^{3+} ions was calculated by using the concentrations of the Fe^{3+} ions in the initial solution and in the equilibrium. Fe^{3+} leakage from the mon-poly(HEMA-MAH) submicron beads was investigated with media pH (6.0–8.0), and also in a medium containing 1.0*M* NaSCN. The nanospheres suspensions were stirred for 24 h at room temperature. Fe^{3+} ion concentration was then determined in the supernatants using an atomic absorption spectrophotometer. It should be also noted that immobilized metal-containing nanospheres were stored at 4°C in the 10 m*M* Tris-HCl buffer (pH 7.4) with 0.02% sodium azide to prevent microbial contamination.

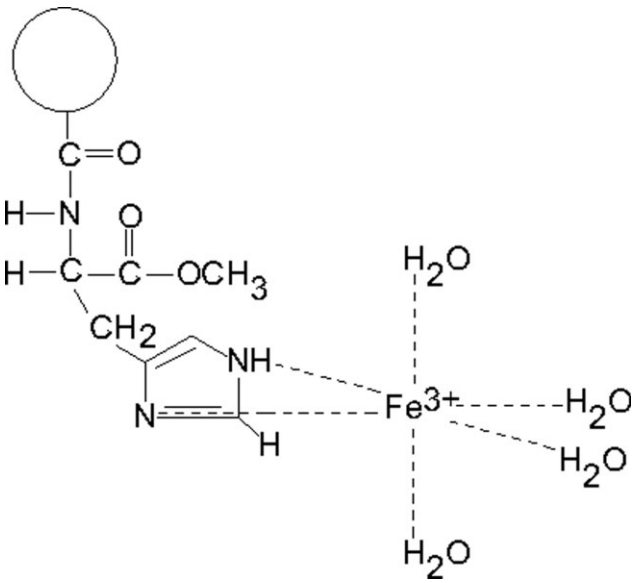

Figure 1 Schematic diagram for the chelation of Fe^{3+} ions through the monosize nanospheres.

Catalase adsorption studies

Catalase adsorption by the Fe^{3+}-chelated monosize poly(HEMA-MAH) nanospheres was studied at various pH values, either in acetate buffer (0.1*M*, pH 4.0–5.5) or in phosphate buffer (0.1*M*, pH 6.0–8.0). Initial catalase concentration was 1.0 mg/mL in the corresponding buffer. The adsorption experiments were conducted for 2 h at 25°C while stirring continuously. At the end of this period, the enzyme-adsorbed nanospheres were removed from the enzyme solution and were washed with the same buffer three times. It was stored at 4°C in fresh

buffer until use. The amount of adsorbed catalase was calculated as:

$$Q = [(C_o - C)V]/m. \quad (2)$$

Here, Q is the amount of catalase adsorbed onto unit mass of nanospheres (mg/g); C_o and C are the concentrations of catalase in the initial solution and in the aqueous phase after treatment for certain period of time, respectively, (mg/mL); V is the volume of the aqueous phase (mL); and m is the mass of the nanospheres used (g). To determine the adsorption capacities of mon-poly(HEMA-MAH)–Fe^{3+} nanospheres, the concentration of catalase in the medium was varied in the range of 0.05–2.0 mg/mL.

Desorption of catalase from Fe^{3+}-chelated nanospheres

To determine the reusability of the mon-poly (HEMA-MAH)–Fe^{3+} nanospheres, the catalase adsorption and desorption cycle was repeated five times. The catalase desorption from mon-poly (HEMA-MAH)–Fe^{3+} nanospheres was carried out with 1.0*M* NaSCN solution at pH 8.0. The beads were washed several times with phosphate buffer (0.1*M*, pH 7.0) and were then reused in enzyme immobilization.

Determination of immobilization efficiency

The amount of protein in the crystalline-enzyme preparation and in the wash solution was determined by spectrofluorimetry (excitation 280 nm and emission 340 nm) using a Shimadzu (Model RF 5301-PC, Japan) spectrofluorimeter.

