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Combined magnetic and chemical covalent immobilization of pectinase on composites membranes improves stability and activity

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Abstract

The idea of preparing the surface of Fe_3O_4 layer preadsorbed on the "spherical polyelectrolyte brush" via layer-by-layer self-assembly approach receives special relevance in enzyme technology. "Spherical polyelectrolyte brush" of core–shell structure were prepared by grafting poly(sodium 4-styrenesulphonate) (PSStNa) from SiO_2 nanoparticles via the surface-initiated atom transfer radical polymerization strategy. The silica-coated $(PEI/Fe_3O_4)_n$ support presents a very simple, mild, and time-saving process for enzyme immobilization. The kinetics of a model reaction catalyzed by the immobilized pectinase was finally investigated by the Michaelis–Menten equation. These particles, premodified with the layer of magnetic nanoparticles to impart a magnetic property and subsequently coated with polyelectrolyte multilayer, were repeatedly used as catalysts following their rapid and easy separation with a magnet. Besides simplicity and versatility, the ease of enzyme regeneration constitutes an additional benefit of this approach.

Keywords: Atom transfer radical polymerization; Polyelectrolyte; Layer-by-layer; Immobilized enzyme

1. Introduction

Pectinases are used abundance in biotechnology applications, for example, in food processing (Kashyap, Vohra, Chopra, & Tewari, 2001). The stability of these enzymes depend on the aqueous medium, and is easily disrupted to the point where the enzymes cannot function appropriately. Immobilization techniques provide a promising approach to retain their stability (Bahar & Elebi, 1998; Khalid et al., 2006; Wu, Lee, & Lee, 1998). Recently, nanostructured chemistry and technology using highly selective enzymatic reactions have been focused on biochemical production and the sensing of target molecules (Yanga, Yanga, Liua, Shena, & Yua, 2004).

Magnetic nanoparticles are additional important materials due to their interesting magnetic properties and have been sophisticatedly employed in many advanced technology areas, including biology, pharmacy, and diagnostics (Dyal et al., 2003; Horák et al., 2001). For example, the magnetic labeled biological materials were rapidly, conveniently, and efficiently separated in an external magnetic field. Recently, Dyal et al. (2003) reported a successful method for immobilizing proteins on naked γ-Fe_2O_3 magnetic nanoparticles for biological use. Also, Kouassi and Irudayaraj (2006) developed gold-coated magnetic nanoparticles for immobilizing oligonucleotide as a DNA sensor. However, the activity of the biomolecule immobilized on theses magnetic particles will depend on the particular nature of the metal/oxide/hydroxide surfaces (acidity, surface coverage, and functional group uniformity). The enzyme desorbs from the particles when they are exposed

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to solution, suggesting that at least some of the reaction was carried out in solution and not on the surface support. This is not the case here, where the enzymes are chemically bonded to the nanoparticles.

To build a stable assembly, the first polyelectrolyte layer was attached to the nanoparticles surface via the surface-initiated atom transfer radical polymerization strategy. Subsequent deposition occurs by multiple electrostatic interactions between the adsorbing poly(styrenesulphonate) (PSStNa) and the oppositely charged layer. Surface-initiated polymerization promises to be an important tool to further develop the chemical and physical properties of nanostructures, by providing a method to covalently attach polymer chains in a well-controlled fashion. Controlled growth of polymer chains may also allow the addition of a polyelectrolyte layer of selectable thickness. Surface-initiated atom transfer radical polymerization (SI-ATRP) has been rapidly developing for its excellent controllability over the molecular weight and polydispersity of graft polymers and their capability of affording an exceptionally high graft density with the robustness and versatility of ATRP retained (Coessens, Pintauer, & Matayjaszewski, 2001; Matayjaszewski & Xia, 2001; Pyun, Kowalewski, & Matayjaszewski, 2003; Shah et al., 2000; Tugulu, Arnold, Sielaff, Johnsson, & Klok, 2005; Xu, Kang, & Neoh, 2005; Xu et al., 2006).

The layer-by-layer (LbL) approach (Georgina, Quinn, Quinn, Tjipto, & Caruso, 2006; Khopade Ajay & Caruso, 2003) provides an easy, low-cost, and versatile method for the fabrication of the silica-coated $(PEI/Fe_3O_4)_n$ support. Briefly; this technique is based on alternating adsorption of oppositely charged polyelectrolytes onto a charged substrate. The major advantage of the layer-by-layer technique is its multifunctionality, allowing the incorporation of a broad variety of functional groups.

