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Polystyrene-based diazonium salt as adhesive: A new approach for enzyme immobilization on polymeric supports

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ABSTRACT

In this work, a new way for enzyme immobilization was explored and properties of the enzyme immobilized on different polymer films were investigated. In the process, a polystyrene-based diazonium salt (PS-DAS) was synthesized and used as molecular adhesive to immobilize β-glucosidase on the polymeric supports (films of polyethylene, polypropylene and poly(ethylene terephthalate)). The immobilization of β-glucosidase on the polymer surfaces was achieved by sequential depositions of a piece of the polymer films in PS-DAS and the enzyme solutions. The surface modification was investigated by X-ray photoelectron spectroscopy (XPS), water contact angle measurement, and atomic force microscopy (AFM). The activity of the immobilized β-glucosidase was evaluated by measuring its enzymatic activity to the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG). The optimized reaction conditions (such as pH and temperature), thermal stability, and reusability of the immobilized enzyme on PE films were assayed by using the enzyme-catalyzed reaction. Results show that the polymeric diazonium salt is firmly adhered on the polymer surfaces and the modified surfaces can react with the enzyme to form covalent bonds. The immobilized enzyme shows changes in the optimized pH and temperature for the hydrolysis reaction catalyzed by β-glucosidase. The kinetic parameter (K_m) of the immobilized β-glucosidase is lower than that of its free counterpart. The immobilized enzyme shows significant enhancement in the thermal stability and reasonable reusability. This new approach can be used as a simple and versatile method for protein immobilization.

1. Introduction

In recent years, immobilization of proteins through covalent bonding on solid supports has attracted great attention for applications in catalytic processes, bioseparations, diagnostics, bioprocessing, new disease therapies among others [1–6]. Enzymes have been well-known as highly efficient biocatalysts for a long time [1]. The multipoint covalent immobilization of enzymes on solid surfaces via short spacers can rigidify the structure of immobilized enzymes to enhance the stability. The immobilized enzymes can be easily isolated from the reaction environments, which can significantly improve the reusability and reduce the cost [4,7–14]. Enzyme immobilization provides feasibility for applications in different reaction environments even under some harsh conditions. Various supports have been used for enzyme immobilization, which include inorganic carriers, natural macromolecules, and synthetic polymers [10,11,15–18]. The enzyme immobilization on supports, such as porous glass, silica, iron oxide, chitosan, cellulose, agarose, carrageenan, epoxy supports and various other polymers, has been well documented and applied in diversified industrial divisions [1,19–21].

For protein immobilization, synthetic polymers can show some advantages such as molecular tailorability, biocompatibility, inertness to microbial attack, excellent processibility and enormous available aggregation forms that can be achieved through different processing and selfassembling methods [22,23]. In recent years, polymer supports have gained more attention for applications in biosensors and biochips because of these advantages [24]. The covalent coupling of enzymes to polymeric supports requires some mild chemical reactions between the amino acid residues of proteins and functional groups of polymers [1]. Only few synthetic polymers or copolymers contain such reactive functional groups, like maleic anhydride, methacrylic acid anhydride, and iodoalkylmethacrylates. For most polymers, even those containing hydroxyl, amino, amide, and carboxyl groups, activation of the supports is required for the enzyme immobilization. To activate the surfaces, various chemical reactions have been investigated and some of them have been commercialized [1–3]. In order to immobilize enzymes and proteins to diversified polymeric carriers, a general molecular adhesive that can firmly adhere to the polymeric

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supports and subsequently react with proteins will be an appealing approaching [25]. In this case, the immobilization of proteins only involves sequential depositions in the adhesive solution and in a protein solution [26,27]. However, reports concerning polymeric adhesive for protein immobilization are still lacking in the literature.

Diazonium salts (DASs) have been widely used as reagents in various organic reactions [28]. The electrophilic substitution of DASs has been used for preparing various dyestuffs and pigments through azo-coupling reactions. The diazonium groups can activate aromatic nucleus through the strong conjugative electron-withdrawing effect, which can then be attacked by nucleophilic reagents. In recent years, the reactions of diazonium salts with carbon nanotube, graphite, metals and others have been intensively investigated [29–34]. Polymeric diazonium salts have been used in the image technologies and microlithography by exploiting the photolytic and coupling reactions [35]. For protein immobilization, the polystyrene-based carriers have been prepared through nitration and reduction to introduce aromatic amino groups on the surfaces. The supports containing the aromatic amino groups are activated through diazotization, which can form azo linkages with phenol (L-tyrosine), imidazole (L-histidine) and other groups of proteins [1]. Although the reactions of DAS with different surfaces have been widely explored, to our knowledge, no systematic study concerning the function of polymeric DAS as polymeric adhesive for enzyme immobilization has been reported.

