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Chitosan-tethered the silica particle from a layer-by-layer approach for pectinase immobilization

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Abstract

"Spherical polyelectrolyte brush" of core–shell structure were prepared by grafting poly (sodium 4-styrenesulphonate) (PSStNa) from SiO_2 nanoparticle via the surface-initiated atom transfer radical polymerization strategy. The colloidal stability was not impeded by the adsorbed proteins despite the fact that up to 316.8 mg of enzyme was adsorbed per gram of the carrier particles. The immobilized pectinase revealed acceptable pH stability over a broad experimental range of 3.0–4.5. The activity half lives for native and bound states of enzyme were found as 13.5 d and 30 d, respectively. The activity of immobilized pectinase adsorbed onto these particles was analyzed in terms of the Michaelis–Menten parameters. Kinetic parameters were calculated as 8.28 and 9.98 g pectin ml^{-1} for K_m and 1.165×10^{-3} g pectin s^{-1} g $enzyme^{-1}$ 1.124×10^{-3} g pectin s^{-1} g $particle^{-1}$ for V_{max} in the case of free and immobilized enzymes, respectively. Enzyme activity was found to be approximately 49.7% for immobilized enzyme after storage for 1 month.
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Keywords: Atom transfer radical polymerization; Polyelectrolyte; Layer by layer; Chitosan; Immobilized enzyme

1. Introduction

The specific characteristics presented by enzymes are high levels of catalytic efficiency and high degrees of specificities including substrate specificity, region specificity and stereospecificity. These properties enable enzymes to catalyze biotransformation reactions, which yield a wide range of useful biological and chemical compounds for pharmaceutical, food and agrochemical derivatives (Koeller & Wong, 2001; Sharma, Chisti, & Banerjee, 2001).

Immobilization of enzymes on solid supports is an important challenge in biotechnology (Bahar & elebi, 1998; Khalid et al., 2006; Wu, Lee, & Lee, 1998). Until now, a great variety of designed systems have been introduced and discussed. The techniques used to immobilize enzymes are key factors for developing reliable biosensors, thus new immobilized schemes and novel materials that can improve the analytical capacities of sensor devices are highly desired (Yanga, Yanga, Liua, Shena, & Yua, 2004).

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.

Chitosan exhibits a considerable protein-binding capacity and a high recovery of enzyme activity, allowing that the enzyme immobilized remains considerably active (Gallifuoco, Dercole, & Alfani, 1998). However, severe shrinkage and deformation could not be easily avoided upon drying

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the chitosan carriers into the corresponding gels (Michael & Arlon, 2001). This can be improved in conjunction with other solid powders to increase their density and strengthen their physical properties, and thus to expand their applications (Bai, Yin, & Wu, 2002). Additionally, amino groups make chitosan a cationic polyelectrolyte ($pK_a \approx 6.5$); It adheres to negatively charged surfaces.

The layer-by-layer (LbL) approach provides an easy, low-cost, and versatile method for the fabrication of the silica-coated chitosan support. Briefly, this technique is based on alternating adsorption of oppositely charged polyelectrolytes onto a charged substrate. By virtue of the attraction of oppositely-charged molecules, chitosan, owing to its cationic polyelectrolyte nature, spontaneously forms water-insoluble complexes with anionic polyelectrolyte (Dumitriu & Chornet, 1998; Kubota & Kikuchi, 1998; Singla & Chawla, 2001).

We report here an observation 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 monodisperse SiO_2 nanoparticle via the surface-initiated atom transfer radical polymerization strategy. In the present work, a cationic polyelectrolyte natural macromolecule, chitosan, was adsorbed onto the "spherical polyelectrolyte brush" from layer by layer assembly approach to fabricate a dual-layer polyelectrolyte nanoparticle support for enzyme immobilization. This simple strategy seems to permit very good results in terms of the activity, catalytic properties, pH and thermal stabilities, and kinetic parameters of the enzymes.

2. Materials and methods

2.1. Materials

Pectin and the enzyme polygalacturonase (pectinase) were obtained from Fluka Chemical Co. (New York, USA), 2-bromopropionyl bromide (BPC, Technical Grade) from Technology of Hongchen Xinxiang He'nan, China. Triethylamine (TEA) (Shanghai Chemical Co., Shanghai, China) was refluxed with *p*-toluenesulphonyl chloride and distilled. The resulting pure TEA was stored over CaH_2. Before use it was refluxed and distilled again. CuBr was purified according to a published procedure (Wang & Matyjaszewski, 1995). SStNa (sodium 4-styrenesulphonate) was supplied by Fluka 99% (New York, USA). Chitosan was supplied by Shanghai Chemical Co. (Shanghai, China). Ultrapure water (resistivity = 18.2 MX, pH 6.82) was used in all experiments. Other reagents and organic solvents for the initiator synthesis and polymerization were purchased from commercial sources and used without further purification.

