Preparation of Chitosan-Coated Magnetite Nanoparticles and Application for Immobilization of Laccase

Nuzhet Ayca Kalkan,[1] Serpil Aksoy,[1] Eda Ayse Aksoy,[2] Nesrin Hasirci[3,4,5,6]

[1]*Department of Chemistry, Faculty of Arts and Sciences, Gazi University, Teknikokullar, 06500 Ankara, Turkey*
[2]*Central Laboratory, Middle East Technical University, Ankara 06531, Turkey*
[3]*Department of Chemistry, Faculty of Arts and Sciences, Middle East Technical University, 06351 Ankara, Turkey*
[4]*Graduate Department of Polymer Science and Technology, Middle East Technical University, Ankara 06531, Turkey*
[5]*Graduate Department of Biotechnology, Middle East Technical University, 06351 Ankara, Turkey*
[6]*Graduate Department of Biomedical Engineering, Middle East Technical University, 06351 Ankara, Turkey*

Received 20 June 2010; accepted 8 March 2011
DOI 10.1002/app.34504
Published online 2 August 2011 in Wiley Online Library (wileyonlinelibrary.com).

ABSTRACT: In this study, immobilization of laccase (L) enzyme on magnetite (Fe_3O_4) nanoparticles was achieved, so that the immobilized enzyme could be used repeatedly. For this purpose, Fe_3O_4 nanoparticles were coated and functionalized with chitosan (CS) and laccase from *Trametes versicolor* was immobilized onto chitosan-coated magnetic nanoparticles (Fe_3O_4-CS) by adsorption or covalent binding after activating the hydroxyl groups of chitosan with carbodiimide (EDAC) or cyanuric chloride (CC). For chitosan-coated magnetic nanoparticles, the thickness of CS layer was estimated as 1.0–4.8 nm by TEM, isoelectric point was detected as 6.86 by zeta (ζ)-potential measurements, and the saturation magnetization was determined as 25.2 emu g^{-1} by VSM, indicating that these nanoparticles were almost superparamagnetic. For free laccase and immobilized laccase systems, the optimum pH, temperature, and kinetic parameters were investigated; and the change of the activity against repeated use of the immobilized systems were examined. The results indicated that all immobilized systems retained more than 71% of their initial activity at the end of 30 batch uses. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 123: 707–716, 2012

Key words: nanoparticle; chitosan; magnetic separation; enzyme immobilization; laccase

INTRODUCTION

In the last decades, applications of nanosized magnetic particles (maghemite, γ-Fe_3O_4 or magnetite, Fe_3O_4) in biology and medicine have been extensively studied especially in the areas of magnetic resonance imaging, hyperthermia generation, magnetically controlled transport of anticancer drugs, RNA and DNA purification, magnetic cell separation and purification, enzyme and protein immobilization.[1] Enzymes, immobilized on solid supports offer many advantages over the use of soluble enzymes in biotechnological practice, because the separation of immobilized enzymes from the reaction mixture by physical methods, such as filtration or sedimentation (centrifugation) is quite easy, and they can be used repeatedly. Magnetic nanoparticles as carriers for the enzymes provide rapid and easy recovery of the biocatalyst from the reaction medium in an external magnetic field and the enzymes separated in this way are subjected to very low mechanical stress compared with centrifugation or filtration.[2,3] In addition, using magnetic nanoparticles as the support of immobilized enzymes also has the following advantages: (1) higher specific surface area for the binding of higher amounts of enzymes, (2) lower mass transfer resistance, (3) less fouling, (4) less diffusion problem, and (5) lower operational cost.[4–6]

To immobilize a bioactive reagent on to nanoparticles, the presence of functional groups on their surface is very crucial. The surface functionalized magnetic particles with reactive groups are desired to increase the loading capacity and stability of the biomolecules immobilized on them.[7] Generally, natural and synthetic polymers are used for the functionalization of magnetic nanoparticles.[3,6,8–10] Chitosan, which is a natural polyaminosaccharide, is one of these polymers with significant biological (biodegradable and bioactive) and chemical properties (polycationic, reactive groups such as —OH and —NH_2). Chitosan based materials are used as supports, in various forms such as powder, flake, and gel, and of different geometrical configurations, for immobilization of several enzymes.[11–15] Also, in the last decades, chitosan containing magnetic supports, especially magnetic microspheres[16–18] were investigated for

Correspondence to: S. Aksoy (seraksoy@gazi.edu.tr).
Contract grant sponsor: Gazi University; contract grant number: Research Foundation Project No: 05/2009-29.

enzyme immobilization by using physical and chemical immobilization techniques.[19,20]

Laccases (benzenediol : oxygen oxidoreductase, EC 1.10.3.2) are extracellular, multi-copper enzymes and are found in plants, insects, bacteria, and more predominantly in fungi. They oxidize various aromatic and nonaromatic compounds by a radical catalyzed reaction mechanism using molecular oxygen. The wide reaction capabilities and broad substrate specificities of laccases make them promising enzymes in various application areas.[21,22] Textile dye bleaching, pulp bleaching, food improvement, bioremediation of soils and water, polymer synthesis, development of biosensors, or biofuel cells are some examples for their applications.[23] For an effective activity and repeated usage of laccases, their immobilization have been achieved on nonmagnetic micron-scaled chitosan-based support materials,[24–26] and some of these immobilized systems were used in industrial applications.[27,28] Although these immobilized systems exhibited good catalytic capability, their removal from the applied media was mostly achieved by filtration or centrifugation methods, which might cause high mechanical stress and loss of activity of the enzyme molecules. To prepare both catalytically active and easily separable immobilized laccase systems, recent developments are focusing on magnetic support materials functionalized with natural and synthetic polymers. Magnetically separable immobilized laccase systems demonstrating an appreciable activity at certain conditions were reported in literature. Some of these systems were prepared in the form of activated polystyrene particles,[3] chitosan microbeads,[29] or hydrophilic macroporous cellulose beads[30] in the presence of magnetic components.

The hypothesis of this study is that, laccase enzyme which is a commercialized industrial catalysts used in bioremediation, would have high catalytic activity and efficient reuse capacity upon immobilization on functionalized magnetic nanoparticles. The objectives of the study are, to functionalize Fe_3O_4 nanoparticles by coating with a functional polymer; to immobilize laccase on these magnetic nanosystems; to measure and compare the activity and reusability of the enzyme containing systems at different conditions. For this purpose, Fe_3O_4 nanoparticles were coated and functionalized with chitosan and the presence and effects of chitosan on Fe_3O_4 was characterized by TEM, FTIR, zeta (ζ)-potential, VSM, and TGA. Laccase enzyme (from *Trametes versicolor*) was immobilized onto chitosan-coated magnetic nanoparticles either by adsorption or by covalent binding by activating the hydroxyl groups of the nanoparticles with carbodiimide (EDAC) and cyanuric chloride (CC). To the best of our knowledge, immobilization of this type of laccase on the chitosan-functionalized magnetite nanoparticles by the mentioned techniques is achieved first time. The presence of laccase on the immobilized laccase systems was detected by SEM/EDS. Activity and stability of the prepared systems at different pH and temperature, kinetic parameters, and reusability capacities were examined against ABTS (2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid) substrate. The results were compared and optimized.

