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Poly(hydroxyethyl methacrylate-co-methacryloylamidotryptophane) nanospheres and their utilization as affinity adsorbents for *porcine pancreas lipase* adsorption

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ABSTRACT

Novel nanospheres with an average size of 350 nm utilizing *N*-methacryloyl-(L)-tryptophane methyl ester (MATrp) as a hydrophobic monomer were prepared by surfactant free emulsion polymerization of 2-hydroxyethyl methacrylate (HEMA), (MATrp) conducted in an aqueous dispersion medium. MATrp was synthesized using methacryloyl chloride and (L)-tryptophane methyl ester. Specific surface area of the non-porous nanospheres was found to be 1902.3 m^2/g. poly(HEMA–MATrp) nanospheres were characterized by Fourier Transform Infrared Spectroscopy (FTIR) and scanning electron microscopy (SEM). Average particle size and size distribution measurements were also performed. Elemental analysis of MATrp for nitrogen was estimated at 1.36 mmol/g nanospheres. Then, poly(HEMA–MATrp) nanospheres were used in the adsorption of *porcine pancreas lipase* in a batch system. Using an optimized adsorption protocol, a very high loading of 558 mg enzyme/g nanospheres was obtained. The adsorption phenomena appeared to follow a typical Langmuir isotherm. The K_m value for immobilized lipase (16.26 mM) was higher than that of free enzyme (10.34 mM). It was observed that enzyme could be repeatedly adsorbed and desorbed without significant loss in adsorption amount or enzyme activity. These findings show considerable promise for this material as an adsorption matrix in industrial processes.

1. Introduction

Enzyme, immobilized on a polymer surface has many advantage over free enzyme including repeated or continuous use, easy separation of the product from reaction system, easy recovery of enzyme, and enhancement in enzymatic stability [1]. Adsorption of an enzyme onto a solid support is probably the simplest way of preparing immobilized enzymes. However, adsorption is generally not very strong and some of the adsorbed protein will desorb during washing and operation. Thus, immobilization via adsorption requires a strong hydrophobic or electrostatic interaction between the enzyme and support. Adsorption of proteins on the hydrophobic supports depends on Van der Waals interactions between uncharged patches on the proteins and surfaces of the support. The number of hydrophobic amino acid side chains on the protein surface (such as valine, tryptophan, phenylalanine, leucine), the degree of hydrophobicity of the support, and the salt type used and its concentration, the medium pH and temperature affect the hydrophobic interaction [2–6].

Recently, the preparation and applications of nanoparticles have attracted considerable attention because of their unusual physical and chemical properties owing to extremely small size and large specific surface area [7,8]. Nanoparticles provide an ideal remedy to the conflicting issues usually encountered in the optimization of adsorbed enzymes: minimum diffusional limitation, maximum surface area per unit mass and high enzyme loading [9]. Non-porous nanoparticles may meet the above requirements.

Lipase (triacylglycerol hydrolase; EC. 3.1.1.3) is one of the most extensively used enzymes that catalyze the hydrolysis of triacylglycerol to glycerol and fatty acids [10]. Depending on the nature of substrate and reaction conditions, lipases can catalyze a wide range of enantio and regioselective reactions, such as hydrolysis, esterifications, transesterifications, aminolysis and ammonialysis [11]. Also, lipases constitute one of the most important groups of industrial enzymes. This is due to their unique ability to hydrolyze fatty acid ester bonds in aqueous environments and synthesize them in non-aqueous medium. These find diverse applications in fats and oil hydrolysis, food industry, detergent industry, peptide synthesis and pharmaceutical industries [12]. There are a number of reports on lipase adsorption on nanoparticles and they have been well characterized [13–15].

In this work, poly(HEMA)-based nanospheres were produced using 2-hydroxyethyl methacrylate (HEMA) and N-methacryloyl-(L)-tryptophan (MATrp) as hydrophobic monomer. Its application for the adsorption and desorption of porcine pancreas lipase was also described.

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2. Materials and methods

2.1. Materials

Lipase (porcine pancreas lipase, E.C. 3.1.1.3), p-nitrophenyl palmitate (p-NPP), L-tryptophan methyl ester and methacryloyl chloride were obtained by Sigma Chemical Co (St. Louis, MO, USA). 2-Hydroxyethyl methacrylate (HEMA) and ethylene glycol dimethacrylate (EGDMA) were purchased from Fluka AG (Switzerland). Poly(vinyl alcohol) (molecular weight: 100,000, 98% hydrolyzed) was obtained from Aldrich (USA). All other chemicals were of analytical grade and were used without further purification.

2.2. Synthesis of N-methacryloyl-(L)-tryptophan (MATrp)

The following experimental procedure was applied for the synthesis of N-methacryloyl-(L)-tryptophan (MATrp). L-tryptophan methyl ester (5.0 g) and $NaNO_2$ (0.2 g) were dissolved in 30 mL of K_2CO_3 aqueous solution (5%, w/v). This solution was cooled down to 0 °C. Methacryloyl chloride (4.0 mL) was slowly poured into this solution under nitrogen atmosphere and this solution was then stirred magnetically at room temperature for 2 h. At the end of this period, pH of the solution was adjusted to 7.0 and subsequently the solution was extracted with ethyl acetate. The liquid phase was evaporated in a rotary evaporator. The residue (MATrp) was crystallized from ether and cyclohexane.