2.2. Synthesis of polyelectrolyte-grafted silica particles

2.2.1. Preparation of silica particles

The monodisperse silica particles in the range of 150 nm were prepared by Stober, Fink, and Bohn, 1968 method. Silica particles were washed with the 'piranha' solution (a mixture of 70 vol% concentrated sulphuric acid and 30 vol% hydrogen peroxide) to remove organic residues. The particles were rinsed with copious amount of deionized water, dried in nitrogen flow.

2.2.2. Immobilization of the initiator on the silica particles surface

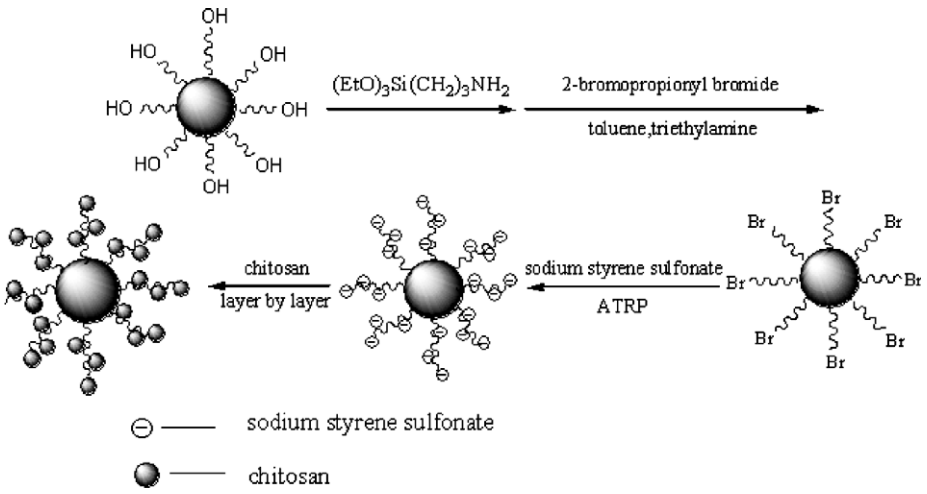
The two-step process of immobilization of the surface initiators was shown in Scheme 1. Firstly, the silica particles were dried over 24 h at 150 °C and aminated silica particles were synthesized by reaction with 3-aminopropyltriethoxysilane in toluene under nitrogen for 12 h at 95 °C. After the reaction, the particles were filtered and washed with methanol. Then, the silica particles were washed carefully with toluene 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 toluene (10 ml) under magnetic stirring. After the above mixture was cooled down to 0 °C, a solution of 2-bromopropionyl bromide (2 ml) and toluene (5 ml) was added drop wise. Four hours later, the silica particles were washed again with toluene and acetone thoroughly, and then dried under vacuum for the subsequent polymerization.

2.2.3. ATRP of sodium 4-styrenesulfonate

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 with the aid of an ultrasonic bath. Sodium 4-styrenesulphonate (539.0 mg) and 2,2′-bipyridine (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 with the aid of an ultrasonic bath. This centrifugation–dispersion cycle was repeated three times to obtain white, purified polyelectrolyte grafted silica particles.

2.3. The silica-coated chitosan supports preparation

The polyelectrolyte-grafted silica particles were then redispersed in 0.2 M acetate buffer of pH 3.5 (about 0.8 g solid content in 30 g solution). A total of 2 ml of 0.5% chitosan in 1% acetic acid was added while stirring. After 8 h, the excess polymer was removed in the supernatant fraction after centrifugation. The particles were washed using 0.002 M acetate buffer of pH 3.8 and ultrapure water for three times alternately.



Scheme 1. Schematic representative for the preparation of the silica-coated chitosan particle.

The SiO_2-coated chitosan particles were cross-linked by treating with 2.5% glutaraldehyde (pH ~ 4) at room temperature for 4 h. The cross-linked particles were brown in colour.