Activity assays of free and immobilized catalase

Catalase activity was determined spectrophotometrically by direct measurement of the decrease in the absorbance of hydrogen peroxide at 240 nm, because of its decomposition by the enzyme. Hydrogen peroxide solutions (5–30 m*M*) were used to determine the activity of both the free and immobilized enzymes. A 4 mL portion of the reaction mixture was preincubated at 25°C for 10 min, and the reaction was started by adding 50 μL of catalase solution (100 μL/mL). The decrease in absorbance at 240 nm was recorded for 5 min. The rate of change in the absorbance (240/min) was calculated from the initial linear portion, with the use of the calibration curve (absorbance of hydrogen peroxide solutions of various concentration (5–30 mM) at 240 nm). One unit of activity is defined as the decomposition of 1 μmol/min of hydrogen peroxide at 25°C and pH 7.0. These activity assays were carried out over the pH range 4.0–8.0 and temperature range 5–60°C to determine the pH and temperature profiles for the free and the immobilized enzymes. The effect of substrate concentration was tested in the 5–30 m*M* hydrogen peroxide range. The results of pH, temperature, and substrate concentration of the medium are presented in a normalized form, with the highest value of each set being assigned the value for 100% activity.

Storage stability

This experiment was conducted to determine the stabilities of free and adsorbed catalase preparations after storage in phosphate buffer (50 m*M*, pH 7.0) for 50 days. The residual activities were then determined as described earlier. Activity of each preparation was expressed as a percentage of its residual activity compared with the initial activity.

Thermal stability

The thermal stability of free and adsorbed catalase was determined by measuring the residual enzymatic activity of two different temperatures (50 and 60°C) in a phosphate buffer (0.1*M*, pH 7.0) for 2 h. After every 15-min time interval, the sample was removed and assayed for enzymatic activity as described earlier. The results were given as activity %.

Repeated use studies

To determine the reusability of the mon-poly(HEMA-MAH)–Fe^{3+} nanospheres, the catalase adsorption and desorption cycle was repeated five times using the same group of monosize nanospheres. Catalase desorption from the mon-poly(HEMA-MAH)–Fe^{3+} nanospheres was carried out with a mixture containing 50% pure ethylene glycol in 1.0*M* NaCl. The desorption medium was stirred magnetically at 100 rpm at 25°C for 120 min. The equilibrium desorption time was found to be 120 min with pre-experiments. The desorption ratio of catalase was calculated by using the following expression:

$$\text{Desorption ratio} = [\text{Enzyme released}/\text{Enzyme adsorbed on the nanospheres}] \times 100. \quad (3)$$

RESULTS AND DISCUSSIONS

Properties of mon-poly(HEMA-MAH) nanospheres

Nanoparticles provide an ideal remedy to the usually contradictory issues encountered in the optimization of adsorbed enzymes: minimum diffusional limitations, maximum surface area per unit mass, and high enzyme loading. In addition to the promising performance features, the unique solution

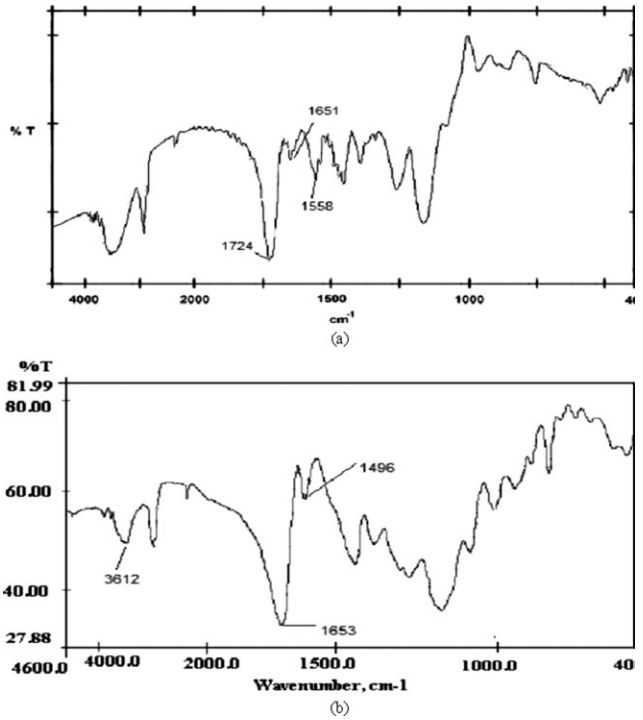

Figure 2 FTIR spectrum of (a) MAH monomer and (b) mon-poly(HEMA-MAH).