EXPERIMENTAL

Materials

Fe_3O_4 nanoparticles (<50 nm, TEM) were purchased from Aldrich (Steinheim, Germany). Chitosan from crab shells (≥75% deacetylated), acetic acid, mineral oil, and 2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were supplied from Sigma (Steinheim, Germany). Ethanol was purchased from Riedel de Haen (Seelze, Germany). Tween 80 was provided from Acros Organics (New Jersey, USA). Glutaraldehyde was obtained from British Drug House (Poole, England). Laccase (E.C.1.10.3.2 with an activity of 22.5 U mg^{-1}) from *Trametes versicolor* was purchased from Sigma-Aldrich (Steinheim, Germany). Bovine serum albumin (BSA), Bradford reagent (0.5 mg mL^{-1}), and NaCl solution (0.15*M*) were provided from Amresco (Ohio, USA). Carbodiimide, EDAC, (*N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride) was obtained from Merck (Hohenbrunn, Germany). Cyanuric chloride, CC was supplied from Merck (Schuchardt, Germany). All reagents used in the present study were of analytical grade.

Preparation of chitosan-coated magnetite nanoparticles

Coating of Fe_3O_4 nanoparticles with CS was achieved by reversed phase suspension technique by modifying the procedure described by Li et al.[31] For this purpose, 200 mg Fe_3O_4 nanoparticles were washed three times with ethanol and added to 50.0 mL mineral oil containing 0.5 mL Tween 80 in a round bottom flask. The mixture was sonicated and chitosan solution (1% w/v, 15.0 mL in 5.0% v/v acetic acid) was added. The Fe_3O_4-chitosan dispersion was sonicated for 30 min, and then stirred at 1500 rpm with a mechanical stirrer. Glutaraldehyde solution (3.0 mL, 25% w/v in water) was added to the medium and stirred for 4 h at room temperature. The resultant chitosan-coated magnetite nanoparticles (Fe_3O_4-CS) were separated from the reaction mixture by a permanent magnet, washed several times with acetone, and dried in vacuum oven at 40°C.

Characterization of chitosan-coated magnetite nanoparticles

The particle size and morphology of Fe_3O_4 and Fe_3O_4-CS nanoparticles were estimated by transmission electron microscope, (TEM, Technai G^2 F30, Hillsboro, USA). The nanoparticles were dispersed in ethanol and sonicated for 5 min. The dispersion was placed onto carbon-coated copper grids and TEM images of the nanoparticles were taken at 100 kV.

The functional groups of Fe_3O_4 and Fe_3O_4-CS nanoparticles were detected by FTIR analysis. The samples were prepared in KBr discs and FTIR spectra were recorded by a FTIR spectrometer (Perkin–Elmer PE 1600, Massachusetts, USA) between 4000 and 400 cm^{-1}.

The zeta (ζ)-potential analysis of Fe_3O_4 and Fe_3O_4-CS nanoparticles were performed at 25°C by a ζ-sizer (Malvern Nano ZS90, Worcestershire, England) in the range of pH 2.0–9.5. The pH value of the medium was adjusted by using 10^{-2} *M* HCl or NaOH.

The magnetization of Fe_3O_4 and Fe_3O_4-CS nanoparticles ($\sim$ 20 mg) versus the applied magnetic field ($\pm$4000 Oe) were investigated by a vibrating sample magnetometer, (VSM, ADE Magnetics Model EV9, Westwood, USA) at room temperature.

The thermal properties of Fe_3O_4 and Fe_3O_4-CS nanoparticles were obtained by a thermogravimetric analyzer (TGA, Perkin–Elmer Pyris 1 TGA, Massachusetts, USA). Samples ($\sim$ 5 mg) were heated from 30 to 700°C at a heating rate of 10°C min^{-1} in N_2 atmosphere.

Enzyme immobilization studies

Activation of Fe_3O_4-CS nanoparticles with EDAC and CC

For the immobilization of laccase by covalent binding, Fe_3O_4-CS nanoparticles were activated by EDAC and CC according to the procedures given below.

EDAC activation. Fifty milligrams of Fe_3O_4-CS nanoparticles was dispersed in 2.0 mL phosphate buffer, (PB, pH 6.0, 0.04*M*) in an ultrasonic bath at 4°C. EDAC solution (0.5 mL, 2.5% w/v, in same buffer) was added, and the system was sonicated for 30 min. The mixture was stirred by a magnetic stirrer at 1000 rpm, at 4°C for 6 h and stored at that temperature overnight. Finally, Fe_3O_4-CS nanoparticles activated by EDAC (Fe_3O_4-CS-EDAC) were collected by a magnet, washed with 2.0 mL PB for five times, and the activated particles were used for laccase immobilization.

CC activation. Fifty milligrams of Fe_3O_4-CS nanoparticles was dispersed in 2.5 mL CC solution (0.5% w/v in 1,4-dioxane) and sonicated for 30 min at room temperature. The mixture was stirred by a magnetic stirrer at 1000 rpm for 6 h and stored overnight. Fe_3O_4-CS nanoparticles activated by CC (Fe_3O_4-CS-CC) were collected by a magnet, washed with 2.0 mL acetone and 2.0 mL PB, each three times, successively. The supernatant was removed and the activated particles were used for laccase immobilization.

Immobilization of laccase

Laccase was immobilized onto Fe_3O_4-CS nanoparticles either by adsorption or by covalent binding on activated nanoparticles (Fe_3O_4-CS-EDAC or Fe_3O_4-CS-CC). For this purpose, 50 mg of each type of magnetic carrier was washed by 2.0 mL PB and 2.0 mL laccase solution (1.0 mg mL^{-1}, in PB, pH 6.0) was added. The system was sonicated for 20 min, stirred magnetically at 1000 rpm for 6 h at 4°C and stored at that temperature overnight. Laccase immobilized systems, Fe_3O_4—CS—L, Fe_3O_4—CS—EDAC—L and Fe_3O_4—CS—CC—L, were separated from the solutions by a permanent magnet and each washed with PB until the unbound enzyme was completely removed. The presence of laccase on magnetic nanoparticles was detected by scanning electron microscope equipped with energy dispersive X-ray detector (SEM/EDS, Quanta 400F Field Emission SEM, Eindhoven, Netherlands).

