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# Soybean peroxidase immobilized onto silica-coated superparamagnetic iron oxide nanoparticles: Effect of silica layer on the enzymatic activity

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ABSTRACT

Peroxidase immobilization onto magnetic supports is considered an innovative strategy for the development of technologies that involves enzymes in wastewater treatment. In this work, magnetic biocatalysts were prepared by immobilization of soybean peroxidase (SBP) onto different silica-coated superparamagnetic iron oxide nanoparticles. The obtained magnetic biocatalysts were tested for the degradation of malachite green (MG), a pollutant often found in industrial wastewaters and with significant drawbacks for the human and environmental health. A deep physicochemical characterization of the materials was performed by means of X-ray diffraction (XRD), Fourier Transform Infrared Spectroscopy (FTIR), High Resolution-Transmission Electron Microscope (HR-TEM) and magnetization measurements among others techniques. Results showed high immobilization yield of SBP onto nanomaterials with excellent properties for magnetic recoverability. A partial loss of activity with respect to free SBP was observed, compatible with the modification of the conformational structure of the enzyme after immobilization. The structural modification depended on the amount (and thickness) of silica present in the hybrid materials and the activity yield of 43% was obtained for the best biocatalyst. Thermal stability and reusability capacity were also evaluated.



## 1. Introduction

Peroxidases are able to catalyse the oxidation of a large variety of aromatic compounds by hydrogen peroxide over a wide range of pH, temperature and ionic strength [1,2]. These features set up a great potential for the use of peroxidases in the decontamination of wastewater containing aromatic compounds that are refractory to conventional treatments. In the last years, soybean peroxidase (SBP) has received much attention because it could be easily obtained in large amount, has a high thermal stability and is more resistant to deactivation than other peroxidases [3,4]. SBP is a class III secretory plant peroxidase characterized by a Fe(III)-heme protoporphyrin IX prosthetic group as active site [5]. The catalytic cycle of plant heme peroxidases involves the two-electron reduction of hydrogen peroxide and the one-electron oxidation of two substrate molecules via the well characterized intermediates Compound I and Compound II [6]. Such mechanism allows the oxidation of several inorganic and organic substrates in a broad range of pH with a maximum activity at pH 5 – 6. Recently, Steevensz et al. [3] successfully applied crude SBP to treat alkyd resin wastewater at pilot scale reaching almost total phenol depletion. The main drawbacks of using soluble enzymes in wastewater treatment are the relatively high extraction cost, difficulties in reuse and enzyme short catalytic lifetime due to the inactivation induced by the polymerization process [7]. Enzyme immobilization was demonstrated

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as an effective approach to overcome these limitations, since the supported enzyme can be recovered at the end of the treatment and reused. Moreover, it has been reported that immobilization can increase thermal resistance and activity in organic solvents [8]. Therefore, an efficient immobilization of enzymes on solid supports should ensure enzyme stability in terms of activity, recoverability of the biocatalyst and allow free diffusion of substrates and reaction products. Various strategies for the enzyme immobilization are reported, including mainly physical adsorption and covalent attachment [9,10]. Comparatively, covalent immobilization can eliminate or significantly reduce leaching of peroxidase and can increase stability [11]. Typical biocatalyst supports include polymers, silica, alumina, titania, and other metal oxides that can be separated by conventional separation techniques such as filtration and centrifugation [12–15]. Magnetic nanoparticles (MNPs) have gained considerable attention as biocatalyst supports because of their response to an applied magnetic field. Magnetic separation has emerged as a robust, highly efficient and rapid catalyst separation tool with many advantages compared to biocatalyst isolation by filtration or centrifugation [16]. Magnetic iron oxides such as magnetite ($Fe_3O_4$) and maghemite ($\gamma$-$Fe_2O_3$) are promising candidates for enzyme immobilization as they offer high specific areas, low cost preparation, facility of reuse and high enzyme loading capability [17]. However, magnetic iron oxides nanoparticles are susceptible to air oxidation and are easily aggregated in aqueous systems [11,18]. Therefore, for the application of these nanoparticles as biocatalyst supports, the stabilization of the iron oxide nanoparticles by surface modification is needed. Since the immobilized enzyme onto magnetic nanoparticles has reported some disadvantages, including lowered activity, conformational change of the enzyme and mass transfer limitations [7], controlling the key parameters in the enzymatic immobilization are critical for the design of magnetically recoverable biocatalysts. Several works reported different methods for the immobilization of a variety of enzymes, such as horseradish peroxidase, laccase, urease and serum albumin, onto magnetic supports [11,17,19–21]. However, to the best of our knowledge, only one recent paper studied the immobilization of SBP on magnetic nanoparticles, reaching higher enzymatic activities than free SBP [7].

