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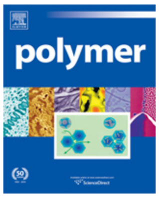

Polystyrene-based diazonium salt as adhesive: A new approach for enzyme immobilization on polymeric supports

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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): $\lambda_{max} = 279$ 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