Immobilization efficiency

Immobilization efficiency of laccase onto chitosan-coated magnetic nanoparticles was estimated by the Bradford assay using bovine serum albumin (BSA) as standard protein.[32] The Bradford protein assay is a spectroscopic analytical method routinely used to measure the concentration of a protein in a solution. To determine the immobilized amount of the laccase, the concentrations of the enzyme in the initial solution and in the washing solutions after the immobilization process were detected by Bradford test measuring the absorbances of the coomassie dye-protein complex at 595 nm using a UV–visible spectrophotometer (UV–vis, Hitachi U-1800, Tokyo, Japan). The enzyme concentrations in the solutions were determined from the BSA calibration curve. The amount of the immobilized enzyme was calculated from the differences of the amounts of initial enzyme and the unbound enzyme detected in washing solutions.

Assay of laccase activity

Free and immobilized laccase activities were determined against ABTS (2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid) substrate at 25°C in a medium of in 0.1*M* citrate/0.2*M* phosphate buffer (CPB, pH

5.0). Formation of green ABTS radical cation ($ABTS^+$) up on oxidation of ABTS by laccase was detected by a UV–visible spectrophotometer (Shimadzu PharmaSpec 1700, Tokyo, Japan) at 414 nm after 1 min.

For the measurement of free laccase activity, 100 μL laccase solution (0.1 mg mL^{-1}, in CPB, pH 5.0) was added into 8.9 mL CPB at 25°C and the enzymatic reaction was started by adding 1.0 mL ABTS solution (0.25 m*M*) into the assay medium.

For the determination of immobilized laccase activity, 50 mg immobilized laccase containing nanoparticles were put into a centrifuge tube, washed three times with CPB, and 9.0 mL CPB was added. Then, 1.0 mL of ABTS solution (0.25 m*M*) was put and the reaction mixture was stirred by a vortex stirrer. After 1 min, laccase immobilized magnetic nanoparticles were separated from the solution by a permanent magnet and the absorbance of the solution was measured. All the experiments were carried out as triplets and all results are obtained as averages of triplicate experiments.

Effect of pH

The effect of pH on the activities of free and immobilized laccase was determined by measuring the laccase activity against 0.25 m*M* ABTS; by using PB (0.1 *M*) at pH 2.5, and by using CPB (0.1 *M*/0.2 *M*) in pH range of 3.0-6.5, at 250°C.

Effect of temperature

The effect of temperature on the free and immobilized laccase activity was determined by assaying the laccase activity at different temperatures in the range of 10–65°C against 0.25 m*M* ABTS at pH 5.0.

Kinetic studies

The effect of substrate concentration on the activities of the free and immobilized laccases was investigated against ABTS within a concentration range of 0.0050–0.0250 m*M* at pH 5.0 and at 25°C. Kinetic parameters: Michealis–Menten constant, K_m, and maximum rate, V_{max}, were determined from Lineweaver–Burk plots. According to Lineweaver–Burk equation, V^{-1} plotted versus $[S]^{-1}$, where V and $[S]$ are the rate and substrate concentration, respectively. The slope of the graph gives K_m/V_{max} and the intercept of the y axis gives $1/V_{max}$ values.

Reusability of immobilized laccases

Reusability of immobilized laccase systems was investigated in a batch type system by using the same immobilized enzyme for 30 times in a day. Before the each cycle, the immobilized laccase was washed three times with CPB and the enzyme activities were measured.

RESULTS AND DISCUSSION

Characterization of Fe_3O_4-CS nanoparticles

The particle size of Fe_3O_4 nanoparticles and thickness of CS on Fe_3O_4-CS nanoparticles were estimated from TEM analysis (Fig. 1). The diameter of uncoated Fe_3O_4 nanoparticles were less than 50 nm [Fig. 1(a) scale bar is 20 nm] and the thickness of CS on Fe_3O_4-CS nanoparticles was $\sim$ 1.0–4.8 nm measured by Image J program. TEM images of the nanoparticles showed that the CS layer is completely covered Fe_3O_4 nanoparticles [Fig. 1(b), scale bar is 20 nm].

Figure 2 shows the FTIR spectra of uncoated Fe_3O_4, CS, and Fe_3O_4-CS nanoparticles. As seen in the FTIR spectrum of CS, the peak between 3650 and 3000 cm^{-1} corresponds to stretching vibrations of O—H and N—H, respectively. Also, the peaks at 2870, 1659, 1074 cm^{-1} relate to —C—H, C=O (in undeacetylated units) and C—O—C vibrations, respectively. The spectrum of Fe_3O_4-CS nanoparticles has peaks $\sim$ 566 and 1648 cm^{-1}, indicating the presence of magnetic component (Fe—O) and the —C=N— vibration resulting from the reaction between amine groups of chitosan and aldehyde group of glutaraldehyde during crosslinking.

The zeta (ζ)-potentials of Fe_3O_4 and Fe_3O_4-CS nanoparticles in aqueous solutions in the range of pH 2.51–9.30 are shown in Figure 3. As shown from this figure, the isoelectric point (pI) of the uncoated Fe_3O_4 nanoparticles was 7.91. For Fe_3O_4-CS nanoparticles, the pI value was shifted 1.05 units towards the lower pH values and measured as 6.86. This also confirms the binding of chitosan and reveals that the chitosan-coated nanoparticles were positively charged below pH 6.86. In literature, a shift of pI value, from 6.70 to 5.95 was reported for Fe_3O_4 nanoparticles after coating with carboxymethylated chitosan.[33]

The magnetic properties, saturation magnetization and superparamagnetism of the Fe_3O_4 and Fe_3O_4-CS nanoparticles were investigated by VSM analysis at room temperature. The mass magnetization curves of Fe_3O_4 and Fe_3O_4-CS nanoparticles versus the applied magnetic field ($\pm$4000 Oe) are shown in Figure 4. The saturation magnetization (σ_s), which is the value of the magnetization to orient the magnetic domains in magnetic nanoparticles to the applied magnetic field, was found to be 74.1 emu g^{-1} for Fe_3O_4 nanoparticles and 25.2 emu g^{-1} for Fe_3O_4-CS. The decrease in the saturation magnetization of nanoparticles after coating can be explained by the