It was further found that precoating the SiO_2 particles with polyelectrolyte/Fe_3O_4 nanoparticles enhanced both the stability (with respect to adsorption) and enzymatic activity of the immobilized pectinase. Multilayer assemblies of polyelectrolyte/Fe_3O_4, for protein immobilization, have been created within the nanoparticles domain. This approach was taken for three reasons: (1) the high surface area can potentially increase the amount of immobilized protein, (2) it is allowed the addition of a polyelectrolyte layer of selectable thickness, and (3) it is shown here that these enzymes, when immobilized on magnetic Fe_3O_4 nanoparticles, can be easily separated from the reaction medium, stored, and reused with consistent results. The fabrication of such colloids opens new avenues for the application of bioparticles and represents a promising route for the creation of complex catalytic particles.

2. Materials and methods

2.1. Materials

Pectin and the enzyme polygalacturonase (pectinase) were obtained from Fluka Chemical Co. (St. Louis, MO, USA). Polyethyleneimine (PEI) and sodium 4-styrenesulphonate (SStNa) were supplied by Fluka (99%, St. Louis, MO, USA). 2-Bromopropionyl bromide (BPC, Technical Grade) from Technology of Hongchen Xinxiang He'nan, China. Triethylamine (TEA) (Shanghai Pharmaceutical Co., Ltd., Shanghai, China) was refluxed with *p*-toluenesulphonyl chloride and distilled. CuBr was purified according to a published procedure (Wang & Matyjaszewski, 1995). Ultrapure water (resistivity = 18.2MX, pH 6.82) was used in all experiments. Other reagents and organic solvents for the initiator synthesis and polymerization were purchased from Shanghai Pharmaceutical Co., Ltd. (Shanghai, China) and used without any further purification. The monodisperse SiO_2 particles in the range of 150 nm were prepared by the method explained by Stober, Fink, and Bohn (1968).

2.2. Synthesis of "spherical polyelectrolyte brush"

2.2.1. Immobilization of the initiator on the silica particles surface

Firstly, the silica particles were dried over 24 h at 150 °C and aminated silica particles were synthesized by reaction with 3-aminopropyltriethoxysilane in tetrahydrofuran (THF) under nitrogen for 12 h at 95 °C. After the reaction, the particles were filtered and washed with methanol. Then, the silica particles were washed carefully with THF and finally dried in vacuum oven for the latter step. Secondly, the above modified silica particles were dispersed in a solution of triethylamine (2 ml) and freshly distilled THF (10 ml) under magnetic stirring. After the above mixture was cooled down to 0 °C, a solution of 2-bromopropionyl bromide (2 ml) and THF (5 ml) was added drop wise. Four hours later, the silica particles were washed again with THF and acetone thoroughly, and then dried under vacuum for the subsequent polymerization.

2.2.2. ATRP of sodium 4-styrenesulphonate

The initiator-grafted silica particles were then used for the ATRP of sodium 4-styrenesulphonate (SStNa) ionic monomers under various reaction conditions. In a typical protocol, the surface-modified silica particles (0.30 g) were dispersed in 6.0 ml of a 3:1 (v/v) water/methanol mixture in an ultrasonic bath. Sodium 4-styrenesulphonate (539.0 mg) and 2,2′-bipyridyl (Bpy, 41.1 mg) were then added to this colloidal dispersion. The mixture was degassed using a nitrogen purge for 30 min with continuous stirring at room temperature. Copper (I) bromide (CuBr, 19.2 mg) was then added under nitrogen. After 3 h, the polymerization was terminated by exposure to air. The reaction mixture was centrifuged at 10,000*g* for 10 min. The supernatant was removed and replaced with doubly distilled, deionized water and the blue ATRP catalyst-contaminated sediment was redispersed in this medium using an ultrasonic bath. This centrifugation–dispersion cycle was repeated three times to obtain white, purified polyelectrolyte grafted silica particles.

2.3. Synthesis of citrate-stabilized magnetic nanoparticles

Citrate-stabilized Fe_3O_4 nanoparticles were synthesized using the method reported by Sahoo et al. (2005). Briefly, the mixture of 0.86 g of $FeCl_2$ and 1.40 g of $FeCl_3$ was mixed in 40 ml of water (degassed while vigorously stirring the mixture), 5 ml of NH_4OH were added using a syringe and heated for an additional 30 min. The supernatant was decanted while the nanoparticles were retained in the reaction flask using a magnet, and then fresh water was added. Citric acid solution (2 ml, 0.5 g/ml) was added, and the reaction mixture was heated to 95 °C for 90 min. The reaction mixture was allowed to cool to room temperature under nitrogen. The nanoparticles suspension were rinsed with deionized (DI) water three times and dialyzed against deionized (DI) water for 72 h. Fe_3O_4 is negatively charged (ζ-potential of −40 mV) under the conditions of adsorption (pH = 5–6).

2.4. Preparation of Fe_3O_4/polycation multilayers on the SiO_2 nanoparticles

The precursor polyelectrolyte film was formed by the adsorption of PEI (Fluka, $M_w < 200{,}000$ Da) from 1 mg ml^{-1} aqueous solutions (containing 0.5 M NaCl) onto the "spherical polyelectrolyte brush". The polyelectrolyte adsorption time was 20 min. Excess polyelectrolyte was removed by four centrifugation (10,000*g*)/wash/redispersion cycles.