behaviors of the nanoparticles also point to an interesting transitional region between heterogeneous and homogeneous catalysis. We suggest here a novel method of introducing a imidazole group into the polymeric monosize nanospheres via polymerization of HEMA and MAH. Then, Fe^{3+} ions covalently incorporated onto nanospheres. The distinctive feature of this method is the elimination of the activation and ligand coupling steps during the preparation of the affinity matrices. Some other advantages over other methods include the use of a known amount of ligand in the polymer preparation mixture and the good reproducibility of the affinity matrix.[5] In addition to this, IMAC is a sensitive and selective method for proteins. The number of locations of surface-exposed electron-donating imidazole and thiol groups and their ability to coordinate with chelated metal ions dictate the adsorption of proteins on metal-immobilized adsorbents.

MAH monomer has the characteristic stretching vibration amide I and amide II absorption bands at 1651 and 1558 cm^{-1}, carbonyl band at 1724 cm^{-1} as shown in Figure 2(a). The FTIR spectrum of mon-poly(HEMA-MAH) has the characteristic stretching vibration band of hydrogen-bonded alcohol, O—H, around 3612 cm^{-1}, carbonyl at 1653 cm^{-1} amide II absorption band at 1496 cm^{-1}, respectively. These data confirmed that the mon-poly(HEMA-MAH) nanospheres were formed with functional groups MAH [Fig. 2(b)].

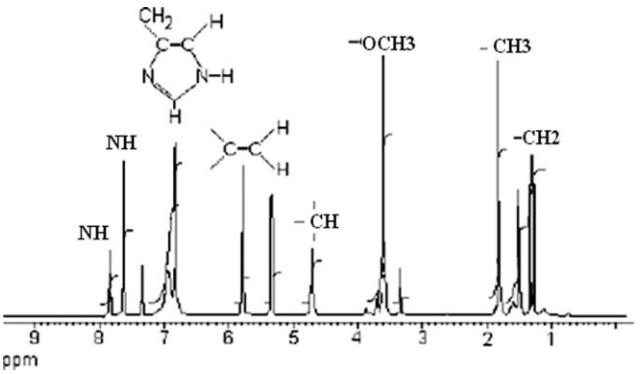

Figure 3 ^{1}H-NMR spectrum of MAH monomer.

^{1}H-NMR was used to determine the synthesis of MAH structure. Figure 3 shows the ^{1}H-NMR spectrum of MAH. ^{1}H-NMR spectrum is shown to indicate the characteristic peaks from the groups in MAH monomer. These characteristic peaks are as follows: ^{1}H-NMR ($CDCl_3$): δ 1.99 (t; 3H, $J = 7.08$ Hz, CH_3), 1.42 (m; 2H, CH_2), 3.56 (t; 3H, —OCH_3) 4.82–4.87 (m; 1H, methin), 5.26 (s; 1H, vinyl H), 5.58 (s; 1H, vinyl); 6.86 (δ; 1H, $J = 7.4$ Hz, NH), 7.82 (δ; 1H, $J = 8.4$ Hz, NH), 6.86–7.52 (m; 5H, aromatic).

As seen in Figure 4, particle size of the mon-poly (HEMA-MAH) nanospheres was measured by Zeta Sizer and about 450 nm with 0.171 polydispersity. The particle size was an average of minimum 30 measurements, and the size distribution was recorded automatically by the software of these repeated measurements.

To evaluate the degree of MAH incorporation into the polymeric structure, elemental analysis of the synthesized mon-poly(HEMA-MAH) nanospheres was performed. The incorporation of the MAH was found to be 0.94 mmol/g polymer using nitrogen stoichiometry.

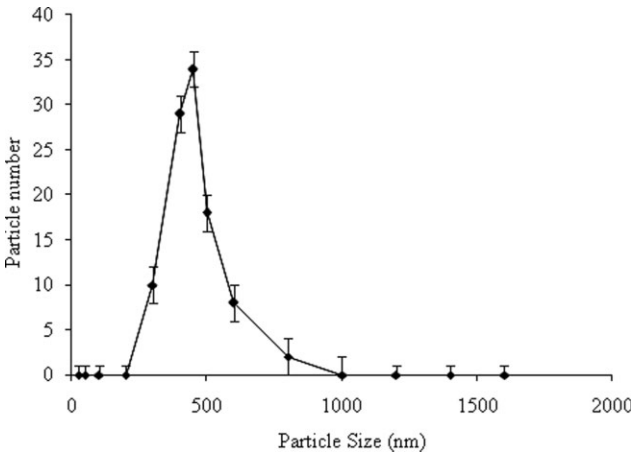

Figure 4 Size analysis of mon-poly(HEMA-MAH) nanospheres.