In this work, the function of polystyrene-based diazonium salt (PS-DAS) as a polymeric adhesive for enzyme immobilization was investigated. β-Glucosidase was used to demonstrate the immobilization process and the effects. β-Glucosidase is well-known as a biocatalyst for the cleavage of glycosidic bonds in oligosaccharides or glycoconjugates. By reversing the normal hydrolytic reaction, it has been used in the synthesis of glycosyl bonds between different molecules [36,37]. β-Glucosidase have many applications in biotechnology and food technology due to its ability of bioconversion of lingocellulosic feedstocks to fuel grade ethanol [38,39]. The synthetic activity of β-glucosidase can be used in the preparation of oligosaccharides and glycoconjugates which has potential use in drugs [40]. Immobilized β-glucosidase can be expected for uses in large-scale processes requiring catalyst recycling and continuous operation. The current research shows that PS-DAS can tightly adhere to surfaces of a variety of polymers such as polyethylene (PE), polypropylene (PP), and poly(ethylene terephthalate) (PET). The modified surfaces show excellent ability to immobilize β-glucosidase just through simple deposition in the enzyme solution. Using this immobilization method, no reactive groups or specific activation processes are required for the polymers, which is a versatile approach for different polymeric surfaces. The experimental details, results and discussion will be presented in the following parts.

2. Experimental section

2.1. Materials

Styrene was purchased from commercial source and purified through distillation under reduced pressure before use. 3-Nitrotoluene was purchased from Sinopharm Chemical Reagent Co. Ltd (China). β-Glucosidase from almonds was obtained from Fluka (USA) in the form of lyophilized powder. *p*-Nitrophenyl-β-D-glucopyranoside (*p*NPG) was purchased from Applichem (Germany). Low density polyethylene (LDPE) (1I2A-1, MI = 2 g/10 min) and polypropylene (PP) (B200, MI = 0.55 g/10 min) as commercial products were purchased from Beijing Yanshan Petrochemical Company (China). PE and PP films were prepared by conventional extrusion method, which possess smooth surfaces with thickness about 0.02 mm and 0.2 mm, respectively. Optical grade films of poly(ethylene terephthalate) (PET) (UX-188, thickness 0.188 mm) were supplied by Chemical&Electronic Chemical Material Manufacturer (Taiwan). The films used for immobilization were cleaned first by sonication in ethanol for 30 min and then washed twice in deionized water before use. Deionized water (resistivity > 18MΩ cm) was obtained from a Milli-Q water purification system. Other chemicals and materials used in this study were commercially purchased and used without further purification.

2.2. Characterization

The UV–vis spectroscopy was recorded by a Perking-Elmer Lambda Bio-40 spectrophotometer. ^{1}H NMR spectra were obtained on a JEOL JNM-ECA300 NMR spectrometer. The molecular weights and their distributions of the polymers were determined by a gel permeation chromatography (GPC) apparatus at room temperature with THF as eluent (1 mL/min). The instrument was equipped with a refractive index (RI) detector (Wyatt Optilab rEX) and fitted with a PLgel 5 μm mixed-D column. The column was calibrated with linear polystyrene standards. Elemental analyses were performed by using an ELEMENTAR Vario EL III Element Analyzer. Surfaces were analyzed both before and after enzyme immobilization by a PHI-5300/XPS instrument (Al/Mg excitation, 45°). The surface morphologies were observed with an atomic force microscope (AFM, Nanoscope IIIa, tapping mode). Silicon tips with the tip curvature radius less than 10 nm were used for the observations. The surface hydrophilicity was characterized with a Dataphysics OCA-20 contact angle instrument. The water contact angles were obtained from CCD images of the water droplets by the equipped software. The droplet sizes were 4 μL and the measurement was carried out in the air-ambient condition.

2.3. PS-DAS synthesis

2.3.1. Polystyrene (PS)

Styrene (50 mL, 0.437 mol) and toluene (50 mL) were added into a polymerization reactor with stirring. Benzoyl peroxide (BPO) (2.2 g, 9.08 mmol) was added into the solution as the initiator. The polymerization was carried out at 80 °C for 7 h under reflux with vigorous stirring. After that, the mixture was poured into 200 mL ethanol. The precipitate was collected by filtration, washed thoroughly with ethanol and dried. White solid powder (24.6 g) was obtained as the product. Yield: 54%. M_n = 12,200, M_w = 22,800, M_n/M_w = 1.87.

2.3.2. Poly(4-nitrostyrene) (PS-NO_2)

PS-NO_2 was prepared through the nitration reaction of PS. PS (4 g) was dissolved in 3-nitrotoluene (40 mL), which was kept at a constant temperature (15 °C). Nitric acid (65% (wt.%), 32 mL) and sulfuric acid (98% (wt.%), 8 mL) were added dropwise into the solution under vigorous stirring. The nitration reaction was carried out at 30 °C for 12 h and then the obtained mixture was transferred into a separating funnel. After the phase separation had occurred, the reddish brown oil phase in the upper layer was separated and gradually added into 100 mL isopropanol under stirring. The yellow precipitate was collected by filtration, repeatedly washed with isopropanol, and dried in the vacuum oven for 48 h at the temperature of 50 °C. Yellow powder (4.6 g) was obtained as the product. Yield: 80%. EA (%): C 64.13, H 4.78, N 9.32. ^{1}H NMR (DMSO-d_6, δ ppm): 1.61 (PS chain CH, CH_2, 3H, br), 6.82 (CH, 2H, br), 7.81 (CH, 2H, br). IR (KBr, cm^{-1}): 3076, 2931, 2854, 1597, 1518, 1452, 1348, 1182, 1111, 1014, 856, 750, 702, 540. UV–vis (DMF): λ_{max} = 279 nm.