2.4. Enzyme immobilization

The silica-coated chitosan 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 at 25 °C in a shaking water bath for 4 h. Microspheres were separated and the unbound enzymes were removed by washing three times with acetate buffer (5 ml). The immobilized enzymes were used freshly or stored at 4 °C between reuses. The whole procedure was summarized in Scheme 1.

2.5. Pectinase assay

Pectinase activity was determined using polygalacturonic acid as a substrate (Bailey & Pessa, 1990). 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-dinitrosalicylic acid method (Miller, 1959).

Different concentrations of pectin solutions prepared in acetate buffer 0.2 mol/l (pH 4.5) were kept in a water bath at 37 °C for 5 min. The immobilized pectinase was immersed in the test tubes and shaken for different incubation times. Then, the immobilized pectinase was removed by centrifugation. After 10 min at 37 °C the reaction was stopped by adding 3,5-dinitrosalicylic acid and colorimetrically after boiling the solutions for 10 min. The reducing sugars were determined from absorbance of the collected solution at 520 nm (measured by using a UV–visible Spectrophotometer, Shimadzu, Kyoto, Japan) with the help of a calibration curve.

2.6. Determination of kinetic parameters

To determine the maximum reaction rate (V_{max}) and the Michaelis–Menten constant (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 acetate buffer 0.2 mol/l (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.

3. Results and discussion

3.1. Characterization of the silica-coated chitosan support

Fig. 1 gave a comparison between the FTIR spectra of SiO_2 and the silica-coated chitosan support. Piron and Domard (1998) indicated that in the IR spectrum of chitosan, characteristic absorptions were displayed at 1656.5 and 1598.6 cm^{-1} attributable to amide bands I and II, respectively. However, the amide bands I peaks did not appear in that of the silica-coated chitosan support, which exhibited instead the C=N stretching absorption at 1644.7 cm^{-1}, indicating its cross-linkage through glutaraldehyde. Compared with the hydroxyl band at 3426.7 cm^{-1} in the IR spectrum of chitosan, the peak of 3438.6 cm^{-1} of the silica-coated chitosan support was wider and stronger demonstrating that it carried some free Si–OH permitted by the incompleteness of TMOS hydrolysis.

The α-bromoester initiator on the silicon gel substrate was prepared by the self-assembly of 3-aminopropyltrieth-oxysilane, followed by amidization with α-bromopropionyl bromide. After this step, X-ray photoelectron spectroscopy (XPS) was used to confirm the formation of the initiator monolayer. The peak of Br3d (the content of Br: 2.2%)

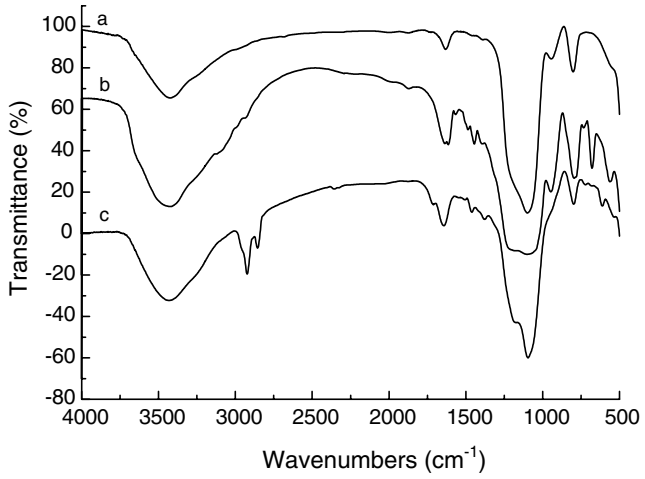


Fig. 1. FTIR spectra of (a) the SiO_2 (b) the SiO_2-grafted sodium 4-styrenesulfonate and (c) the SiO_2-coated chitosan.

was observed around the binding energy (BE) at 71 eV in the wide scan XPS spectra (inset Fig. 2a) of the above modified silicon particles surface and it comprises a major peak component at the binding energy of about 399.4 eV, attributable to the two N 1s peak components at the lower binding energies suggest the presence of the Si–N bonded species, $–C_6H_4–NSi_2$ and $–C_6H_4–NHSi$ (Xu, Zhong, Yung, & Kang (2004)).The above result is the robust proof of the formation of the initiator monolayer. Then surface-initiated ATRP was carried out from the above initiator-modified particles as soon as possible.