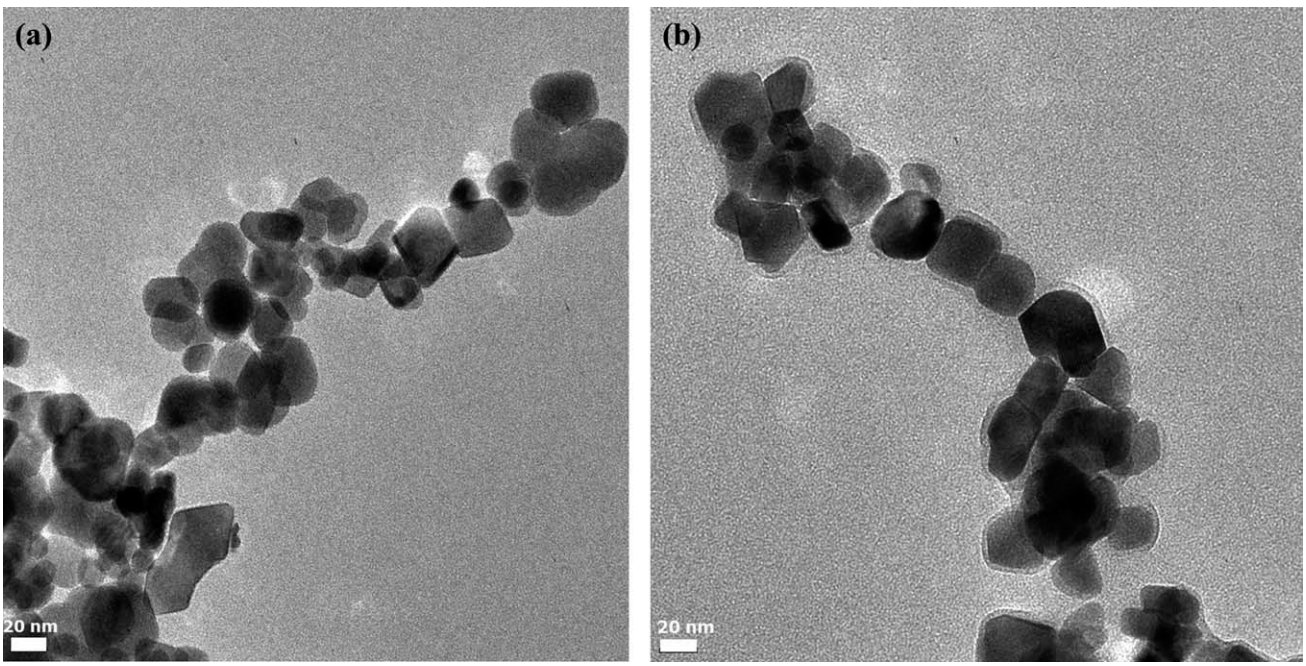

Figure 1 TEM images of nanoparticles. (a) Fe_3O_4, (b) Fe_3O_4-CS (scales are 20 nm).

decrease in the amount of the magnetic moments per unit weight due to the diamagnetic contribution of CS shell. Similar decreases in σ_s value were reported by other researchers when magnetic nanoparticles were coated with different polymers or modifying agents.[31,34,35] For Fe_3O_4 nanoparticles, the magnetization curve exhibits a little remenance and coercivity. For Fe_3O_4-CS nanoparticles, these values are significantly lower than Fe_3O_4 nanoparticles. This can be explained as suspension of the domains of the Fe_3O_4 nanoparticles after chitosan coating which causes a decrease in the magnetic forces between them in the absence of an external magnetic field. This trend reveals that the prepared Fe_3O_4-CS nanoparticles are almost super paramagnetic.

The chitosan content of Fe_3O_4-CS nanoparticles was estimated from TGA. For Fe_3O_4 nanoparticles, the weight loss between 30 and 700°C was detected $\sim$ 1%. This might be due to the loss of residual

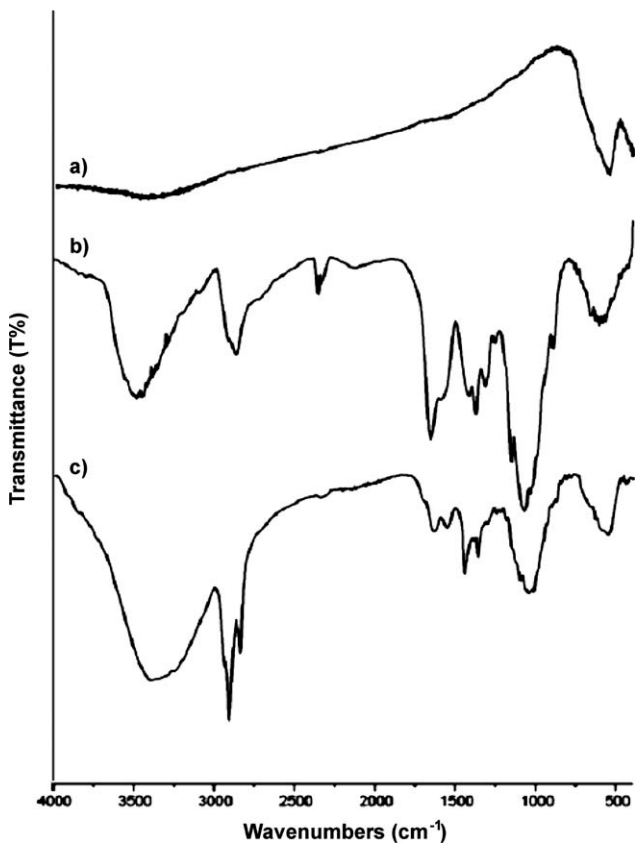

Figure 2 FTIR spectra of samples. (a) Fe_3O_4, (b) CS, (c) Fe_3O_4-CS.

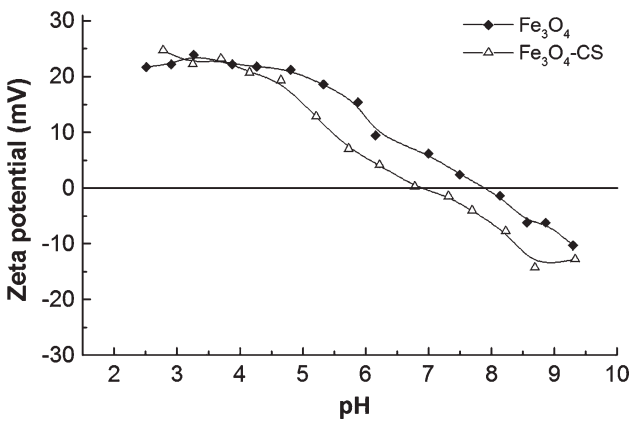

Figure 3 Zeta (ζ)-potentials of Fe_3O_4 and Fe_3O_4-CS nanoparticles at different pHs.

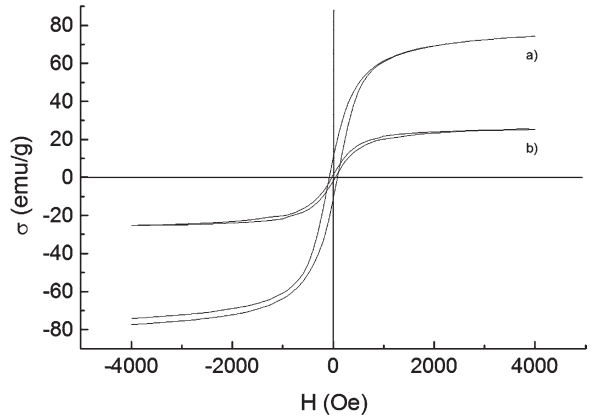

Figure 4 Magnetization versus magnetic field for nanoparticles. (a) Fe_3O_4, (b) Fe_3O_4-CS.

water from the sample. For pure chitosan, a sharp decrease in weight was observed in the temperature range of 300–450°C (data not shown). It was observed that more than 75% of chitosan removed when heated up to 700°C. This demonstrates all chitosan was degraded at ~ 400°C. On the other hand, for Fe_3O_4-CS nanoparticles, the weight loss was ~ 5.7% below 200°C, because of the removal of absorbed water by physical and chemical attractions, similarly to Fe_3O_4.[31] But, a significant degradation was detected between 200 and 550°C which corresponds to the breakdown of the main chitosan chains covering the magnetic nanoparticles. There was no significant change after 550°C, implying the presence of iron oxide above this temperature. From these data, the chitosan content of Fe_3O_4-CS nanoparticles was estimated as 51.7%.