2.3.3. Poly(4-animostyrene) (PS-NH_2)

PS-NH_2 was prepared through the reduction of PS-NO_2 by using Tin powder as catalyst. Tin powder (8 g) was added into

hydrochloric acid (45 mL) and refluxed with stirring at 60 °C. PS-NO_2 (2 g) dissolved in *N*, *N*-dimethylformamide (25 mL) was added dropwise into the above solution with vigorous stirring. The reaction was carried out at 100 °C for 24 h and the solution was poured into 400 mL ethanol. The oily precipitate at the bottom was collected and dissolved in 40 mL methanol. Then, the solution was added dropwise into 120 mL ethanol. The white precipitate was collected by filtration, washed with ethanol for several times, and dried in vacuum at 25 °C for 3 h. White powder (1.134 g) was obtained as the product. Yield: 71%. EA (%): C 51.48, H 6.14, N 6.68. ^{1}H NMR (D_2O, δ ppm): 1.30 (PS chain CH CH_2, 3H, br), 6.45 (CH, 2H, br), 6.84 (CH, 2H, br). UV–vis (H_2O): $\lambda_{max} = 290$ nm.

2.3.4. Polystyrene-based diazonium salt (PS-DAS)

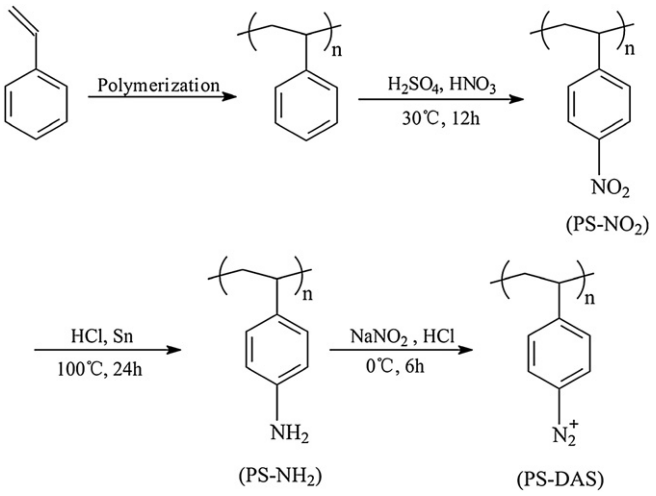
The PS-NH_2 powder (1.134 g) was dissolved in deionized water (22.7 mL) and the solution was stored in a refrigerator at 4 °C before this step reaction. Hydrochloric acid (0.3 mL) was added into the aqueous solution of PS-NH_2 (2 mL). The solution temperature was controlled to be 0 °C with ice-bath cooling. Sodium nitrite (0.12 g) dissolved in water (1 mL) was added dropwise into the PS-NH_2 solution. The reaction was carried out at 0 °C with stirring for 6 h. PS-DAS obtained as an aqueous solution was stored in a refrigerator at 4 °C before it was used for enzyme immobilization.

2.4. Enzyme immobilization

The β-glucosidase solution was prepared by dissolving the enzyme (3 mg) in 0.2 M phosphate buffer (10 mL, pH 7.0). The immobilization processes were carried out by using PE, PP and PET films as supports. Typically, a piece of the polymer films (1 cm^2) was first dipped into the PS-DAS solution (about 0.25 mmol/mL) with stirring at 4 °C for 100 min. Then, the film was washed several times with deionized water and dried by air-stream blowing. After that, the film was dipped into the solution of β-glucosidase (5 mL, 0.3 mg/mL) at 4 °C for 10 h. The film was washed with 0.2 M phosphate buffer (pH 7.0) until no protein was detected in the washings. The film was properly dried and kept in a refrigerator at 4 °C before the assays.

2.5. Enzyme activity and stability assays

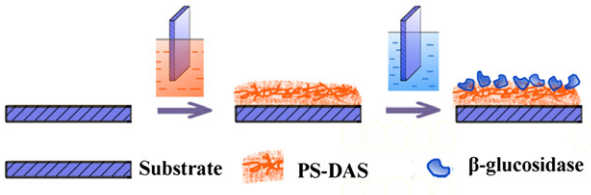
The activity of immobilized β-glucosidase was determined by measuring the hydrolysis conversion of *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) catalyzed by the enzyme [41]. A piece of the polymer films (PE films in a typical case) with immobilized β-glucosidase was immersed in 1 mL *p*NPG solution (10 mm *p*NPG in 0.2 M phosphate buffer, pH 7.0) at 40 °C for 4.5 min and then 1 M sodium carbonate solution (2 mL) was added. The hydrolysis conversion was determined by detecting the absorbance of the solution at 405 nm (the absorbance of *p*-nitrophenyl) with reference to a working curve that was obtained from the standard *p*-nitrophenol solutions. One enzyme unit is defined as the amount of biocatalyst for liberating 1 μmol of *p*NPG per minute in the conditions mentioned above. These activity assays were carried out over the pH range 4.0–8.0 and temperature range 30–50 °C to determine the pH and temperature profiles of the reactions using PE films as support. The initial rate of enzymatic reaction as a function of *p*NPG concentration was determined for immobilized enzyme on PE films over different substrate concentrations. Parametric identification of Michaelis–Menten constants (K_m) and maximum velocity (V_{max}) of free and immobilized enzyme were used to characterize the initial reaction velocity. For thermal stability evaluation, the immobilized β-glucosidase on PE films was kept at 60 °C for different time periods. The activities measured after different time intervals were used to characterize the thermal stability of the enzyme. For comparison, the properties of the β-glucosidase solution (0.2 M phosphate buffer, pH 7.0) were also obtained by similar methods and procedures. The protein concentration in the solution before and after enzyme immobilization was determined by the Bradford method using BSA as standard protein, the enzyme immobilization capacity was defined as the amount of protein (μg) per square centimeters of the polymer films [42]. All experiments mentioned above were carried out at least twice to ensure the reproducibility.