Fe_3O_4/polycation multilayer on the SiO_2 nanoparticles were formed by adding 0.4 ml of Fe_3O_4 magnetic fluid ($\sim 1.5 \times 10^{14}$ nanoparticles) to the polyelectrolyte-coated SiO_2 nanoparticles dispersed in 0.5 ml water, allowing 20 min for Fe_3O_4 adsorption, removing excess Fe_3O_4 by four repeated centrifugation (4000*g*)/wash/redispersion cycles, and subsequently depositing polycation (1 mg ml^{-1}/0.5 M NaCl). This process was repeated until the desired numbers of multilayers were formed. When the positively charged PEI polyelectrolyte formed the outermost layer, the SiO_2-coated $(PEI/Fe_3O_4)_5$/PEI particles were cross-linked by treating with 2.5% glutaraldehyde (pH ~ 4) at room temperature for 4 h.

2.5. Enzyme immobilization

The silica-coated $(PEI/Fe_3O_4)_5$/PEI supports were added to the enzyme solution (5, 10, or 20 U enzymes per 1 ml acetate buffer at 4.5 pH) and the immobilization reaction was carried out in a shaking water bath for 4 h. Particles were separated and the unbound enzyme was removed by washing three times with 0.2 ml/l acetate buffer (pH = 4.5). The immobilized enzymes were used freshly or stored at 4 °C between reuses.

2.6. Determination of immobilized protein amount

Quantification of reducing sugars was performed by the 3,5-dinitrosalicyclic acid (DNS) method (Miller, 1959). The amount of reducing sugar formed was estimated by the 3,5-dinitrosalicyclic acid method (Miller, 1959). The standard compound used for the calibration curve for determining pectinase activity using 3,5-dinitrosalicylic acid was D-(+)-galacturonic acid monohydrate.

Pectinase (40 mg, Fluka, St. Louis, MO, USA) was dissolved in 1 ml 0.2 mol/l acetate buffer at 4.5 pH, added to the solutions of the silica-coated $(PEI/Fe_3O_4)_5$/PEI particles (100 mg/ml), and stirred for 24 h at 4 °C temperature to avoid microbial growth. The surplus of nonadsorbed enzyme was then removed by centrifugation (10,000*g* for 10 min). The amount of nonadsorbed enzyme was determined from absorbance of the eluate at 520 nm (measured by using a UV–visible Spectrophotometer, SHIMADZU CO Ltd., Kyoto, Japan) with the help of a calibration curve.

2.7. Pectinase activity assay

Pectinase activity was determined using polygalacturonic acid as a substrate (Miller, 1959). One unit of activity was defined as the amount of enzyme required to hydrolyze 1.0 mol of pectin per min under the described conditions. The amount of reducing sugar formed was estimated by the 3,5-dinitrosalicyclic acid method (Bailey & Pessa, 1990).

2.8. Determination of kinetic parameters

Enzyme activities, in the free and immobilized forms, were evaluated using the classical Michaelis–Menten kinetics:

$$V = \frac{V_{max}[S]}{K_m + [S]}$$

where $[S]$ is the substrate concentration, V_{max} is the maximum reaction rate attained at infinite substrate concentration, and K_m is the Michaelis–Menten constant.

To determine V_{max} and the K_m, the activity assay was applied for different substrate (pectic) concentrations. Pectin solutions (0.05/0.1/0.2/0.4/0.6/1.0/1.5 mol/l) were prepared in 0.2 mol/l acetate buffer (pH = 4.5) and kept in a water bath at 37 °C for 5 min, and then the immobilized pectinase or free enzyme solution was added to the test tubes and shaken for different incubation times. Both the free and immobilized enzyme concentrations were 1.0 mg/ml.

2.9. Determination of the storage stability of free and immobilized pectinase

The activity of the immobilized enzyme was measured daily while standing at 20 °C for 40 days. The remaining percentage of immobilized enzyme activity was calculated in each determination.