Immobilization of laccase

The effective use of an enzyme in enzymatic processes can be enhanced by immobilizing the enzyme on a solid support.[36] In this study, laccase enzyme which has many industrial applications, such as decolorization of textile industry effluents and delignification of pulp, was immobilized onto magnetically separable Fe_3O_4-CS nanoparticles by adsorption and two different covalent binding methods. The operational stabilities and efficiencies of the prepared immobilized laccase systems against free laccase were investigated.

The immobilization of laccase onto Fe_3O_4-CS nanoparticles by adsorption was achieved at pH 6.0. As stated previously, the isoelectric point, pI, of Fe_3O_4-CS nanoparticles was founded as 6.86 by ζ-potential measurements. In literature, pI value of laccase from *Trametes versicolor* was reported as 3.0,[36] revealing that the net charge of the protein is negative above pH 3.0. Therefore, the enzyme can be

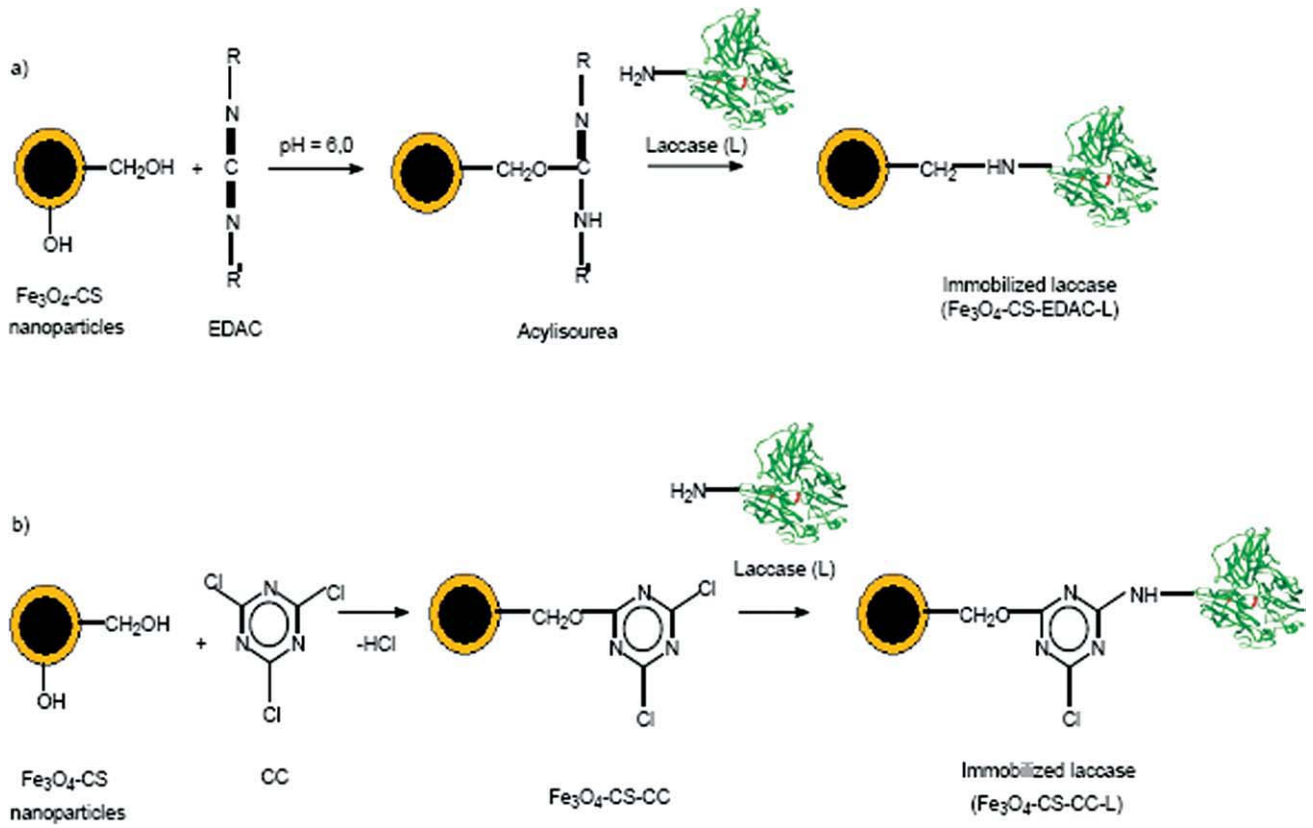

Figure 5 Scheme of laccase immobilization onto Fe_3O_4-CS nanoparticles (a) by EDAC and (b) by CC. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