Scheme 1. Synthetic route of PS-DAS.

3. Results and discussion

In this study, polystyrene-based diazonium salt (PS-DAS) was used as a polymeric adhesive for enzyme immobilization. PS-DAS was synthesized through the nitration, reduction and diazotization steps (Scheme 1) [43]. PS was prepared through radical polymerization in solution and had the number average molecular weight (M_n) of 12,200 with the polydispersity index of 1.87. PS-NO_2 was obtained through the nitration of PS with a mixture of nitric acid and sulfuric acid using 3-nitrotoluene as the solvent. The ^{1}H NMR analyses indicates high conversion of the reaction as the resonance of the protons at the *p*-position of the benzene rings totally disappears after the reaction. The degree of functionalization is nearly 100% calculated from this data. The reduction of PS-NO_2 was carried out by using Tin/HCl catalytic system [44]. The degree of functionalization of the amino group is nearly 100% calculated from the peak area of the ^{1}H NMR spectrum. The product PS-NH_2 with a suitable solubility in water was used to prepare PS-DAS through the diazotization. PS-DAS as an aqueous solution could be stably stored at low temperature (such as 4 °C) for at least 72 h. We found in this study that PS-DAS possesses a very strong adhesion to different polymeric supports and the adsorbed PS-DAS maintains the ability to react with protein at the same time. Therefore, the



Scheme 2. Sketch illustration of enzyme immobilization using PS-DAS as molecular adhesive.

Table 1
XPS elemental concentrations of pristine PP film and PP surfaces after PS-DAS deposition and enzyme immobilization.

Film	C_{1s} (%)	N_{1s} (%)	O_{1s} (%)
PP	93.25	0.29	6.46
PP with surface PS-DAS layer[a]	86.45	1.57	11.97
PP with immobilized enzyme	72.28	7.83	19.89

[a] Before testing, the film was dried and stored in air for two days.

enzyme immobilization can be carried out simply by dipping the surface-modified polymeric carriers in the enzyme solutions. The immobilization of enzyme on polymer surfaces involves preparing active PS-DAS layers on the polymer surfaces and coupling reaction between PS-DAS and enzymes (Scheme 2). In the following sections, the results obtained from β-glucosidase immobilization study will be presented in detail.

3.1. Adsorption of PS-DAS on polymer surfaces

The adsorption of PS-DAS was carried out by dipping a piece of the polymer films into the PS-DAS solution at 4 °C for 100 min. In order to characterize the surface modification, the films obtained through the deposition and properly drying were characterized by X-ray photoelectron spectroscopy (XPS), contact angle measurement, and atomic force microscopy (AFM). The results were compared with those obtained from the pristine polymer films under the same condition. The adsorption of PS-DAS did not show distinction for the PE, PP and PET surfaces. As the rigid PP films and smooth-surfaced PET films are more suitable for surface characterization, the results are given here for further discussion. Table 1 gives the XPS data of a pristine PP film and the PP film treated with the PS-DAS solution. The pristine PP film shows the characteristic C1s peak (Binding Energy = 284.7 eV) and the O1s peak (Binding Energy = 532.7 eV). The O1s peak is due to the oxygen surface contamination arising from manufacture and processing. After dipped in the PS-DAS solution for 100 min, dried and stored in air for two days, XPS survey of PP film shows the characteristic peak of N1s (Binding Energy = 400.1 eV) in addition to the C1s and O1s peaks. The N1s peak is attributed to the nitrogen atoms in PS-DAS. The water contact angles of pristine PET and PP films are 61.4° ± 3.7° (Fig. 1(a)) and 91.4° ± 1.2° (Fig. 1(e)). After the adsorption of PS-DAS, the contact angles change to 72.8° ± 3.6° (Fig. 1(b)) and 75.3° ± 0.8° (Fig. 1(f)). The contact angle changes indicate that PS-DAS has adsorbed on the polymer surface. After stored in air for two days, the contact angles of the films change to 88.1° ± 2.8° (Fig. 1(c)) and 92.4° ± 1.4° (Fig. 1(g)). This result could be caused by the slow decomposition of the diazonium groups of PS-DAS under the conditions. Therefore, the nitrogen content given in Table 1 should be understood as the contribution from the remaining diazonium groups. Fig. 2 shows some representative AFM images of the PET films. Obvious morphology change can be seen by comparing the topographical images of the pristine PET film (Fig. 2(a)) with the PET film covered with PS-DAS layer (Fig. 2(b)).