3. Results and discussion

3.1. Formation and characterization of layer-by-layer assembled magnetic nanoparticles

A strategy for the fabrication of composite magnetic core–shell particles, where the shell consisted of magnetic (Fe_3O_4) nanoparticle/polyelectrolyte multilayers and the colloidal core was SiO_2 nanoparticles, from layer-by-layer approach was proposed. This strategy was confirmed by the successful synthesis of very strong charge controlled "spherical polyelectrolyte brush" of core–shell structure, which was prepared by grafting poly(sodium 4-styrenesulphonate) (PSStNa), a negatively charged polyelectrolyte, from monodispersed SiO_2 nanoparticles via the surface-initiated atom transfer radical polymerization strategy. In the present work, a cationic polyelectrolyte macromolecule, polyethyleneimine (PEI), was adsorbed onto the "spherical polyelectrolyte brush" from layer-by-layer assembly approach to fabricate the dual-layer polyelectrolyte nanoparticles. Magnetic nanoparticles were alternately deposited with polyethyleneimine (PEI) on the 500 nm diameter SiO_2 nanoparticles. The introduction of a magnetic function to SiO_2 provides particles which may be of importance in various technologies (e.g., separations, biomedicine, etc.). Alternate depositions of Fe_3O_4 and PEI on the precoated SiO_2 nanoparticles, under conditions where the nanoparticles and polyelectrolyte were oppositely charged (pH $\sim$ 5–6), produce magnetic nanoparticle/polyelectrolyte multilayer.

The chemical composition of the modified silicon surfaces was determined by X-ray photoelectron spectroscopy (XPS). The XPS spectra were recorded on a Shimadzu ESCA 3400 spectrometer (digital instruments, SHIMADZU Co. Ltd., Kyoto, Japan) equipped with an Mg KR X-ray source. The XPS spectra were recorded for the silica-coated $(PEI/Fe_3O_4)_5$ particles. Fig. 1 shows the survey spectra for the silica-coated $(PEI/Fe_3O_4)_5$ particles. The characteristic peaks for O1s, C1s, Si2p, N1s and Fe2p were observed in the spectrum at 533, 285, 104, 400 and 743 eV, respectively.

Formation of the magnetic shell covering the SiO_2 nanoparticles can be directly visualized by transmission electron microscopy (TEM). Specimens for inspection by TEM were prepared by drop-casting dilute solutions of SiO_2 and hybrid nanoparticles onto carbon-coated copper grids, followed by evaporation of the solvent in air. Fig. 2 shows TEM images of polyelectrolyte-modified SiO_2 nanoparticles covered with nanocomposite Fe_3O_4/polyelectrolyte multilayer shells at the 100,000 magnification. The uncoated particles were spherical in shape and exhibited a smooth surface. Average particle size and shell thickness of the nanospheres, determined from TEM images. The polyelectrolyte-modified SiO_2 nanoparticles covered with nanocomposite Fe_3O_4/polyelectrolyte multilayer shells are shown in Fig. 2. The presence of a Fe_3O_4/PEI shell clearly resulted in increased surface roughness. The Fe_3O_4 nanoparticles distribution on the surface of the SiO_2 nanoparticles was somewhat uneven and less than monolayer coverage. Aggregates of the magnetic particles can also be seen. The aggregation of these nanoparticles on the surface may be ascribed to a combination of the electrostatic attraction between the outer polycation layer and Fe_3O_4, and the magnetic interactions between the nanoparticles themselves.

Microelectrophoresis experiments were conducted to qualitatively follow the formation of the Fe_3O_4 multilayers on the colloids (Caruso, Lichtenfeld, Möhwald, & Giersig,

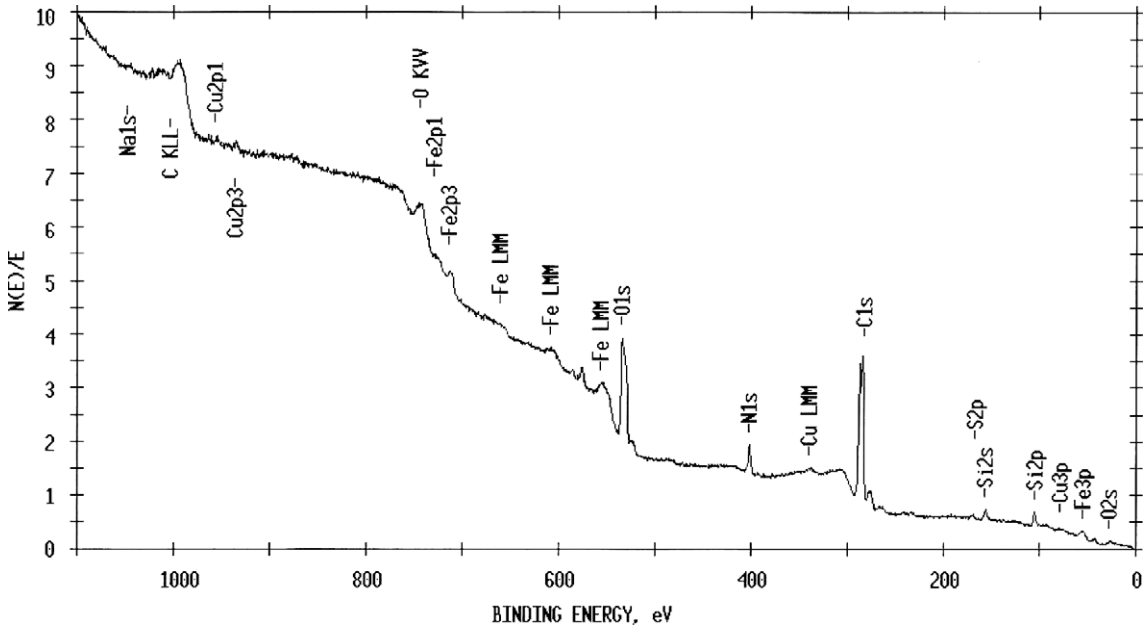

Fig. 1. XPS of the silica-coated $(PEI/Fe_3O_4)_5$ particles.

