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Characterization of horseradish peroxidase immobilized on PEGylated polyurethane nanoparticles and its application for dopamine detection

Maurícia B. Fritzen-Garcia[a,b,*], Fabíola F. Monteiro[a], Tatiane Cristofolini[a], José Javier S. Acuña[c], Betina G. Zanetti-Ramos[d], Inês Rosane W.Z. Oliveira[e], Valdir Soldi[f], André A. Pasa[g], Tânia B. Creczynski-Pasa[a,*]

[a] GEIMM, Department of Pharmaceutical Science, Federal University of Santa Catarina, Florianopolis 88040-900, Brazil
[b] Material Science and Engineering Program, Federal University of Santa Catarina, Florianopolis 88040-900, Brazil
[c] Central Laboratory of Electronic Microscopy, Federal University of Santa Catarina, Florianopolis 88040-900, Brazil
[d] Department of Research, Development and Innovation - Nanovetores, Florianopolis 88033-000, Brazil
[e] Department of Chemistry, Federal Rural University of Rio de Janeiro, Seropedica 23890-000, Brazil
[f] Department of Chemistry, Federal University of Santa Catarina, Florianopolis 88040-900, Brazil
[g] LFFS, Department of Physics, Federal University of Santa Catarina, Florianopolis 88040-900, Brazil

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ABSTRACT

In this work, we present a study of peroxidase adsorption on PEGylated polyurethane (PU-PEG) nanoparticles using a purified horseradish peroxidase (HRP). The influence of the immobilization on the catalytic activity of the enzyme was also investigated and the system was applied as a modified carbon paste electrode to dopamine determination in pharmaceutical products. HRP adsorption onto PU-PEG nanoparticles followed the Langmuir isotherm model reaching an adsorption plateau at the concentration of 22.5 μg HRP mg^{-1} nanoparticles. An estimation theoretical calculation was done regarding the number of proteins molecules immobilized per nanoparticle, based on the molecular weight of the protein and the nanoparticles size. It was found that about 4400 HRP molecules were adsorbed to one PU-PEG nanoparticle, which corresponds to about 45% of the surface area of the nanoparticle. Transmission electron microscopy (TEM) performed on immuno-gold labeled samples showed the HRP adsorbed onto the surface of PEGylated polyurethane nanoparticles. HRP-adsorbed PU-PEG nanoparticles dispersions retained 50% of the enzyme activity even after 50 days of storage. Square-wave voltammetry (SWV) experiments were carried out to investigate the performance of the modified carbon paste electrode containing HRP-adsorbed PU-PEG nanoparticles and the analytical curve was linear for dopamine concentrations from 1.7×10^{-5} up to 1.9×10^{-3} mol L^{-1} ($r = 0.9997$), with a detection limit of 2.0×10^{-6} mol L^{-1}. The recovery of dopamine from pharmaceutical samples ranged from 93% to 107% and the results obtained using the modified carbon paste electrode and those obtained by the official method are in agreement at the 95% confidence level.

1. Introduction

Enzymes have a wide range of applications in chemistry, biochemistry, biomedical, pharmaceutical and industrial areas. They exhibit a number of features such as high catalytic efficiency, high selectivity, low toxicity and water solubility [1]. However, these molecules in their free forms have a short lifetime, which limits its biotechnological use. It is important to study methods of enzymes immobilization because it is known that their stability can be improved and thus reducing the amount of material required in many processes. Immobilization also improves the control of the reaction, avoids product contamination by the enzyme (especially relevant in food chemistry) and permits the use of different reactor configurations [2]. In the case of the use of enzymes in the construction of biosensors, one can obtain a higher sensitivity and relative stability of the device [3].

Physical adsorption has been used more frequently as a method for enzymes immobilization [4–11]. The advantages of this method consist in its simplicity and in a wide variety of support materials that can be used for the adsorption. The success and efficiency of adsorption of an enzyme on a support depend on various conditions such as protein size, surface area of the support, porosity, and pore size. The efficiency of immobilization also depends on

* Corresponding authors at: GEIMM, Department of Pharmaceutical Science, Federal University of Santa Catarina, Florianopolis 88040-900, Brazil.
Tel.: +55 48 37212212; fax: +55 48 3234 0599.
E-mail addresses: mgarcia.ufsc@gmail.com (M.B. Fritzen-Garcia), tania.pasa@ufsc.br (T.B. Creczynski-Pasa).

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the enzyme concentration. The amount of enzyme adsorbed on the support increases with increasing concentration of the biocatalyst, reaching a level of saturation. The adsorption process is usually performed at a constant temperature and the obtained adsorption isotherms usually follow prevailing models such as the equations proposed by Langmuir and by Freundlich [12].

The use of nanostructured materials may improve the efficiency of enzyme immobilization because small particles provide a larger surface area, leading to an increased amount of immobilized enzyme per unit mass of particles. Therefore, polymeric nanoparticles are an ideal alternative of nanostructured material for enzymes immobilization and may also offer a minimum diffusion limitation in the case of biosensor applications [13].

Polyurethanes are widely used in many industrial applications and also for the development of biomedical devices or drug delivery systems, due to their excellent physical properties and good biocompatibility [14]. Recently, it was reported the synthesis by the miniemulsion technique of biodegradable polyurethane nanoparticles based on a natural triol monomer (castor oil) [15]. In some of the formulations it was added polyethylene glycol (PEG 400) as co-monomer for hydrophilic and PEGylated nanoparticles. PEG has been extensively used in biomedical applications due to its excellent ability to reduce protein adsorption on the PEG modified surface and superior biocompatibility, with greatly reduced immunogenicity and toxicity. It has been known that steric repulsion between proteins and PEG modified surface plays a major role in reducing the protein adsorption. However, the protein repellant ability of PEG-containing surfaces depends on molecular weight of PEG chain, interfacial PEG chain density and PEG chain architecture [16]. Although PEGylated nanoparticles made by high molecular weight PEG may not be an ideal candidate for immobilization of enzymes because their physical properties, there are evidences in the literature, which indicate that in some cases, depending on the molecular weight PEG does bind to proteins [17].