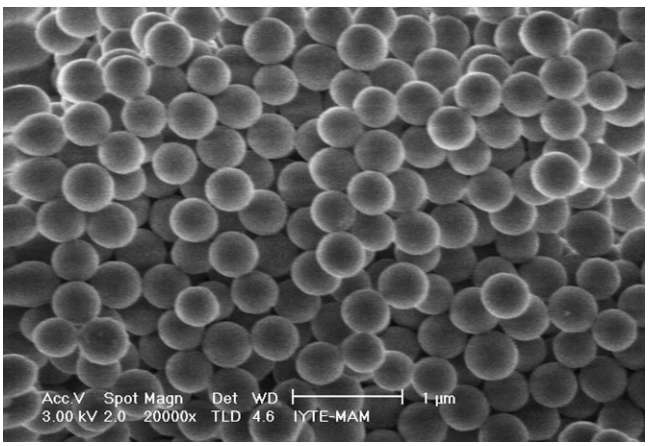

Figure 5 SEM micrographs of mon-poly(HEMA-MAH) nanospheres.

Nanospheres can produce larger specific surface area and therefore may result in high enzyme loading. Therefore, it may be useful to synthesize nanospheres with large surface area and utilize them as suitable carriers for the adsorption of enzymes. The specific surface area was calculated as 604 m^2/g for poly(HEMA) and 651.5 m^2/g for mon-poly(HEMA-MAH) nanospheres. As seen in Figure 5, the SEM micrographs clearly showed the spherical and monosize character of poly(HEMA-MAH) nanospheres. In addition, the total monomer conversion was determined as 97.4% (w/w) for mon-poly(HEMA-MAH) nanospheres.

Adsorption efficiency and retention of activity

The effect of pH on the adsorption of catalase onto mon-poly(HEMA-MAH)–Fe^{3+} nanospheres was studied in the pH range 4.0–9.0 and the effects of pH on adsorption are presented in Figure 6. The decrease in the protein adsorption capacity in more acidic and more alkaline regions can be attributed to electrostatic repulsion effects between the opposite charged groups. Proteins have no net charge at their isoelectric points, and therefore the maximum adsorption from aqueous solutions is usually observed at their isoelectric points. The isoelectric pH of catalase is 6.35. In this study, the maximum adsorption was not at this pH, but had slightly shifted toward more acidic pH values. This could be due to preferential interactions between catalase molecules and Fe^{3+}-incorporated polymeric matrix at neutral pH.

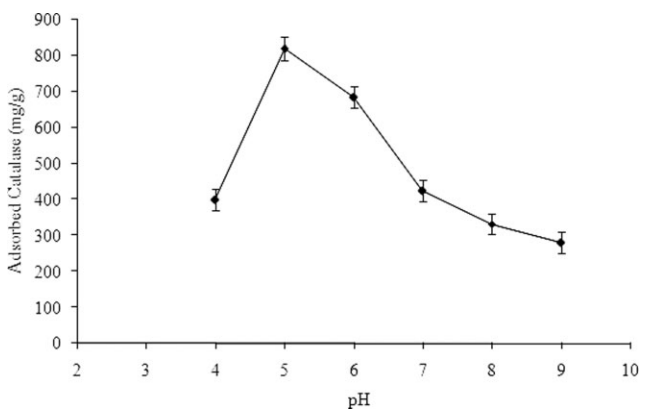

Figure 6 Effect of pH on catalase adsorption onto mon-poly(HEMA-MAH)–Fe^{3+} nanospheres; MAH content: 0.94 mmol/g; catalase concentration: 1.0 mg/mL; *T*: 35°C.