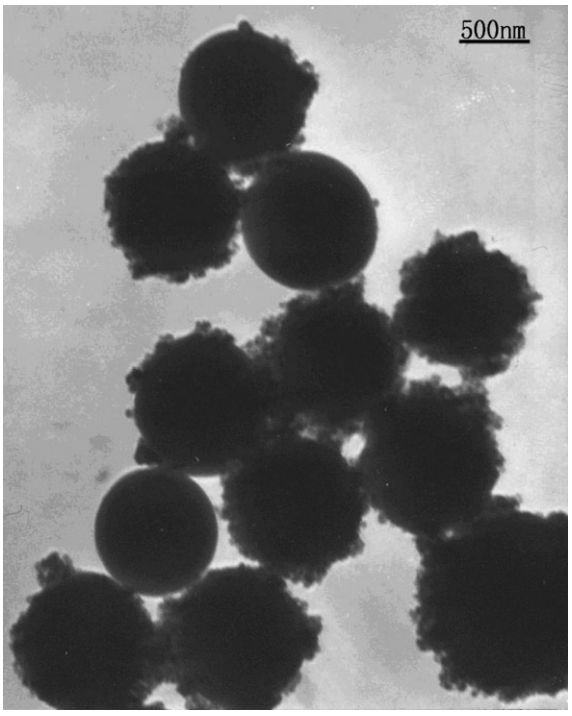

Fig. 2. Transmission electron microscopy (TEM) micrographs of the silica-coated $(PEI/Fe_3O_4)_5$ particles.

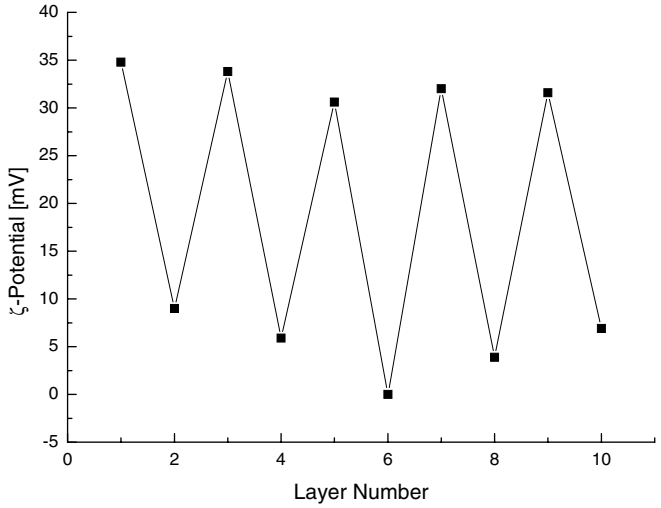

Fig. 3. ζ-Potential of the SiO_2 surface deposited by five bilayers of PEI/Fe_3O_4 with the Fe_3O_4 as the outermost layer.

1998, 1999; Caruso & Möhwald, 1999). The ζ-potentials of the coated particles were calculated from the mobilities that were measured after deposition of each layer. Fig. 3 shows the ζ-potential of SiO_2 particles coated with multilayers of PEI/Fe_3O_4 when the positively charged PEI polyelectrolytes formed the outermost layer; positive ζ-potentials were measured. The slight differences in the measured values can be attributed to the different polyelectrolytes or contributions from the layer underneath, arising due to different conformations and distribution of the polymers at the surface. The ζ-potentials measured for the coated particles when Fe_3O_4 formed the outermost layer were mostly close to zero and occasionally slightly positive. The values obtained most probably reflect variations in surface coverage of Fe_3O_4 on the polyelectrolyte surface.