XPS spectra were recorded for the polyelectrolyte-grafted silica particles and the silica-coated chitosan particles. Fig. 2 shows the survey spectra for (a) the poly (SStNa)-grafted silica particles and (b) the silica-coated chitosan particles. The former spectrum contained five signals, which were assigned to O1s, C1s, Si2p, S2p, and Na1s. In contrast, characteristic peaks for O1s, C1s, Si2p, and N1s were observed in the latter spectrum at 533, 285, 104, and 400 eV, respectively. Integration of the peak areas of the elements of interest allowed the surface chemical composition to be calculated for each sample; see Table 1.

Transmission electron microscopy (TEM) micrographs of the silica nanoparticles are shown in Fig. 3a. A basically core–shell structure (dark colored core for SiO_2 and light-colored shell for sodium 4-styrenesulfonate) was obtained (Fig. 3b). Average particle size and shell thickness of the nanospheres, determined from TEM images. The SEM images (inset Fig. 3c) revealed the significant change of

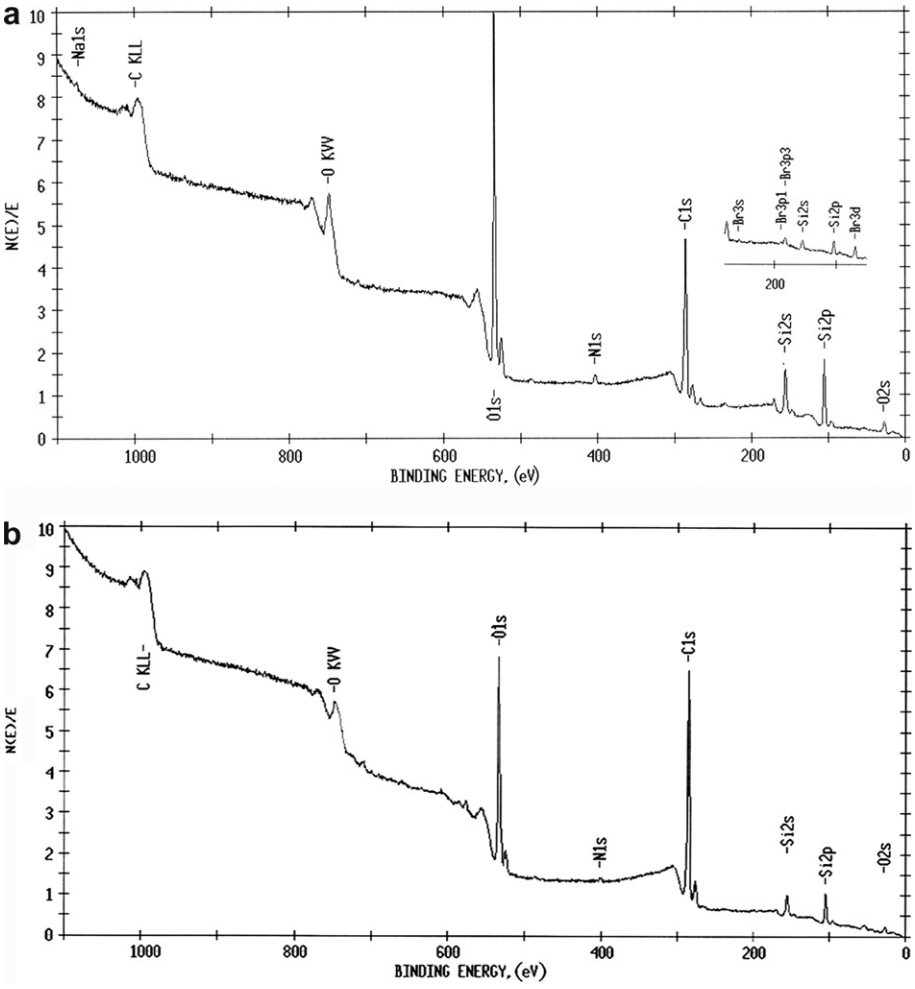


Fig. 2. XPS of (a) the SiO_2-grafted sodium 4-styrenesulfonate and (b) the SiO_2-coated chitosan.

Table 1
XPS surface compositions of the silica-coated chitosan particle

Sample type	Element surface compositions determined by XPS (atom %)				
	Si 2p	O 1s	C 1s	N 1s	Na 1s
SiO_2-grafted poly (SStNa)	13.97	30.05	53.77	1.97	0.24
SiO_2-coated chitosan	7.00	23.20	68.95	0.85	

the morphology polymer modified particles, and the polymer modified particles owned core–shell structure.