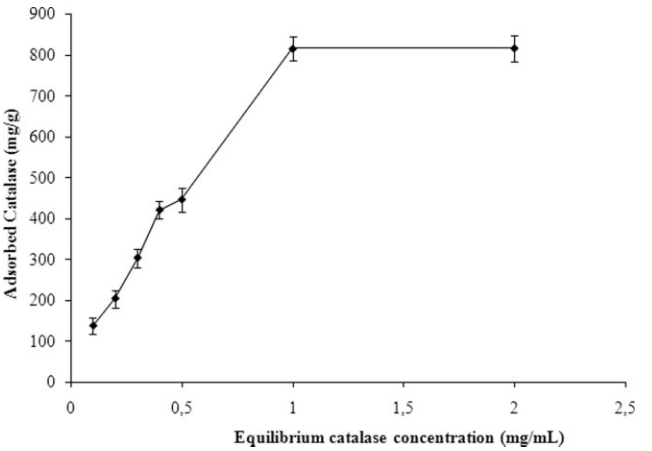

Figure 7 Effect of catalase concentration on the adsorption efficiency of mon-poly(HEMA-MAH)–Fe^{3+}; MAH content: 0.94 mmol/g; pH 5.0; *T*: 35°C.

Effect of catalase concentration on the adsorption efficiency of mon-poly(HEMA-MAH)–Fe^{3+} nanospheres is shown in Figure 7. An increase in catalase concentration in the adsorption medium led to an increase in adsorption efficiency but this leveled off at a catalase concentration of 1.0 mg/mL. Maximum catalase adsorption was obtained for mon-poly (HEMA-MAH) nanospheres (814.0 mg/g). This could be due to the specific interactions between catalase and chelated-Fe^{+3} ions, because catalase has an iron protoporphyrin prosthetic group.

Kinetic parameters, Michaelis constants K_m and V_{max} for free and immobilized catalase were determined using Lineweaver-Burk plot in Figures 8 and 9. Hydrogen peroxide was used as the substrate. For the free enzyme, K_m was found to be 45.5 mM, whereas *V*max was calculated as 6.4×10^4 U/mg protein. Kinetic constants of the immobilized catalase were also determined in a batch system. K_m values were found to be 18.2 m*M* for mon-poly(HEMA-MAH)–Fe^{3+}-catalase preparation. The V_{max} values of immobilized enzyme for mon-poly(HEMA-MAH)–Fe^{3+}-catalase preparation were estimated from the data as 1.69×10^3 U/mg of adsorbed protein onto mon-poly(HEMA-MAH) nanospheres. As expected, the K_m and V_{max} values were significantly affected after adsorption onto the Fe^{3+}-incorporated mon-poly(HEMA-MAH) nanospheres.

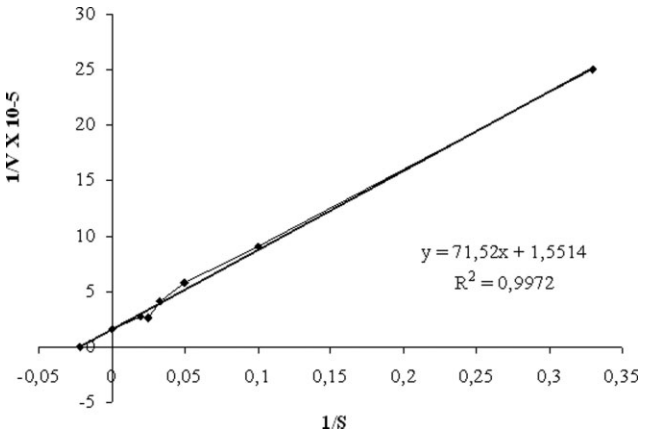

Figure 8 Lineweaver-Burk plot for free catalase.

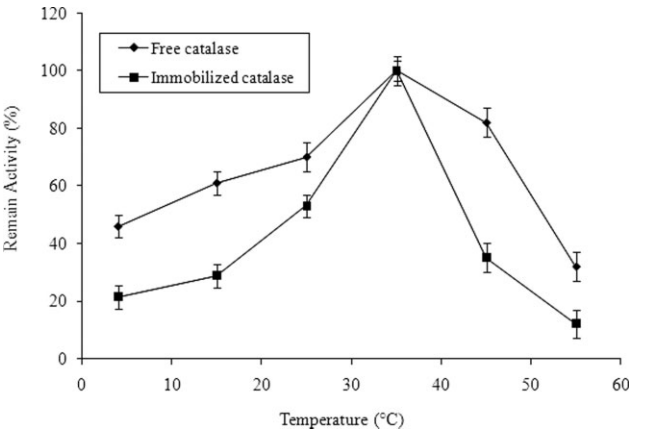

Figure 10 Temperature profiles of the free and adsorbed catalase; MAH content: 0.94 mmol/g; catalase concentration: 0.5 mg/mL; pH 5.0.