Atomic force microscopy (AFM) experiments were performed in tapping mode using the WET-SPM-9500-J3 (digital instruments, SHIMADZU Co. Ltd., Kyoto, Japan) scanning probe microscope. The samples were applied onto freshly cleaved mica surface and left to dry in air at room temperature. Images show a preference for particles to self-associate, probably due to magnetic attraction, the absence of a strong interaction with the substrate, and the procedure of sample preparation (Fig. 4a). The AFM image for pectinase immobilized on a precursor film of $(PEI/Fe_3O_4)_5$/PEI on the PSStNa-treated SiO_2 sphere is shown in Fig. 4b. The $1.25 \times 1.25\ \mu m^2$ area was well covered with pectinase. Most of the pectinase on the surface have a diameter in the range of 20–50 nm. In contrast to the multilayer film where the interlayer was (PEI/

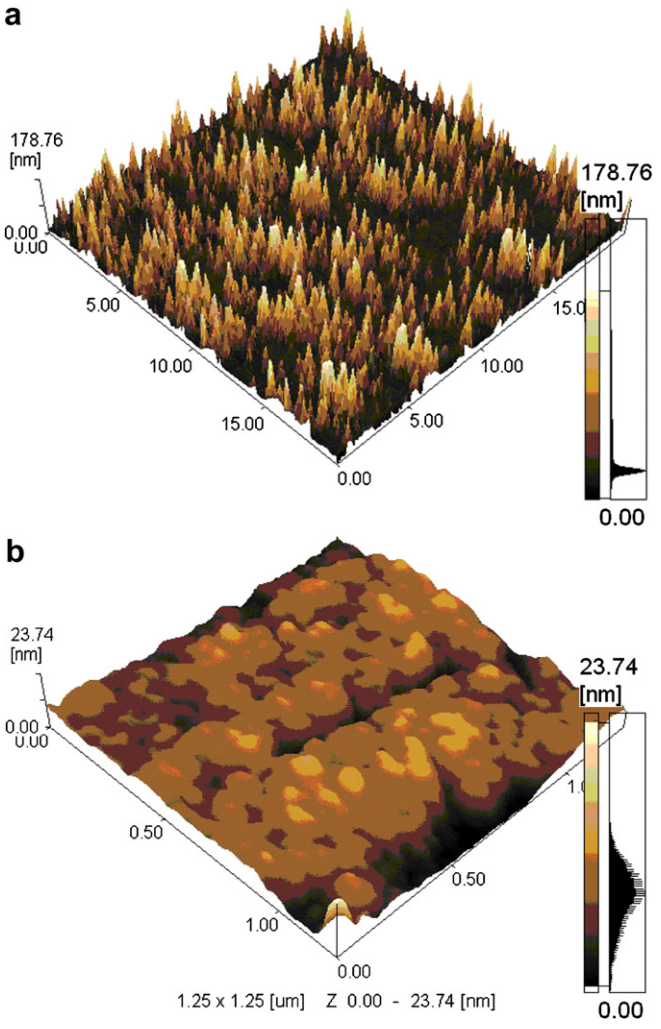

Fig. 4. AFM image (tapping mode) of (a) the SiO_2 surface deposited by five bilayers of PEI/Fe_3O_4 with the Fe_3O_4 as the outermost layer and (b) TM AFM image of the pectinase immobilized on the $(PEI/Fe_3O_4)_5$ multilayer film on SiO_2 nanoparticles pretreated with PSStNa.

$Fe_3O_4)_5$/PEI; pectinase aggregation is clearly evident in this image. Some smaller structures of diameter of ca. 100 nm and height of ca. 23 nm were also seen in the image; these too were aggregates of pectinase. Thus, in this film, individual pectinase molecules were not imaged. A noticeable feature of this image was the nonuniform coverage of the surface by pectinase.

3.2. Immobilization of pectinase on the silica-coated Fe_3O_4 particles

The amounts of bound protein are 416.8 mg/g, without any negative effect on the expressed activity. In comparison with the free enzyme, the immobilized pectinase under its optimum reaction condition retains 80.2% of activity. In case of pectinase–alginate system, activity retention of immobilized pectinase was 56% (Munishwar & Meryam, 2003). After immobilization, a decrease of enzyme activity was commonly observed. This could be explained by the minor modification in the three-dimensional structure of the enzyme that leads to the distortion of amino acid residues involved in catalysis, the presence of random immobilization which causes the analytic approach to the active site of the enzyme hindered, and the limitations imposed by slow mass transfer of substrate or product to or from the active site of the enzyme. The activity of immobilized enzymes was dependent on the type of outer layer used for loaded and on the number of layers deposited. For the same number of layers, the enzyme activity was lower when Fe_3O_4 outer layers were used for loaded as compared to the PEI outer layer cross-linked by treating with 2.5% glutaraldehyde (Fig. 5). The numbers of the layers on SiO_2 particles has a significant influence on the rate of enzyme immobilization. For the silica-coated (PEI/$Fe_3O_4)_n$/PEI supports and coated with 1, 2, 3, 4 and 5 bilayers of PEI/Fe_3O_4 supports, pectinase adsorption reached saturation at ca. 24 h, 17 h, 10 h, 4 h and 1 h, respectively. It seems that pectinase has a rapid immobilization on the silica-coated (PEI/$Fe_3O_4)_5$ supports.

3.3. Storage stability

This system offers a relatively simple technique for separating and reusing enzymes over a longer period than that for free enzymes alone and for enzymes which were immobilized by electrostatic immobilization and covalent immobilization.