3.2. Immobilization of pectinase on the silica-coated chitosan support

Table 2 shows the activity and kinetic parameters of the free and immobilized pectinase under the optimum reaction conditions. The amounts of bound protein are 316.8 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 74% activity. In case of pectinase–alginate system, activity retention of immobilized pectinase was found just to be 56% (Munishwar & Meryam, 2003).

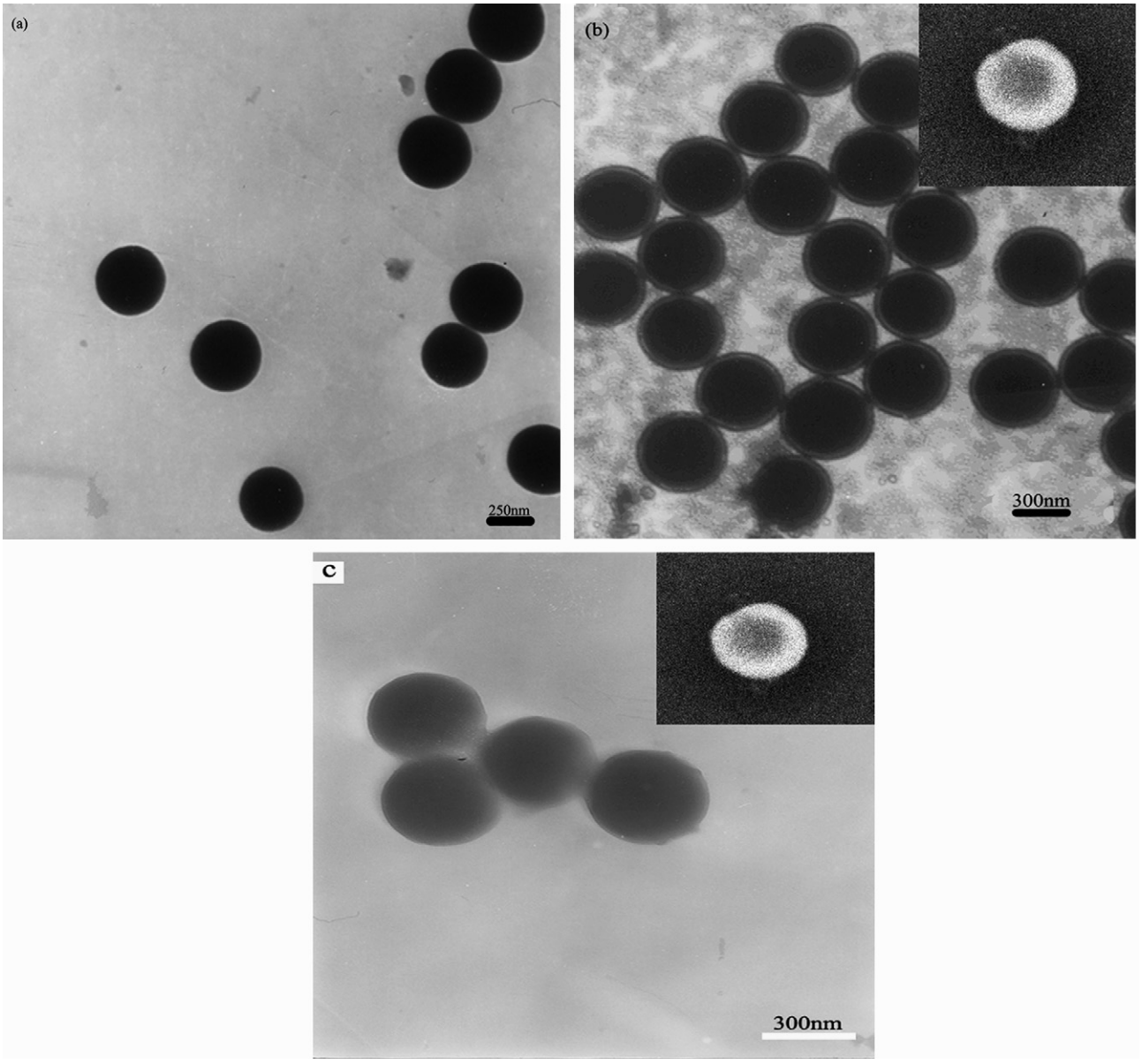


Fig. 3. Transmission electron microscopy (TEM) micrographs of (a) the SiO_2 (b) the SiO_2-grafted sodium 4-styrenesulfonate and (c) the SiO_2-coated chitosan.