Effect of temperature on the catalytic activity

In general, the effects of changes in temperature on the rates of enzyme-catalyzed reactions do not provide much information on the mechanism of catalysis. However, these effects could be important in indicating structural changes in enzymes. In Figure 10, the relative activities of both the free and the immobilized catalase as a function of temperature are reported, together with that of the free enzyme for comparison. The optimum temperature for the free and immobilized preparation of mon-poly(HEMA-MAH)–Fe^{3+}-catalase is 35°C.

Effect of pH on the activity

The pH effect on the activity of the free and immobilized catalase preparations for hydrogen peroxide degradation was studied. The effect of pH on the free and the immobilized preparations was investigated in the pH range between 4.0 and 9.0 in acetate and phosphate buffers and the results are presented in Figure 11. The data show that mon-poly(HEMA-MAH)–Fe^{3+}-catalase preparation has the same optimum as the free enzyme (pH: 7.0). The immobilized preparations gave a significantly broader profile than that of the free enzyme. The pH profiles of the immobilized enzymes were much broader with respect to the free enzyme, probably because of the production of oxygen, forming bubbles, and causing external diffusional limitations on the enzyme-polymer beads surface.

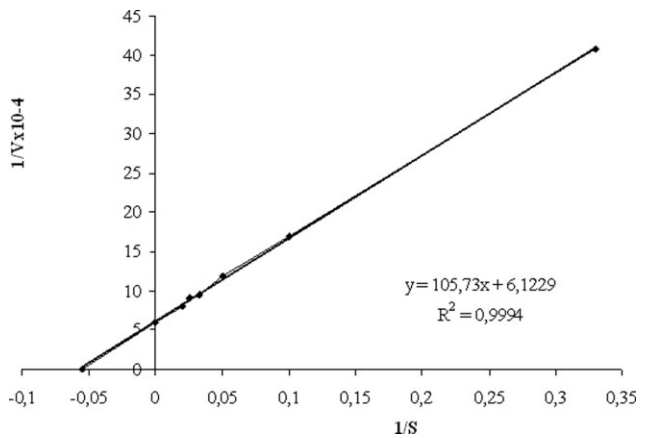

Figure 9 Lineweaver-Burk plot for immobilized catalase.

Storage stability

Free and immobilized catalase preparations were stored in a phosphate buffer (0.1*M*, pH 7.0) at 4°C, and the activity measurements were carried out for a period of 50 days (Fig. 12). No enzyme release was observed. The free enzyme lost all of its activity within 25 days. Immobilized preparation of mon-poly(HEMA-MAH)–Fe^{+3}-catalase lost 18% of its activity during the same period. This decrease in enzyme activity was explained as a time-dependent natural loss in enzyme activity and this was prevented to a significant degree upon immobilization.

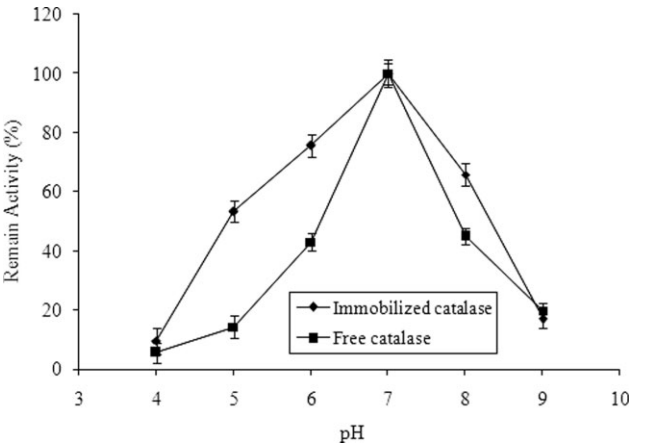

Figure 11 Effect of pH on the free and adsorbed catalase; MAH content: 0.94 mmol/g; catalase concentration: 0.5 mg/mL; *T*: 35°C.