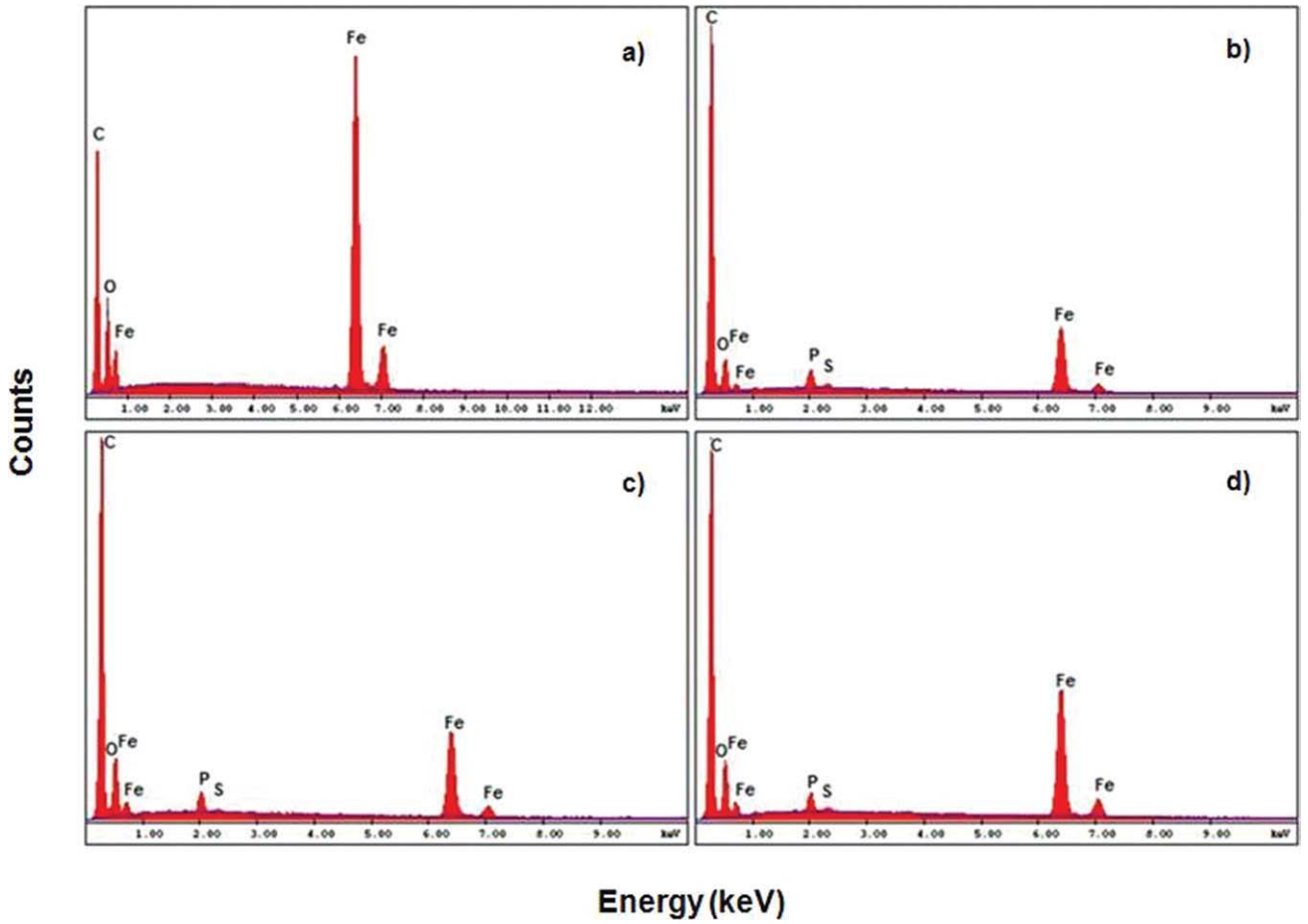

Figure 6 SEM/EDS spectra of samples (a) Fe_3O_4-CS, (b) Fe_3O_4-CS-L, (c) Fe_3O_4-CS-EDAC-L, and (d) Fe_3O_4-CS-CC-L. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

adsorbed onto Fe_3O_4-CS nanoparticles at these conditions.

The covalent immobilization of laccase onto Fe_3O_4-CS nanoparticles was carried out by EDAC and CC activation. The scheme for the activation and laccase immobilization onto Fe_3O_4-CS nanoparticles is presented in Figure 5. The activation of chitosan with EDAC and immobilization of the enzyme was performed at pH 6.0,[37] because the highest immobilized enzyme activity was detected at this pH, compared with the experiments achieved at pH 4.0 and pH 5.0.

CC is a well-known —OH group activating agent, which was widely used in our previous studies for the activation of the polymeric supports for enzyme immobilization.[38,39] The concentration of CC was an important parameter during the activation step, because decomposition of CS layer of Fe_3O_4-CS nanoparticles were observed if the CC concentrations were higher than 0.5% (w/v).

The presence of enzyme on the prepared immobilized laccase systems, Fe_3O_4-CS-L, Fe_3O_4-CS-EDAC-L, and Fe_3O_4-CS-CC-L was detected by SEM/EDS analysis. The SEM/EDS spectra of these three immobilized systems and Fe_3O_4-CS nanoparticles are presented in Figure 6. From these spectra, it was observed that the signal intensity of the C atom for Fe_3O_4-CS nanoparticles (atomic percentage of C is 76.23%) increased to more than 84.90% for all immobilized systems. Also, the signal indicating S atoms were observed for the immobilized systems, arising from the presence of the enzyme on these nanoparticles.

Immobilization efficiency

Immobilization efficiencies of laccases were found to be 93.76% for Fe_3O_4-CS-L, 94.22% for Fe_3O_4-CS-EDAC-L, and 94.36% for Fe_3O_4-CS-CC-L where all values are higher than 93%. These results indicated that significant amount of enzyme was immobilized onto Fe_3O_4-CS nanoparticles by adsorption and by covalent binding.

Effect of pH

The effect of pH on the activities of free and immobilized laccases were investigated within pH range

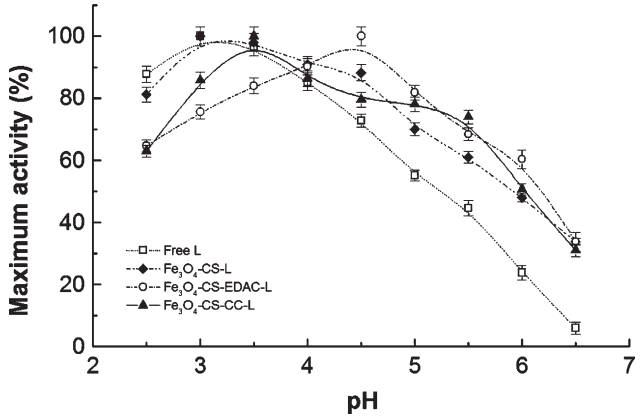

Figure 7 Effect of pH on the activities of free and immobilized *Trametes versicolor* laccases. Reaction conditions: 25°C, 0.25 m*M* ABTS.

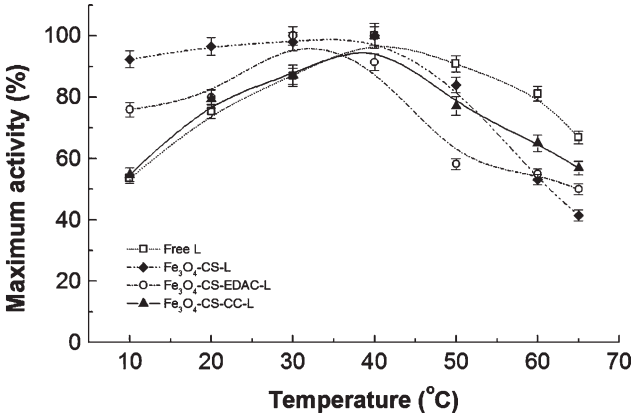

Figure 8 Effect of temperature on the activities of free and immobilized *Trametes versicolor* laccases. Reaction conditions: pH 5.0, 0.25 m*M* ABTS.

of 2.5–6.5 at 25°C and the obtained relative activities are presented in Figure 7. The optimum pH for free laccase (L) and the immobilized laccases, Fe_3O_4-CS-L, Fe_3O_4-CS-EDAC-L, and Fe_3O_4-CS-CC-L were found to be 3.0, 3.0, 4.5, and 3.5, respectively. Immobilization caused 1.5 or 0.5 unit shift to higher pH values for the systems prepared by covalent immobilization. This is most probably because of the variations in the conformation of the enzyme upon covalent bonds formation and change in microenvironment upon immobilization. In literature, a shift in the optimum pH toward higher values was reported for the lipase immobilized onto chitosan microspheres activated by carbodiimide[37] and invertase immobilized onto poly(2-hydroxyethyl methacrylate) activated by CC.[38] In this study, all immobilized laccases retained more than 70% of their original activities between pH 3.0 and 5.0 while for free laccase activity was decreased to 55% at pH 5.0. A high maximum activity of the immobilized enzyme systems is expected, because immobilization makes the enzyme more stable against pH changes. Results show that immobilized laccases can be used more efficiently and repeatedly in the defined pH region compared with the free laccase.