Table 2
Activity parameters of the free and immobilized pectinase under optimum reaction conditions

Samples	Temp (°C)	pH	Bound protein (mg/g)	Specific activity (U/mg)	Activity retention (%)
Free pectinase	50	4.0		1.654	100
Immobilized pectinase	50	3.5	316.8	1.224	74

3.3. Effect of reaction pH and temperature on enzyme activity

Effect of the pH of reaction medium. Changes in free and immobilized enzymes activities with the pH of the reaction medium are compared in Fig. 4. The optimum pH of immobilized enzyme was moved to 3.5, 0.5 U lower than that of free enzyme and the relative activity of immobilized enzyme above the optimum pH was a little higher than that of free enzyme whereas the behavior of activity below optimum pH was almost same as that of the free enzyme. Therefore, the microenvironment of the immobilized enzyme on the silica-coated chitosan support might have been buffered and immobilized enzyme was less affected from the acidity of the solution. The immobilized pectinase revealed acceptable pH stabilities over a broad experimental range.

Chitosan belongs to the polycationic polymer, so that the pH optimum of the immobilized enzyme on the silica-coated chitosan particles shifted slightly in the acidic region relative to the pH of the free enzyme. It is known that polyionic matrices cause the partitioning of protons between the bulk phase and the enzyme microenvironment causing a shift in the optimum pH value. The shift depends on the enzyme reaction as well as on the structure and the charge of the matrix. For pectinase immobilization, shifts towards the acidic directions have been observed.

Effect of the temperature of reaction medium. The effect of temperature on the activity of free and immobilized pectinase in the temperature range of 20–80 °C is shown in Fig. 5. It was found that the optimum temperatures for free and immobilized pectinase were obtained at 50 °C. The rate of enzymatic reactions increases with increase of reaction temperature to a certain value in general. At a certain temperature protein denaturation occurs and so the reaction rate decreases.

3.4. Storage stability

Enzymes are not stable during storage in solutions and their activities decrease gradually by time. Immobilization of enzyme may overcome this constraint and material could be stored, in some cases, at room temperature without much loss of enzyme activity. This is very important for the application of an enzyme on a commercial scale. The storage stability was investigated by measuring the enzyme activities at certain time intervals and the results are given in Fig. 6. After 10 d, there were tiny differences between the activities of the immobilized enzyme and the free enzyme. There was a significant decrease in the activity of the immobilized and free enzyme over a 25 d period, but immobilized enzyme provided a prominence advantage in stability over free enzyme, especially at longer durations. The activity half lives for native and bound states of enzyme were found as 13.5 d and 30 d, respectively. The enhancement in thermal stability provided by this immobilization method is approximately twofold.

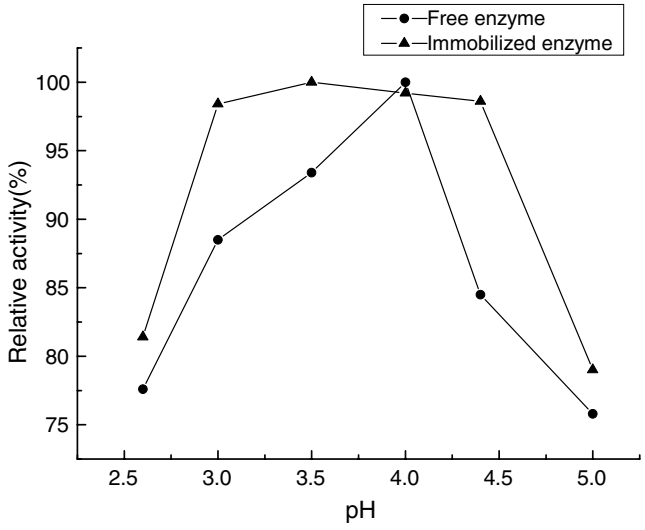


Fig. 4. The activity of free and immobilized pectinase at different reaction medium pH values. (●) Free enzyme; (▲) immobilized enzyme.