Above results and those obtained from the other polymer films all indicate that the PS-DAS molecules can adhere to the polymer film surfaces. This point can be further confirmed by the protein immobilization study reported in the following sections. In recent year, the covalent bonding of low-molecular-weight diazonium salts on different surfaces has been intensively investigated, which covers metals, semiconductors, oxides, and polymers [45–48]. Although the adsorption of polymeric diazonium groups on the polymer surfaces has rarely been reported, the strong interaction of PS-DAS with the surfaces can be understood by considering the high density of the diazonium groups in its side-chain positions.

3.2. Enzyme immobilization

Using the polymer films treated with the PS-DAS solution, the enzyme immobilization can be simply carried out by dipping the films in the enzyme solutions. The XPS elemental concentration of the enzyme-immobilized PP surface is given in Table 1. After deposition in β-glucosidase solution and dry, N1s peak (Binding Energy = 400.1 eV) of the XPS analysis shows a significant increase in the nitrogen content resulted from the immobilized enzyme. The immobilization of β-glucosidase on the polymeric films is also evidenced by the change of the contact angle of the water droplets on the surfaces. After enzyme immobilization, the water contact angle of PET and PP are 58.0°± 1.4° (Fig. 1(d)) and 67.5°± 0.5° (Fig. 1(h)). The surfaces become more hydrophilic after the enzyme immobilization. Fig. 2(c) shows a typical topographical AFM image of a piece of the PET films after enzyme immobilization. The morphology change can be perceived by comparing it with the films before the enzyme immobilization. Protein concentrations in the solutions before and after enzyme immobilization were estimated by the Bradford method using BSA as standard protein. The amounts of enzyme immobilized on the PE, PP, and PET films were calculated to be 4.76 μg/cm^2, 3.11 μg/cm^2 and 5.00 μg/cm^2. The difference of the water contact angles on the enzyme-immobilized PET and PP films could be attributed to the relatively higher immobilization amount on the PET surfaces.

To understand the adsorption dynamics, the relationship between the dipping time and the relative activity of immobilized enzyme was investigated. The PE films were used as a typical specimen to carry out the experiment. In the process, several pieces of the PE films were first dipped into the PS-DAS solution for

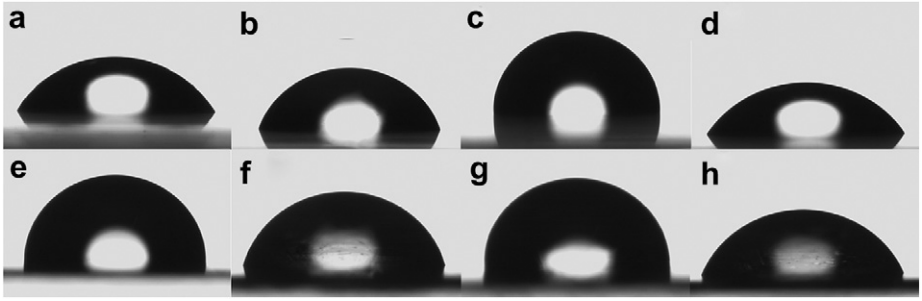


Fig. 1. CCD images of the water droplets on different surfaces: (a) pristine PET film; (b) PET film with PS-DAS surface layer; (c) PET film with PS-DAS surface layer, after stored in air for two days; (d) PET film with immobilized enzyme; (e) pristine PP film; (f) PP film with PS-DAS surface layer; (g) PP film with PS-DAS surface layer, after stored in air for two days; (h) PP film with immobilized enzyme.