In the present work we investigated the covalent immobilization of SBP onto different modified magnetic nanoparticles at low enzyme concentration using silica as surface coating to protect magnetic iron oxide core and a functionalized outer coating with (3-aminopropyl) triethoxysilane (APTES). In particular, the effect of the surface coating agents on both the SBP immobilization yield and the enzymatic activity was analyzed. The resulting biocatalysts were characterized by means of several experimental techniques, including FTIR spectroscopy, HR-TEM, XRD, gas-volumetric adsorption of $N_2$ at 77 K and magnetization curves. Finally, the activity and stability properties of immobilized SBP were tested toward the removal of Malachite Green (MG) from aqueous solution. MG, a triarylmethane dye that can cause mutagenic and carcinogenic effects [22], is an effective antimicrobial and antifungal agent in food industry and very used for dyeing wool, silk, cotton, leather, etc. [23].

## 2. Materials and methods

### 2.1. Reagents

Soybean peroxidase (SBP, EC 1.11.1.7) RZ = 2.0 was purchased from Bio-Research Products Inc. (activity 1269 U/mg, lot# SBP-B275P154); glutaraldehyde (25%), tetraethylorthosilicate (TEOS, 98%), 3-aminopropyltriethoxysilane (APTES, 98%), $NH_4OH$ (33%) and $FeCl_3$ (97%) from Sigma-Aldrich, ethanol (99.8%) and $FeSO_4.7H_2O$ (99.5%) from Fluka; Malachite Green from Anedra and $H_2O_2$ (30%) from Cicarelli. All chemicals were used without further purification.

### 2.2. Preparation of magnetic materials

#### 2.2.1. Synthesis of magnetic iron oxide nanoparticles (MNP)

Iron oxide nanoparticles were prepared by co-precipitation method [9] in nitrogen atmosphere using 5.13 g of $FeCl_3$ and 2.67 g of $FeSO_4{\cdot}7H_2O$ solubilised in 100 mL of water. The solution, constantly kept under stirring, was heated at 90 °C and then 10 mL of $NH_4OH$ 30% and 50 mL of water were added. The reaction was kept occurring for 30 min, after that the solution was cooled down at RT and the black solid product was magnetically separated from the supernatant using a magnet. The solid was washed with deionised water four times, dried in a rotavapor at 80 °C and subsequently stored under $N_2$ until use for the syntheses of the different composite materials.

#### 2.2.2. Functionalization of MNP with APTES (NPA)

MNP were functionalized with APTES by a modification of the Keziban procedure [11]. 3.0 g MNP were dispersed in 150 mL of ethanol/water (1:1) solution. The suspension was kept under nitrogen and sonicated for 10 min, after that 11.12 mL of APTES were added and the mixture was stirred for 2 h at 40 °C. The material obtained (NPA) was washed three times with ethanol/water solution and dried in rotavapor at 80 °C.

#### 2.2.3. Synthesis of silica coated MNP with APTES surface modification (NPTAI and NPTAII)

MNP were coated with silica by sequential reactions with TEOS and APTES [9]. In brief, 2 gr of MNP were dispersed in 400 mL of ethanol and sonicated for 10 min. TEOS (2 or 5.5 mL), $NH_4OH$ (30 mL) and $H_2O$ (60 mL) were added and the mixture was kept under stirring for 5 h. After the reaction, the solid was washed with ethanol/water solution and dried in rotavapor at 80 °C. Successively, 2 g of these materials were suspended in ethanol, sonicated for 10 min, and 60 mL of APTES were added during stirring and kept reacting for 2 h at 25 °C and 2 h at 50 °C. Finally, the samples were washed for three times in ethanol/water and dried in rotavapor at 80 °C. The samples prepared with 2 and 5.5 mL of TEOS were named NPTAI and NPTAII, respectively.

#### 2.2.4. SBP immobilization

SBP was immobilized onto the surface of the synthesized materials using glutaraldehyde as spacer [14] (Fig. 1). 1 g of MNP, NPA, NPTAI or NPTAII was introduced in a 100 mL solution of glutaraldehyde (2.5%) in phosphate buffer (0.1 M at pH 7). The mixture was stirred for 1 h in the dark and the products were magnetically separated and washed with the buffer solution twice. Then, each material was incubated with 40 mL of SBP solution $4.96\times10^{-6}$ M in phosphate buffer for 15 h at 4 °C and continuous stirring. Quantification of SBP immobilized was determined from the difference of absorption at 403 nm ($\varepsilon_{SBP,403nm}$ = 96400 $M^{-1}cm^{-1}$ [24]) in the supernatant solution before and after the contact of SBP with the nanomaterials. All the obtained solids were washed with 4 mL of phosphate buffer solution twice. Possible release of SBP from the nanomaterial to the aqueous media during the washing process was monitored by UV–vis spectroscopy. In all cases, no absorption at 403 nm was detected in the washing solution, indicating no release of SBP. Depending on the nanomaterial used for the enzyme immobilization (MNP, NPA, NPTAI and NPTAII), the SBP containing materials were named MNP-SBP, NPA-SBP, NPTAI-SBP and NPTAII-SBP, respectively.