Effect of temperature

The effect of temperature on the activities of free and immobilized laccases were examined in the range of 25–65°C and the maximum activities of all systems against temperature are represented in Figure 8. The optimum temperature for free L, Fe_3O_4-CS-L, and Fe_3O_4-CS-CC-L was determined as 40°C, whereas for Fe_3O_4-CS-EDAC-L it was found as 30°C. Changes in the optimum temperature value of the immobilized enzymes compare with the free ones depend on the immobilization method and the interactions between the enzyme and the support. In literature, shifts in the optimum temperature of the immobilized laccases prepared by physical and chemical immobilization techniques towards higher[28,40] or towards lower values[41] were reported. It was observed that in the range of 10–40°C, all of the immobilized systems demonstrated higher activity than that of the free laccase.

Kinetic studies

The effect of substrate concentration on the kinetics of the reaction catalyzed by free and immobilized laccases was studied by using ABTS as substrate at 25°C and at pH 5.0. K_m and V_{max} values estimated from Lineweaver–Burk plots are presented in Table I. An increase in K_m values for immobilized laccases was observed, in agreement with other investigators reported significant affinity decrease for immobilized enzymes.[29,42] These variations can be attributed to conformational changes, steric hindrances, and partitioning effects of protein.[42] V_{max} values also demonstrated an increase upon immobilization. Catalytic efficiency (V_{max}/K_m) of free laccase was calculated as 1.35 min^{-1} while for the immobilized systems, Fe_3O_4-CS-L, Fe_3O_4-CS-EDAC-L, and Fe_3O_4-CS-CC-L catalytic efficiencies were found to be 1.31, 1.30, and 1.24 min^{-1}, respectively. The immobilized laccase system prepared by adsorption, Fe_3O_4-CS-L,

TABLE I
Kinetic Parameters for Free and Immobilized Laccases

System	K_m (10^{-2} m*M*)	V_{max} (10^{-2} m*M* min^{-1})	V_{max}/K_m (min^{-1})
Free L	5.69	7.70	1.35
Fe_3O_4—CS—L	10.69	14.00	1.31
Fe_3O_4—CS—EDAC—L	15.97	12.29	1.30
Fe_3O_4—CS—CC—L	11.41	14.11	1.24

Reaction conditions: 25°C, pH 5.0; 0.00–0.25 m*M* ABTS.

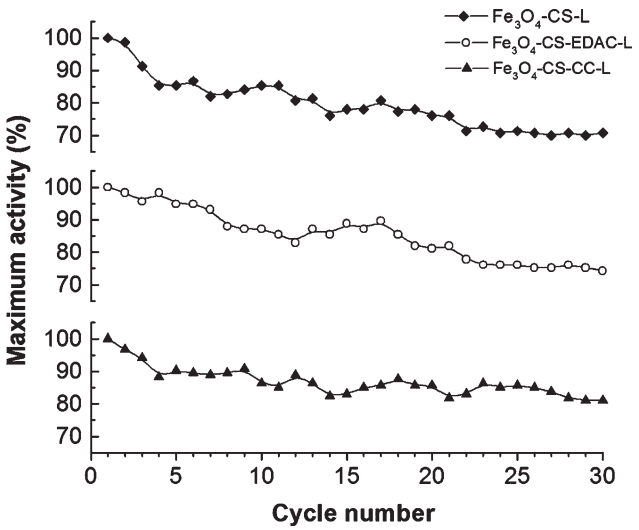

Figure 9 Effect of reuse number on activities of immobilized *Trametes versicolor* laccases. Reaction conditions: pH 5.0, 25°C, 0.25 m*M* ABTS.

exhibited the highest catalytic efficiency, whereas the other systems containing covalently bound enzymes presented slightly lower values. The conformational changes of the 3D structure of the enzyme molecules and the alterations of the microenvironments upon immobilization process can be effective on the decrease of the catalytic efficiencies for this immobilized laccase system.

Reusability of immobilized laccases

Reusability of immobilized enzymes is one of the most important aspects for industrial applications, because immobilized enzymes decrease the cost of production due to their repeated continuous and batch uses.[43] In this study, the reusability was examined by using the immobilized laccases repeatedly 30 times in 1 day in batch type system and maximum activities are presented in Figure 9. After 10th use, the retained activities for Fe_3O_4-CS-L, Fe_3O_4-CS-EDAC-L, and Fe_3O_4-CS-CC-L systems were found more than 85% for all and at the end of 30th use, these values were found as 71%, 74%, and 81%, respectively. The higher values were obtained for covalently immobilized systems (Fe_3O_4-CS-EDAC-L and Fe_3O_4-CS-CC-L), due to the strong chemical bonds between the enzyme and the support. In literature, there are retained activity values reported as 80% after 10 batch uses for the laccase immobilized by adsorption and crosslinking onto magnetic microspheres[29] and 70% after 10 consecutive operations for laccase immobilized on magnetic mesoporous silica spheres by covalent binding method.[44] Therefore, the obtained activities higher than 71% for all systems after 30 uses are very effective values for the laccase immobilized systems.

CONCLUSIONS

Chitosan-coated magnetite nanoparticles with a high magnetic property were prepared by reversed phase suspension technique, and the structural and magnetic properties of these nanoparticles were characterized. TEM results revealed that the thickness of chitosan layer covered the surface of magnetite nanoparticles were less than 5 nm. Isoelectric point of these nanoparticles determined by zeta (ζ)-potential measurements were found to be 6.86 and the saturation magnetization of chitosan-coated magnetite nanoparticles was determined as 25.2 emu g^{-1} by VSM analysis indicating that these nanoparticles were almost superparamagnetic. Laccase from *Trametes versicolor* was immobilized on these magnetically separable chitosan-coated magnetite nanoparticles through adsorption and covalent binding methods. The dependence of immobilized laccase activities against pH and temperature demonstrated that enzyme became more stable upon immobilization compared with the free form. Kinetic studies showed that; catalytic efficiencies of the immobilized enzymes were also quite close to the free enzyme. All the immobilized systems prepared either by adsorption or covalent binding exhibited more than 71% activity at the end of 30th repeated use. Therefore, it can be concluded that all laccase immobilized magnetic nanosystems prepared in this study can be good candidates to be used in industrial applications in terms of catalytic activity and reuse capacity.