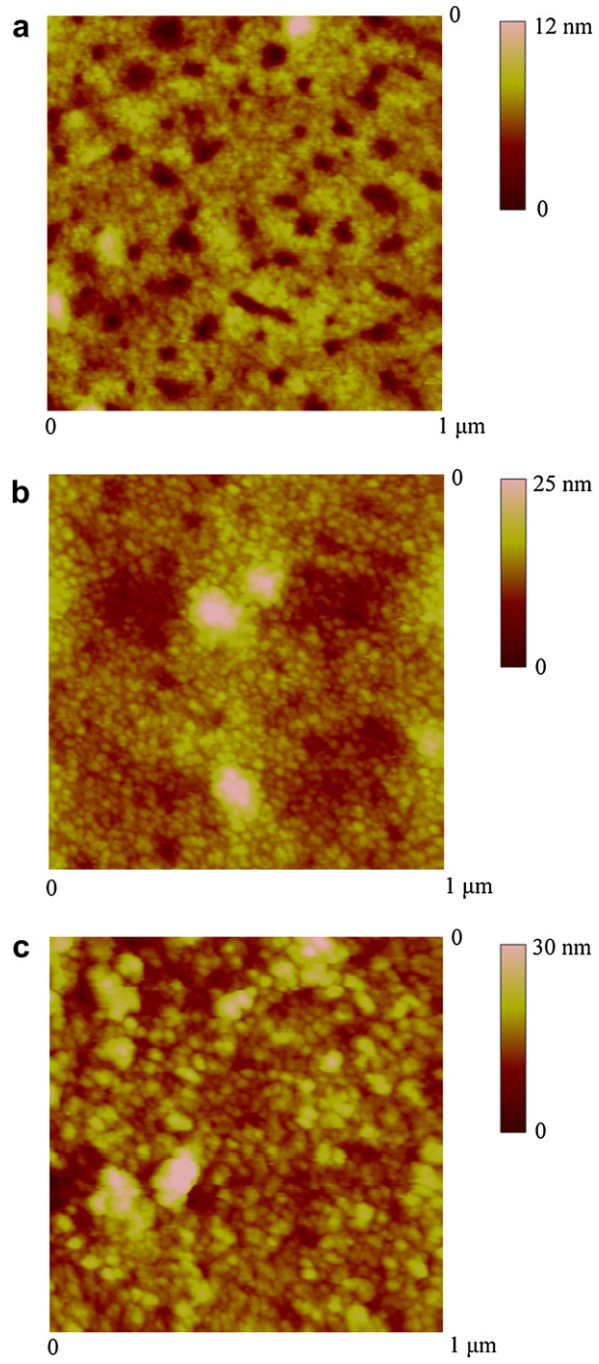


Fig. 2. Typical AFM images of the surfaces: (a) pristine PET film, (b) PET film with PS-DAS surface layer, (c) PET film with immobilized enzyme.

100 min to ensure the saturated adsorption of PS-DAS. Then, the PE films after washing and drying were dipped into the enzyme solution for different time periods. Fig. 3 shows the relative activity of the immobilized β-glucosidase on the PE film as a function of the dipping time. It can be seen that the relative activity increases as dipping time increases and it needs about 150 min to reach the saturation. It takes relatively longer time to reach the saturation compared with simple physical adsorption. This point can give some clue for the possible immobilization mechanism, which will be discussed in the following sections. To ensure the completion of the immobilization reaction, the deposition time of 10 h was used in this work for the following study.

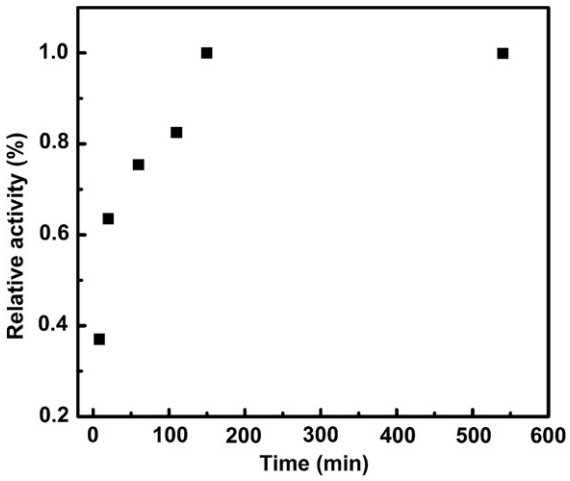


Fig. 3. Relationship between the dipping time and enzyme loading amount on PE film.

3.3. Properties of immobilized enzyme

The enzymatic activity of the immobilized β-glucosidase on supports was determined by measuring the hydrolysis conversion of *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) catalyzed by the enzyme. The enzyme units on supports were calculated to be 0.126 U/cm^2, 0.124 U/cm^2 and 0.207 U/cm^2 for the PE, PP and PET films. By using the immobilized amounts estimated from the Bradford method, the activity of immobilized enzyme was calculated to be 26.5 U/mg, 39.9 U/mg, and 41.4 U/mg for PE, PP and PET films, which is smaller than that of free enzyme (>50 U/mg). The differences in loading capacity and activity of the immobilized enzyme can be attributed to the different surface properties such as roughness and hydrophilicity of these three types of polymeric supports. As a control, after dipped in β-glucosidase solution for 10 h, the polymeric supports that were not pre-treated with the PS-DAS solution did not show enzymatic activity in the same assay process.

The influence of pH values and temperatures was studied by measuring the relative activity of the free and immobilized β-glucosidase on the PE film under different conditions. The pH of the reaction solution shows a significant influence on the enzyme activity (Fig. 4). The optimized pH value is about 7 for the free β-glucosidase. For β-glucosidase immobilized on PE films, it shows

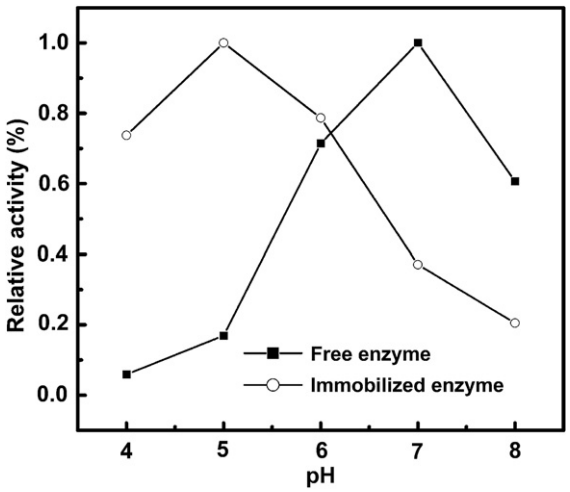


Fig. 4. Relationship between the solution pH and the enzymatic activity of the free and immobilized β-glucosidase on PE film.