2.3. Synthesis of poly(HEMA–MATrp) nanospheres

poly(HEMA–MATrp) nanospheres were produced by surfactant free emulsion polymerization. For the synthesis of poly(HEMA–MATrp) nanospheres, the following experimental procedure was applied: 0.5 g of poly(vinyl alcohol) was dissolved in 45 mL of de-ionized water and added to the glass sealed polymerization reactor. Then, 0.6 mL of HEMA, 0.3 mL of EGDMA and 80 μL of MATrp comonomer added this solution and slowly shaked for 30 s. 0.0198 g potassium peroxodisulphate (in 45 mL of water)was added in the reactor and was conducted at 70 °C for 7 h. Polymerization was verified with the occurrence of white color of medium. After completion of the polymerization, the reactor contents were cooled to room temperature. Nanospheres were cleaned by washing with ethanol and water several times to remove the unreacted monomers. For this purpose, the nanospheres were precipitated and collected with the help of a centrifuge at 18000 *g* for 1 h and resuspended in ethanol and water several times. After that poly(HEMA–MATrp) nanospheres were further washed with de-ionized water. Poly (HEMA) nanospheres were produced by same formulation without MATrp comonomer.

2.4. Characterization of poly(HEMA–MATrp) nanospheres

Characterization of poly(HEMA–MATrp) nanospheres was investigated with Fourier Transform Infrared Spectroscopy (FTIR), scanning electron microscopy (SEM) and Elemental Analysis. The characteristic functional groups of the poly(HEMA–MATrp) nanospheres were analyzed by using an FTIR spectrophotometer (Varian FTS 7000, USA). For this, dry nanospheres (0.1 g) were mixed with KBr (0.1 g) and pressed into a pellet form. The FTIR spectrum was recorded.

The average bead diameter, size distribution, and surface morphology of the beads were obtained by scanning electron microscopy (SEM, Philips XL-30 S FEG, Netherland). A drop of dilute nanospheres dispersion in ethanol was spread on the glass surface and dried in a dust-free environment at room temperature. The dried sample was then sputtered under vacuum with a thin layer (ca. 4 nm) of gold before viewing under SEM and photographed.

The amount of MATrp incorporation in the poly(HEMA–MATrp) nanospheres was evaluated using an elemental analysis instrument, (CHNS-932, Leco, USA) by nitrogen stoichiometry.

The surface area of poly(HEMA–MATrp) nanospheres was calculated using the following expression [16]:

$$N = 6 \times 10^{10} S / \pi \rho_s d^3$$

Here N is the number of nanospheres per milliliter; S the % solids; ρ_s the density of bulk polymer (g/mL); d is the diameter (nm). The number of nanosphere in mL suspension was determined by utilizing from the mass–volume graph of nanosphere. From all these data, specific surface area of poly(HEMA–MATrp) nanospheres was calculated by multiplying N and surface area of one nanosphere.

2.5. Adsorption of porcine pancreas lipase onto poly(HEMA–MATrp) nanospheres

Adsorption of porcine pancreas lipase on the poly(HEMA–MATrp) nanospheres was performed in a batch experimental set-up. Adsorption experiments were conducted for 120 min at 25 °C with continuous stirring. The effect of pH, temperature and initial lipase concentration on adsorption of lipase onto poly(HEMA–MATrp) nanospheres were investigated. Additionally, lipase adsorption studies were investigated ionic strength range of 0–1.0 M and different salt type [NaCl, $(NH_4)_2SO_4$, Na_2SO_4]. The amount of adsorbed lipase on the nanospheres was determined by measuring the initial and final concentrations of protein. In order to determine the reusability of the poly(HEMA–MATrp) nanospheres, the lipase adsorption and desorption cycle was repeated five times using the same group of nanospheres. Lipase desorption from the nanospheres was performed with 50% of ethylene glycol solution.

2.6. Activity assay of free and immobilized porcine pancreas lipase

The activities of free and immobilized lipase were determined according to the method reported by Ye et al. [17]. In this purpose, reaction mixture containing 14.4 mM p-nitrophenyl palmitate (p-NPP) and 1.0 mL of phosphate buffer saline (PBS) (50 mM, pH 7.5) was prepared and the reaction was started by addition of 0.1 mL lipase preparation. The mixture was incubated at 37 °C under reciprocal agitation at 120 strokes per minute. After 5 min of reaction, agitation was stopped, and then the reaction was terminated by adding 2.0 mL of 0.5 N Na_2CO_3 followed by centrifuging for 10 min (10 000 rpm). 0.5 mL of supernatant was diluted 10-folds with de-ionized water. The amount of released p-nitrophenol was measured by a UV–vis spectrophotometer (UV-1601, Shimadzu, Japan) at 410 nm against a blank without enzyme. The activity for lipase was calculated using a standard calibration curve of p-nitrophenol. One unit (U) of lipase activity was defined as the amount of enzyme necessary to produce 1 μmol of p-nitrophenol per min under the assay conditions. Specific activity was defined as the number of enzyme unit per mg of protein.

These activity assays were performed over the pH range 3.0–8.0 and temperature range 4.0-55.0 °C to determine the pH and temperature profiles for the free and immobilized lipase. The effect of substrate concentration was investigated at p-NPP concentration from 1 to 15 mM.

2.7. Thermal stability

The thermal stability of free and adsorbed lipase were investigated by measuring the residual enzymatic activity at 55 °C in acetate buffer (0.1 M, pH 4.0) for 5 h. After time interval, a sample was removed and assayed for activity. The results were given as activity %.