The authors thank to UNAM, Institute of Materials Science and Nanotechnology, for TEM images; Middle East Technical University, Department of Materials Science and Engineering for VSM analysis; and Middle East Technical University, Central Laboratory and Department of Chemistry for other characterization tests.

References

1. Selim, K. M. K.; Ha, Y.-S.; Kim, S.-J.; Chang, Y.; Kim, T.-J.; Lee, G. H.; Kang, I.-K. Biomaterials 2007, 28, 710.
2. Turkova, J. Bioaffinity Chromatography, Chapter 5: Solid Matrix Supports; Amsterdam, the Netherlands: Elsevier, 1993; pp 171–211.
3. Pich, A.; Bhattacharya, S.; Adler, H.-J. P.; Wage, T.; Taubenberger, A.; Li, Z.; van Pee, K.-H.; Böhmer, U.; Bley, T. Macromol Biosci 2006, 6, 301.
4. Halling, P. J.; Dunnill, P. Enzyme Microb Tech 1980, 2, 2.
5. Liao, M.-H.; Chen, D.-H. Biotechnol Lett 2001, 23, 1723.
6. Konwarh, R.; Karak, N.; Rai, S. K.; Mukherjee, A. K. Nanotechnology 2009, 20, 1.
7. Yong, Y.; Bai, Y.; Yanfeng, L.; Lin, L.; Cui, Y.; Xia, C. J Magn Magn Mater 2008, 320, 2350.
8. Dresco, P. A.; Zaitsev, V. S.; Gambino, R. J.; Chu, B. Langmuir 1945 1999, 15.
9. Huang, S.-H.; Liao, M.-H.; Chena, D.-H. Sep Purif Technol 2006, 51, 113.
10. Gaihre, B.; Khil, M. S.; Lee, R. D.; Kim, H. Y. Int J Pharm 2009, 365, 180.

11. Edwards, W.; Leukes, W. D.; Rose, P. D.; Burton, S. G. Enzyme Microb Tech 1999, 25, 769.
12. Ghanem, A.; Skonberg, D. J Appl Polym Sci 2002, 84, 405.
13. Bindhu, L. V.; Abraham, E. T. J Appl Polym Sci 2003, 88, 1456.
14. Chang, M.-Y.; Juang, R.-S. Biochem Eng J 2007, 35, 93.
15. Krajewska, B. Enzyme Microb Tech 2004, 35, 126.
16. Denkbaş, E. B.; Kiliçay, E.; Birlikseven, C.; Öztürk, E. React Funct Polym 2002, 50, 225.
17. Zhang, J.; Zhang, S.; Wang, Y.; Gao, S. J Appl Polym Sci 2007, 103, 3045.
18. Li, P.; Zhu, A. M.; Liu, Q. L.; Zhang, Q. G. Indust Eng Chem Res 2008, 47, 7700.
19. Peniche, H.; Osorio, A.; Acosta, N.; de la Campa, A.; Peniche, C. J Appl Polym Sci 2005, 98, 651.
20. An, X.; Su, Z.; Zeng, H. J Chem Technol Biotechnol 2003, 78, 596.
21. Thurston, C. F. Microbiology 1994, 140, 19.
22. Alcalde, M. In Industrial Enzymes; Polania, J., MacCabe, A. P. Eds.; Dordrecht, the Netherlands: Springer, 2007, Chapter 26.
23. Kunamneni, A.; Plou, F. J.; Ballesteros, A.; Alcalde, M. Recent Patents Biotechnol 2008, 2, 10.
24. Delanoy, G.; Li, Q.; Yu, J. Int J Biol Macromol 2005, 35, 89.
25. Yang, W. Y.; Min, D. Y.; Wen, S. X.; Jin, L.; Rong, L.; Tetsuo, M.; Bo, C. Process Biochem 2006, 41, 1378.
26. Lu, L.; Zhao, M.; Wang, Y. World J Microbiol Biotechnol 2007, 23, 159.
27. Araújo, J. H. B.; Uemura1, V. O.; Moraes, F. F.; Barbosa, A. M.; Zanin, G. M. Braz Arch Biol Techn 2005, 48, 1.
28. D'Annibale, A.; Stazi, S. R.; Vinciguerra, V.; Mattia, E. D.; Sermanni, G. G. Process Biochem 1999, 34, 697.
29. Jiang, D.-S.; Long, S.-Y.; Huang, J.; Xiao, H.-Y.; Zhou, J.-Y. Biochem Eng J 2005, 25, 15.
30. Rotková, J.; Šuláková, R.; Korecká, L.; Zdražilová, P.; Jandová, M.; Lenfeld, J.; Horák, D.; Bílková, Z. J Magn Magn Mater 2009, 321, 1325.
31. Li, G.; Jiang, Y.; Huang, K.; Ding, P.; Chen, J. J Alloy Compound 2008, 466, 451.
32. Bradford, M. M. Anal Biochem 1976, 72, 248.
33. Chang, Y.-C.; Chen, D.-H. J Colloid Interf Sci 2005, 283, 446.
34. Wu, Y.; Jia, G.; Yang, W.; Wang, C.; Fu, S. Polymer 2006, 47, 5287.
35. Yamamura, M.; Camilo, R. L.; Sampai, L. C.; Macedo, M. A.; Nakamura, M.; Toma, H. E. J Magn Magn Mater 2004, 279, 210.
36. Claus, H.; Faber, G.; König H. Appl Microbiol Biotechnol 2002, 59, 672.
37. Chiou, S.-H.; Wu, W.-T. Biomaterials 2004, 25, 197.
38. Altinok, H.; Aksoy, S.; Tümtürk, H.; Hasirci, N. Russ Chem Bull 2006, 55, 1860.
39. Tümtürk, H.; Aksoy, S.; Hasırcı, N. Starch/Stärke 1999, 51, 211.
40. Kunamneni, A.; Ghazi, I.; Camarero, S.; Ballesteros, A.; Plou, F. J.; Alcalde, M. Process Biochem 2008, 43, 169.
41. Lante, A.; Crapisi, A.; Krastanov, A.; Spettoli, P. Process Biochem 2000, 36, 51.
42. Georgieva, S.; Godjevargova, T.; Portaccio, M.; Lepore, M.; Mita, D. G. J Mol Catal B: Enzyme 2008, 55, 177.
43. Yamak, O.; Kalkan, N. A.; Aksoy, S.; Altinok, H.; Hasirci, N. Process Biochem 2009, 44, 440.
44. Zhu, Y.; Kaskel, S.; Shi, J.; Wage, T.; van Pée, K.-H. Chem Mater 2007, 19, 6408.