highest enzymatic activity in the solution with pH about 5. The observation suggests a significant alteration of enzyme microenvironment upon immobilization on the PE surface. The change of the optimized pH could be caused by a relatively lower proton concentration near surface than that in bulk solution, which is related with the electric double layers near the PS-DAS surfaces. However, the exact explanation for this phenomenon is still unclear at the current stage. Fig. 5 shows the effect of temperature on the activity of the free and immobilized β-glucosidase when pH of the solution is 7.0. It can be seen that after the immobilization, the temperature for β-glucosidase to show highest enzymatic activity increases from about 40 °C for free enzyme to 45 °C for the immobilized enzyme. This could be attributed to the increased stability of the immobilized enzyme, where the conformational change was restricted by the supports. As the density of functional groups in PS-DAS is rather high, the enzyme could be immobilized on the supports through multipoint covalent bonds which could greatly enhance the stability of the enzyme [7–9]. On the other hand, the substrate was more difficult to approach the immobilized enzyme through diffusion. A higher reaction temperature could enhance the substrate diffusion. For other polymeric carriers, the immobilized β-glucosidase also shows the optimized activity at a higher reaction temperature in comparison with the free enzyme [49].

The immobilized β-glucosidase shows a significantly improved thermal stability. The thermal stability of the free and immobilized β-glucosidase on the PE film is characterized by comparing the residue enzymatic activity measured after set at 60 °C for the different time periods (Fig. 6). It can be seen that the immobilization can significantly increase the thermal stability of β-glucosidase. The free β-glucosidase loses about 70% of its initial activity within about 40 min. On the contrary, the immobilized β-glucosidase maintains 70% of its initial activity under the same condition. It has been indicated by the previous study that thermal stability of the immobilized enzyme can be significantly improved through multipoint covalent bonding with the supports [8]. The enhanced thermal stability also evidences the multipoint covalent bonds between the PS-DAS and enzyme molecules.

Kinetic constants of the immobilized β-glucosidase (K_m, and V_{max}) were assayed at different substrate concentrations. The estimated K_m and V_{max} of the immobilized β-glucosidase are 2.58 mM and 0.122 μm/(min cm^2) while the K_m of free β-glucosidase is 9.89 mM. The reduced K_m value can be attributed to the strong binding of the enzyme on the substrates, which could limit the molecule collision

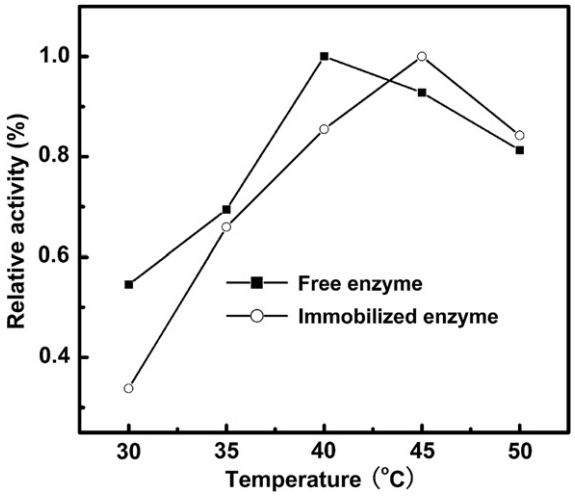


Fig. 5. Relationship between the temperature and the enzymatic activity of the free and immobilized β-glucosidase on PE film.

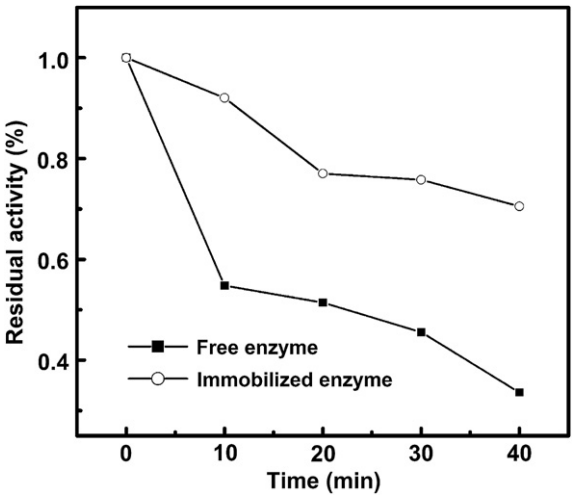


Fig. 6. Thermal stability of the free and immobilized β-glucosidase on PE film at 60 °C.

and reduced the rate to form the enzyme–substrate complex. The lower K_m is consistent with a previous report about the property of β-glucosidase immobilized on smectite clays [49].