There is a direct relationship between enzyme stability and layer number and the type of outer layer for the silica-coated (PEI/$Fe_3O_4)_n$/PEI support particles. The silica-coated (PEI/$Fe_3O_4)_n$/PEI support particles with pectinase and coated with 1, 2, 3, and 4 bilayers of PEI/Fe_3O_4 yielded an activity of 22%, 43%, 67%, and 76%, respectively, after 120 min (Fig. 6).

The storage stability was investigated by measuring the enzyme activities at certain time intervals and the results are given in Fig. 7. Free pectinase was inactivated immediately, losing its entire activity within ca. 20 days (a). When the silica-coated (PEI/$Fe_3O_4)_5$ support particles were used for immobilized pectinase, inactivation was slightly slower (55% activity after 20 days) (b). Notably, the enzyme stability was significantly higher for the silica-coated (PEI/$Fe_3O_4)_5$/PEI support particles which the PEI outer layer was cross-linked by 2.5% glutaraldehyde (50% of the original activity was retained after 30 days). The activity half lives for native and bound states of enzyme (b) were found as 13.5d and 30d, respectively. The enhancement in thermal stability provided by this immobilization method was approximately two fold.

3.4. Reuse stability of the immobilized lipase

Reuse stability for the immobilized enzyme is very important in economics, and an increased stability can make the

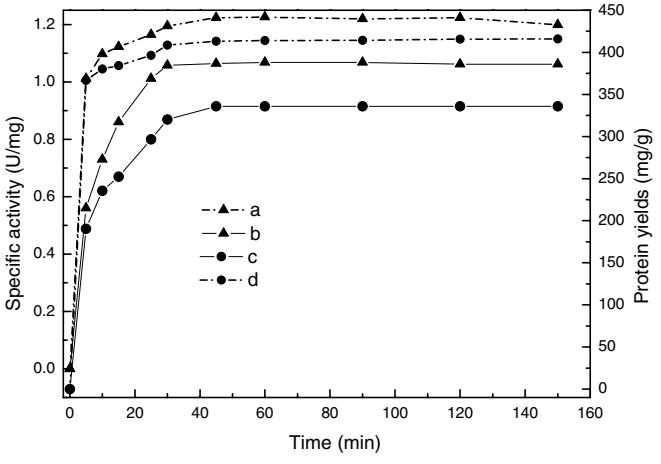

Fig. 5. (a) and (b) Protein yields and immobilized enzyme activity of the silica-coated (PEI/$Fe_3O_4)_5$/PEI; (c) and (d) protein yields and immobilized enzymes activity of the silica-coated (PEI/$Fe_3O_4)_5$.

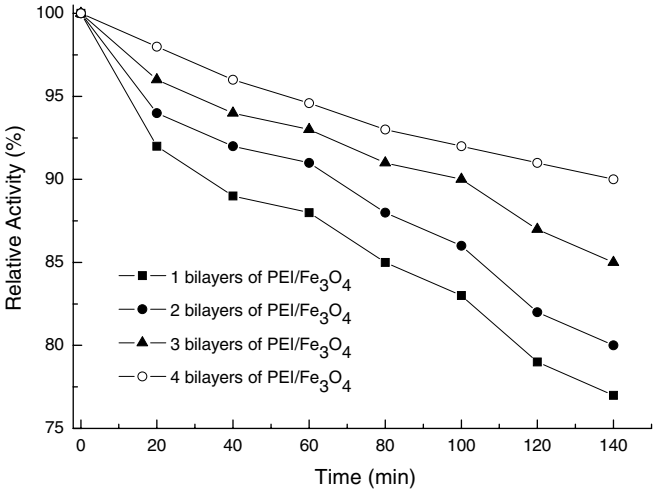

Fig. 6. Stability of immobilized enzymes of the silica coated with (■) one PEI/Fe_3O_4 bilayers; (●) two PEI/Fe_3O_4 bilayers, (▲) three PEI/Fe_3O_4 bilayers, and (○) four PEI/Fe_3O_4 bilayers.

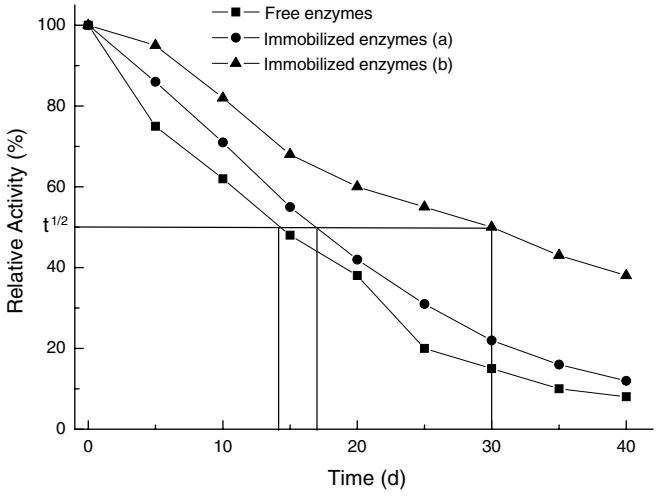

Fig. 7. Storage stability of free and immobilized enzymes. (■) Free enzyme; (●) immobilized enzyme of the silica-coated $(PEI/Fe_3O_4)_5$ particles (a); and (▲) immobilized enzymes of the silica-coated $(PEI/Fe_3O_4)_5$/PEI particles (b).