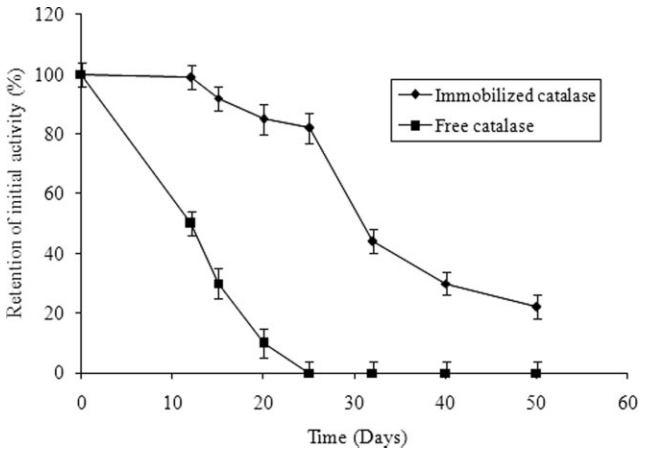

Figure 12 Storage stability of immobilized catalase; MAH content: 0.94 mmol/g; catalase concentration: 0.5 mg/mL; pH 5.0; *T*: 35°C.

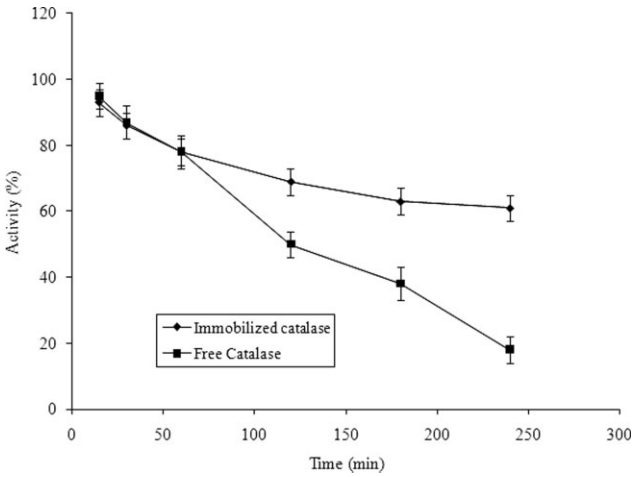

Figure 14 Activity profiles of the free and immobilized catalase at 50°C; MAH content: 0.94 mmol/g; catalase concentration: 0.5 mg/mL; pH 5.0.

Thermal stability

The effect of temperature on the stability of the free and adsorbed catalase is shown in Figures 13 and 14. The pattern of heat stability indicated that a smaller rate of thermal inactivation was observed for the adsorbed catalase on the mon-poly(HEMA-MAH) nanospheres than that of the free enzyme. At 40°C, the free enzyme retained 23.4% of its initial activity after a 240 min of heat treatment, whereas the adsorbed enzyme showed significant resistance to thermal inactivation (retaining about 66.3% of its initial activity after the same period). At 50°C, the free enzyme retained 18.9% of its initial activity after a 240 min of heat treatment, whereas the adsorbed enzyme showed significant resistance to thermal inactivation (retaining about 65.6% of its initial activity after the same period). These results suggest that the thermostability of immobilized catalase becomes significantly higher at higher temperature. If the heat stability of enzymes increased upon adsorption, the potential application of these enzymes would be extended. Increased thermal stability has been reported for a number of adsorbed enzymes, and the polymer network and multipoint attachment in covalent adsorption method are supposed to preserve the tertiary structure of enzyme.

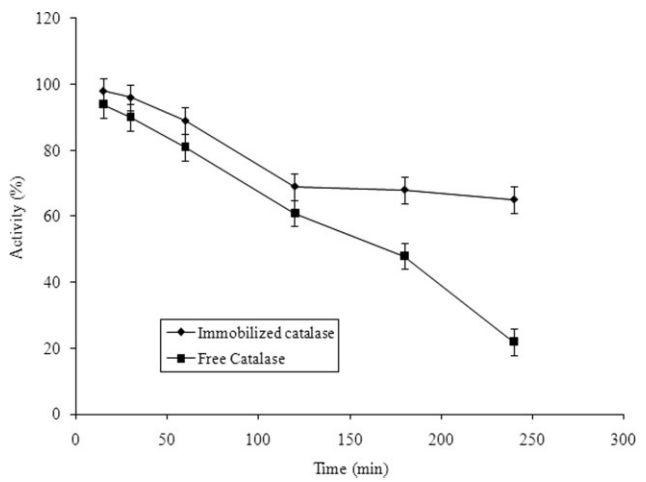

Figure 13 Activity profiles of the free and immobilized lipase at 40°C; MAH content: 0.94 mmol/g; catalase concentration: 0.5 mg/mL; pH 5.0.