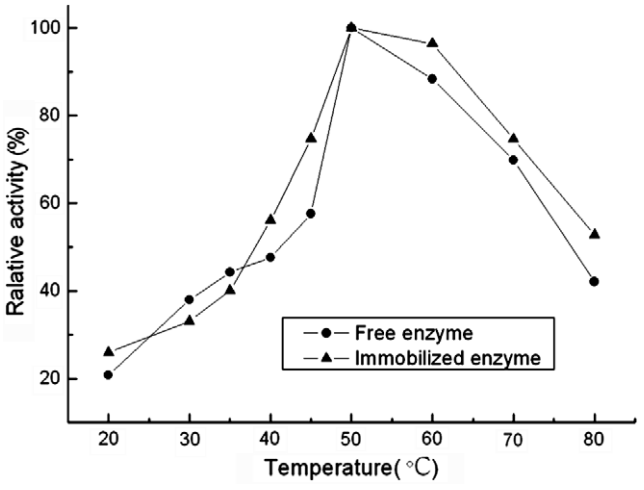


Fig. 5. The activity of free and immobilized pectinase at different temperature. (●) Free enzyme; (▲) immobilized enzyme.

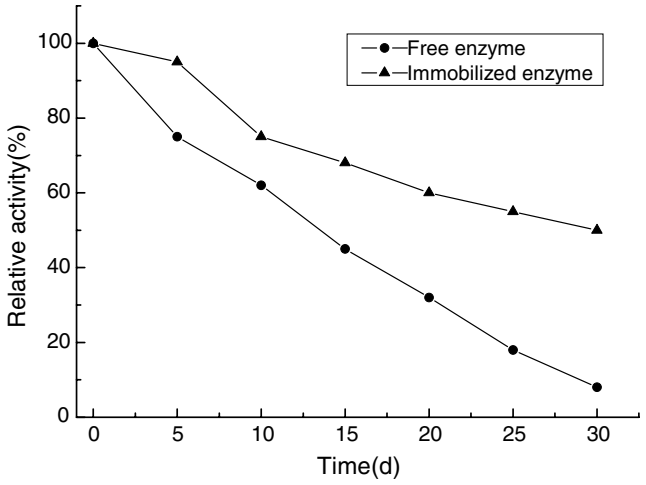


Fig. 6. Storage stability of immobilized enzymes. (●) Free enzyme; (▲) immobilized enzyme.

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. V_{max} defines the highest possible rate when all the enzyme is saturated with substrate, therefore, this parameter reflects the intrinsic characteristics of the immobilized enzyme, but may be affected by diffusional constraints. K_m is defined as the substrate concentration that gives a reaction rate of 1/2 V_{max}. This parameter reflects the effective characteristics of the enzyme and depends upon both partitioning and diffusional effects. K_m and V_{max} calculated from the equations of these plots are summarized at Table 3.

The apparent Michealis–Menten constant for pectinase was affected after immobilization. Diffusion effects, caused by the three dimensional structure of the support and the polymer chains, may be mainly responsible for the slightly increase in K_m value. Other reasons that may be invoked include the modeling of the process of adsorption.

The modeling of the process of adsorption must therefore be divided into two distinct steps: (a) the first step is an equilibration of the free and the bound protein and (b) the adsorbed protein is rearranged within the brush so that the process of desorption becomes slow on the time scale of the present experiment (Arslan, Kıralp, Toppare, & Bozkurt, 2006). In that case, the mutual interaction between the immobilized proteins within the brush layer may become important.

In this study, for pectinase, the immobilized enzyme achieved the same rate as the free enzyme when saturated with the substrate. K_m value of immobilized enzyme was higher than that of free enzyme, as expected.

The results demonstrate that the enzyme must remain in its native active state even if adsorbed onto the silica-coated chitosan support. This is in accord with previous findings showing that the secondary structure of the desorbed proteins is practically unchanged (Haupt, Neumann, Wittemann, & Ballauff, 2005).

Table 3
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	9.98
V_{max}[a]	1.165	1.124

[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.

4. Conclusions

A strategy for the fabrication of the silica-coated chitosan support from layer by layer approach was proposed. This strategy was confirmed by the successful synthesis of the silica-coated chitosan support to immobilized pectinase. The silica-coated chitosan supports were treated with glutaraldehyde for stability in both alkaline and acidic media. The pectinase immobilized on the silica-coated chitosan support exhibited improved resistance against pH denaturation. This immobilized enzyme could be applied in a wide pH range (3.0–4.5) without significant loss in its activity. The present work demonstrated a promising application potential of the composite particles for enzyme immobilization.

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