3.4. Stability and reusability of the immobilized β-glucosidase

To evaluate the adhesive stability of PS-DAS on polymer supports, two pieces of PE films were first dipped into the PS-DAS solution for 100 min, and then were immersed in the enzyme solution for 2 h and 18 h, respectively. After carefully washed and dried, the activity of the immobilized enzyme for these two supports didn't show obvious difference. This result indicates that when the adsorption is saturated, the immobilized enzyme cannot be washed away in the processes. One of most significant advantage of the enzyme immobilization is the reusability, which can significantly reduce the cost in the real industrial processes. To evaluate the reusability of the immobilized enzyme obtained by the above method, the enzymatic activity of β-glucosidase immobilized on PE films was assayed after repeatedly washing with phosphate buffer (0.2 M, pH 7.0). After each washing and drying step, the PE films were immersed into a fresh *p*NPG solution and the catalytic activity was evaluated. Fig. 7 shows the enzymatic activity of the immobilized β-glucosidase up to 8 washing cycles. After each cycle, the activity of the immobilized β-glucosidase decreases gradually. The decrease is due to the inactivation of the enzyme caused by the gradual protein denaturation and leakage of protein from the PE surface [50]. After 8 cycles, the residual activity of the immobilized enzymes is about 70%. For the immobilized β-glucosidase, the reusability achieved is comparable with other covalent-binding immobilization methods [1].

3.5. Discussions

Diazonium groups have been used for enzyme immobilization for years. In those reported processes, the diazonium groups are introduced into the polymeric supports through diazotization reaction, which is only suitable for polymers containing aromatic amino groups on surfaces. Typically, macroporous or macroreticular resins of polystyrene are used as the carriers and the protein immobilization needs nitration, reduction, diazotization and azo-coupling steps. Distinct from the conventional method, PS-DAS is used in this work as a polymeric adhesive and the diazonium groups are introduced through the PS-DAS adsorption on polymeric supports. This new method requires neither the pretreatment of

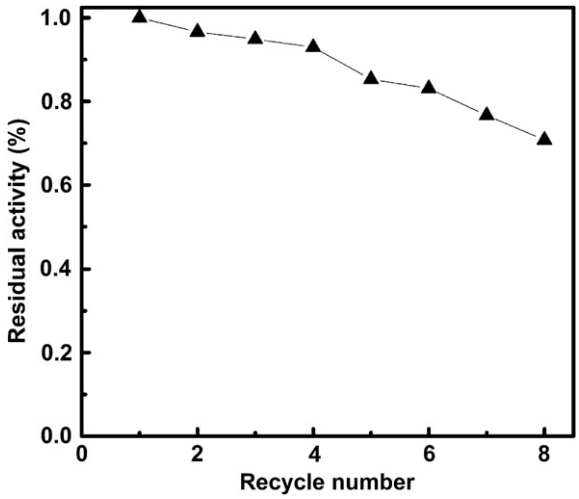


Fig. 7. Reuse stability of the immobilized β-glucosidase on PE film.

polymer surfaces nor the existence of active groups on polymer surfaces. It is well-known that most polymers provide a chemically inert surface, which is the major drawback for enzyme immobilization applications. There are four concurrently occurring factors involved in the modification of the surface: introduction of functional groups, cross-linking of near-surface groups, degradation of polymer molecules, and etching of the surfaces [51]. However, it has also been reported that the modified surfaces are unstable for storage [52]. The present study reveals that PS-DAS can be used for the protein immobilization through the simple two-step deposition process. Surface activation is realized by adsorption of PS-DAS on the polymer surfaces and the modified surfaces can interact with enzymes to achieve the stable immobilization.

The properties of the immobilized β-glucosidase, such as the optimized pH value and temperature range, the reduced reaction rate, the increased thermal stability and reusability, all indicate that the enzyme molecules have been firmly immobilized on the polymer supports. The interaction between PS-DAS layer and protein can be attributed to the covalent bonding between PS-DAS and β-glucosidase, which are formed through the azo-coupling reaction between the diazonium salts and the phenol (L-tyrosine), imidazole (L-histidine) and other groups of the enzyme [1]. Such reaction, which has been well documented in the literature, is the main cause for the enzyme immobilization observed in the current studies. On the other hand, although this study shows that PS-DAS can be firmly adsorbed on a variety of polymeric surfaces, the nature of this strong interaction is still not fully understood. In recent years, many reports show that diazonium groups are very reactive to different surfaces such as carbon, metals and semiconductors [45–48]. In most of the cases, the formation of chemical bond has been confirmed. However, for the tight adhesion of PS-DAS on polymer surfaces reported here, whether chemical reactions occur at polymer surfaces will need further investigation.

4. Conclusions

This study demonstrates a new approach for enzyme immobilization on polymeric supports, which is characterized by using a polymeric diazonium salt as polymeric adhesive. The immobilization of β-glucosidase on the polymer surfaces was achieved by sequential depositions in the PS-DAS and the enzyme solutions. The surface adsorption was characterized by XPS, AFM, and the water contact angle measurement. Significant surface modifications were indicated by the analyses. The activity of immobilized enzyme was measured to be 26.4 U/mg, 39.8 U/mg, and 41.4 U/mg for those immobilized on the PE, PP and PET films. The immobilized β-glucosidase shows some changes in the optimized temperature and pH for the hydrolysis reaction as well as a decreased initial reaction rate in comparison with that of its free counterpart. The immobilized enzyme showed the significantly improved thermal stability and reasonable reusability. The method developed in this work offers a simple way for protein immobilization, which could be used for industrial applications.

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