Repeated use

Desorptions of catalase from Fe^{+3}-chelated mon-poly (HEMA-MAH) nanospheres were carried out in a batch system. Mon-poly(HEMA-MAH)–Fe^{+3}-catalase preparation was placed within the desorption medium containing 0.1*M* NaSCN (pH 8.0) at room temperature for 2 h. It was then repeatedly used in adsorption of catalase. The catalase adsorption capacity was not change during the five successive adsorption–desorption (Fig. 15). Enzyme activities of preparations did not significantly change during these adsorption–desorption cycles. These results

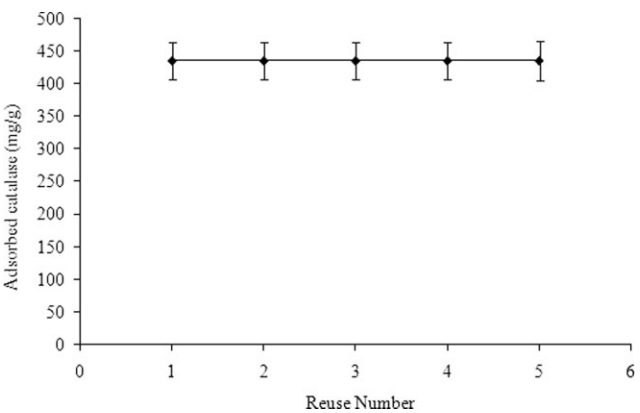

Figure 15 Repeated use of mon-poly(HEMA-MAH)–Fe^{3+} nanospheres; MAH content: 0.94 mmol/g; catalase concentration: 0.5 mg/mL; pH 5.0; *T*: 35°C.

showed that Fe^{+3}-incorporated, novel mon-poly (HEMA-MAH) nanospheres can be repeatedly used in enzyme immobilization as matrix, without detectable losses in their initial adsorption capacities.

CONCLUSIONS

Various nanostructured materials are more affordable for a broader range of applications with recent development in nanotechnology. In addition to this, various nanostructures have been examined as hosts for enzyme adsorption via approaches including enzyme adsorption, covalent attachment, enzyme encapsulation, and sophisticated combinations of methods.[32] Various nanostructures, generally providing a large surface area for the adsorption of enzyme molecules, have been actively developed for enzyme stabilization. Only limited work has been published on the application of nanosized particles in the adsorption of enzymes.[15]

The time-consuming and high cost of metal-chelating procedure has inspired a search for suitable low-cost adsorbents. The main advantage of IMAC consists in its simplicity, universality, stability, and cheapness of the chelating supports.[32,33] In addition, the IMAC supports ensure the milder elution conditions of proteins, retaining their biological activity. In this study, a novel *N*-methacryloyl-(L)-histidine methyl ester (MAH)-containing monosize nanosized affinity adsorbent for the immobilization of catalase was prepared. This new approach for the preparation of metal-chelating adsorbent has many advantages over conventional techniques. An expensive and critical step in the preparation process of metal-chelating adsorbent is coupling of a chelating ligand to the adsorption matrix. In this procedure, comonomer MAH acted as the metal-chelating ligand, and there is no need to activate the matrix for the chelating-ligand immobilization. In addition to this, nanosized and submicron particles, which synthesized in this study, can produce a larger specific surface area and, therefore, may result in high adsorption capacity for enzymes. Another major issue is that of slow release of these covalently bonded chelators from the matrix. Metal-chelating ligand release is a general problem encountered in any immobilized metal-chelate affinity adsorption technique, which causes a decrease in adsorption capacity. Also, it is well known that metal-chelating ligand leakage from the adsorbent causes contaminations that will interfere with analysis of the purified protein. Metal-chelating ligand immobilization step was also eliminated in this approach. Metal-chelating ligand and/or comonomer MAH was polymerized with HEMA and there is no leakage of the ligand. The monosize poly(HEMA-MAH) nanospheres revealed good adsorption properties as a new matrix and will be useful in the enzyme adsorption technology.

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