immobilized enzyme more advantageous than its free counterpart. To confirm the reusability of the immobilized pectinase the enzyme was tested for the same hydrolysis reaction, magnetically precipitated, collected, and washed extensively with the 0.2 mol/l acetate buffer solution (pH = 4.5). After that the immobilized enzymes were retested under the same conditions using different substrate concentrations. The activity of pectinase immobilized on the SiO_2 particles with $(PEI/Fe_3O_4)_5$/PEI layers which the PEI outer layer was cross-linked using 2.5% glutaraldehyde decreased by only ca. 8% after recycling five times. Significantly more activity (ca. 22.5%) was lost if the enzyme was only immobilized on the SiO_2 particles with $(PEI/Fe_3O_4)_5$ layers. After 25 successive batch reactions, the free pectinase and the immobilized pectinase showed activities of 30% and 73%, respectively. The lower activity observed for the immobilized pectinase was explained by the inactivation of the enzyme caused by the denaturation of the protein and the enzyme leakage when the multiple soaking, separation, and washing steps employed during the recycling process.

Thus, the reusability of the nanomagnetic composite hosted enzyme is clearly demonstrated using different substrate concentrations. The high reproducibility of the reused enzyme (separated and purified) for a new batch of substrate after magnetic separation suggests the clear separation and facile reusability advantages of the immobilized enzyme. It is noted that the procedures for magnetic separation were as follows: enzyme-bound magnetic nanocomposites (colloidal stable) are recovered from the product mixture by placing the bottle of a test tube on a permanent magnet with a magnetization of ~6000 G. All magnetic particles settled at the bottom of the test tube to give a clear top solution within 30 s–1 min. This clearly supports the fact that the pectinase is magnetically recoverable when the enzyme is immobilized on the silica-coated $(PEI/Fe_3O_4)_5$/PEI support particles which the PEI outer layer was cross-linked using 2.5% glutaraldehyde. It is noted that for industrial applications reusability and durability of magnetic catalyst are very important issues which warrant further detailed investigation.

Table 1
Determination of kinetic parameters for free and immobilized pectinase at 37 °C

Kinetic parameters	Free enzyme	Immobilized enzyme
K_m (g pectin ml^{-1})	8.28	9.86
V_{max}[a]	1.165	1.112

[a] $\times 10^{-3}$ g pectin s^{-1} g enzyme^{-1} and $\times 10^{-3}$ g pectin s^{-1} g particle^{-1} for free and immobilized enzymes, respectively.

3.5. Kinetic parameters

The activities of free and immobilized enzymes for various substrate concentrations are plotted in Lineweaver–Burk graph, from which maximal activities (V_{max}) and Michealis–Menten constants (K_m) values are calculated. K_m and V_{max} calculated from the equations of these plots are summarized in Table 1.

In this study, for pectinase, V_{max} values for free and immobilized enzymes were not different, thus demonstrating almost no change upon immobilization. This shows that the immobilized enzyme achieves the same velocity as the free enzyme when saturated with the substrate. K_m value of immobilized enzyme was higher than that of free enzyme. This increase in the K_m values was either due to the conformational change of the enzyme resulting in a lower possibility of forming a substrate–enzyme complex, or to the lower accessibility of the substrate to the active sites of the immobilized enzyme caused by the increased diffusion limitation. Other reasons that may be invoked include the modeling of the process of adsorption.

4. Conclusions

The present work was the first detailed study to characterize the silica-coated $(PEI/Fe_3O_4)_5$/PEI support particles which the PEI outer layer was cross-linked using 2.5% glutaraldehyde as a support for enzyme immobilization. Pectinase was successfully immobilized on the silica-coated $(PEI/Fe_3O_4)_5$/PEI particles by using quite simple method. The enzyme retained its activity even after the immobilization. Magnetic handling and transport of the immobilized enzyme could be performed in reactor application. The storage stability, reusability and kinetic properties of free and immobilized pectinase were investigated. The most significant advantage of our samples was their long-term stability. This (so far unreported) long-term stability illustrates the advantage of attaching the enzymes by the physicochemical approach to the nanoparticles. Such stability might make economically viable the use of expensive enzymes and hence opens a new horizon for enzymatic catalysis in biotechnology.

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