We demonstrated in a previous work the immobilization of peroxidase obtained from pine kernel on PEGylated polymeric nanoparticles [10]. However, because in that work we used crude extract, i.e., a non purified enzyme as the enzymatic source, it was not possible to study the specific points of the adsorption process such as the amount of adsorbed enzyme per nanoparticle. The main goal of our work is to obtain more data about enzyme adsorption process, in this case, of a known protein on a nanostructured system little investigated. Thus, in this paper, we present a study of the horseradish peroxidase (HRP) adsorption behavior onto PEGylated polyurethane (PU-PEG) nanoparticles. The influence of immobilization on its catalytic activity and the characterization of the adsorption behavior of HRP on PU-PEG nanoparticles were evaluated through adsorption isotherms and transmission electron microscopy images. The enzyme adsorbed on the nanoparticles was used in the construction of a modified carbon paste electrode and the electrochemical properties of the system were evaluated by voltammetric techniques and used for dopamine determination.

2. Experimental

2.1. Reagents, solutions and other supplies

Horseradish peroxidase (HRP; MW ~44,000 g mol^{-1}, 113 U mg^{-1}, EC 1.11.1.7) was purchased from Sigma–Aldrich Co. (St. Louis, USA). Guaiacol (Sigma–Aldrich) stock solution was prepared freshly in 0.1 mol L^{-1} phosphate buffer (pH 6.5) and the hydrogen peroxide used was purchased from Merck Co. (Whitehouse Station, USA). The carbon paste electrodes were prepared using an Acheson 38 graphite powder (Fisher Scientific Inc., Pittsburgh, USA) and mineral oil (Sigma–Aldrich). Bovine serum albumin (BSA) (Sigma Chemical Co., USA), mouse polyclonal to HRP (ABCAM INC, USA) and anti-mouse IgG 10-nm gold particle conjugate (Sigma Chemical Co., USA) were used for the immunolabeling experiments. Other chemicals were of analytical grade and used without further purification. Water used throughout all experiments was purified with the Millipore system (Millipore®, Billerica, Massachusetts, USA) with resistivity not less than 18 MΩ cm.

2.2. Apparatus

The zeta potential measurements were obtained in a Zetasizier Nano ZS (Malvern Instruments, Worcestershire, UK) ZEN 3600 model, equipped with a laser of 633 nm (He–Ne, 4 mW). TEM images were obtained with a JEM 1011 (JEOL Ltd., UK) transmission electron microscope operating at 100 kV. The absorption spectra were recorded on a UV-VIS spectrophotometer (Spectro Vision UV/VIS, China). Electrochemical measurements were carried out on a potentiostat/galvanostat Autolab PGSTAT30 (Eco Chemie, Utrecht, Netherlands) using a conventional three-electrode system with an electrode named modified carbon paste electrode as working electrode, a platinum wire as auxiliary electrode, and a Ag/AgCl/3 M KCl as a reference electrode.

2.3. Preparation and characterization of PU-PEG nanoparticles

The nanoparticles were prepared by miniemulsion technique, as described in a previously work [15]. Briefly, a monomer mixture containing the diisocyanate (isophorone diisocyanate), the natural polyol, poly (ethylene glycol) (PEG) and the hydrophobic agent (olive oil) was added to an aqueous solution containing the surfactant at room temperature. Nanodroplets of monomers were obtained using an Ultra-Turrax® T18 homogenizer at 18,000 rpm for 15 min. The resulting dispersions were maintained under mechanical stirring (800 rpm) at 60 °C for 4 h to allow the complete polyurethane nanoparticle formation. The whole monomer concentration in the polymerization medium was 5 wt %. Tween 80 was employed as surfactant at concentration of 10 wt% (versus monomer). The PEG 400 was added as a co-monomer in substitution to 50 mol% of natural triol. These nanoparticles were characterized by dynamic light scattering (DLS) and the average hydrodynamic radius of the particles was determined using Stokes–Einstein relation and it is equal to 131 nm. The mean particle number density (N_p) was calculated considering the particle mean diameter determined by DLS, solid content in 1.00 mL of dispersion and polymer density as 1.00 g cm^{-3}.

2.4. Adsorption evaluation of HRP on PU-PEG nanoparticles

Different concentrations of HRP (0.5–2 mg mL^{-1}) were added to a phosphate buffer (pH 6.5) containing nanoparticles of PU-PEG to a final concentration of 20 mg mL^{-1}. The mixture was stirred and incubated for 4 h at 4 °C. After incubation, the mixture was centrifuged at 8000 × g for 30 min at 4 °C and the absorbance at λ = 403 nm of the supernatant was measured in a UV–vis spectrophotometer and compared with a calibration curve (see Supporting Information) to determine the concentration of the enzyme that was not adsorbed to the nanoparticles. By subtracting the obtained values from the initial concentrations, the concentration of adsorbed peroxidase $[HRP]_{ads}$, was calculated. Desorption tests were performed by re-dispersing nanoparticles containing the adsorbed enzyme in 1.0 mL of phosphate buffer (pH 6.5). After 24 h, the dispersions were centrifuged at 8000 × g for 30 min at 4 °C and the concentration of free enzyme in the supernatant was determined again spectrophotometrically at 403 nm. The HRP activity was determined before and after immobilization