



Artificial enzyme with magnetic properties and peroxidase activity on indoleamine metabolite tumor marker



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ABSTRACT

Stable and easily-handled synthetic materials mimicking natural enzymes activity would find important biotechnological applications. This article describes the synthesis and characterization of magnetic molecularly imprinted catalytic polymers that exhibit peroxidase-like activity towards 5-hydroxyindole-3-acetic acid (5-HIAA) oxidation. This multifunctional material is obtained from highly crystalline magnetite nuclei coated with a silica layer to protect the iron nucleus from oxidation and to provide anchoring for hydroxyl surface groups. After acrylic functionalization via sol–gel process, a molecularly imprinted polymer with heme as catalytic center and 5-HIAA as template has been successfully attached to the structure. The resulting hybrid composite is magnetically separable and possesses excellent catalytic ability for the selective oxidation of the indoleamine metabolite tumor marker, showing Michaelis–Menten kinetics with this molecule but not towards other structural analogs. Therefore, it can be considered an artificial peroxidase enzyme.

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1. Introduction

The design and synthesis of new materials that can mimic natural enzymes has attracted widespread attention of scientists from a variety of disciplines, including sensors development. Among natural enzymes taken as model, heme-proteins are very attractive since the presence of heme (iron (III) protoporphyrin IX) in an appropriate environment provides a great variety of functions such as electron transfer, substrate oxidation or metal ion storage [1]. Heme-peroxidases that convert peroxide to water, are quite abundant in nature and catalyze the oxidation of a range of different substrates, thus opening the door for a number of biotechnological and analytical applications [2]. Several original strategies have been explored to synthesize new materials mimicking peroxidases. Catalytic activity on hydrogen peroxide oxidation of organic compounds can be achieved by incorporating the redox-active heme center to polymeric structures [3]. In this way, an effective supported catalyst can be obtained, with good stability and activity, but lacking substrate and regioselectivity, which are the essential characteristics of natural enzymatic proteins. The use of molecularly imprinted polymers (MIPs) is providing exciting opportunities for enhancing selectivity in this

kind of materials [4,5]. MIPs are obtained by polymerization around a molecule that acts as template and catalytically active MIPs can be evolved by templating with transition state analogs [6], usually incorporating some metallic centers as active groups [7,8]. Alternatively, the substrate can be employed as template [9]. In the particular case of peroxidase-mimicking, the incorporation of heme as a polymerizable monomer along with the substrate as template give the opportunity not only to create a cavity with a defined shape, corresponding to the shape of the substrate, but also to introduce the desired catalytic functionality within this cavity, providing the means to make a biomimetic material, with some degree of chemical selectivity [9,10].

For biotechnological applications, artificial enzymes have to be obtained in a form that facilitates its handling. In this sense, functional magnetic nanocomposites offer innovative prospects [11] as they allow combining their easy manipulation by an external magnetic field with catalytic activity. Some approaches can be found using magnetic particles (MPs) as carriers of porphyrin groups in order to obtain supported catalyst with peroxidase activity [12,13] or to incorporate an enzyme providing hydrolase activity [14]. On the other hand, MPs have been used by the first time combined with an affinity MIP by Mosbach et al., in 1998 [15]. Since then, efforts have been made to obtain magnetic MIPs by using different polymerization techniques, from the classical free radical polymerization (FRP) [16–18] and sol–gel processes [19–21] to the

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newest living radical polymerization such as atom transfer radical polymerization (ATRP) [22–24] and reversible addition-fragmentation chain transfer polymerization (RAFT) [25]. Applying these techniques, various synthetic approaches have been established in order to obtain tailor-made affinity magnetic MIPs [26]. From the simple entrapment of the MPs into the growing imprinted polymer [27] or the conjugation of both types of nanoparticles through a photocoupling process [28] to the core–shell approach where the magnetic material is coated with a layer of the imprinted polymer [29], affinity magnetic MIPs keep on improving step-by-step. However, the employment of a magnetic core in the preparation of a catalytic molecularly imprinted polymer incorporating hemin as catalytic center has thus far not yet been reported.

The present work describes an efficient route for obtaining such material. We have selected as a model substrate an indoleamine metabolite, 5-hydroxyindole-3-acetic acid (5-HIAA), as it plays an important role in diagnostics acting as a biomarker of carcinoid tumors [21]. An imprinted microgel obtained in a FRP process is combined with magnetic nanocomposites prepared in a core–shell approach based on sol–gel processes, giving rise to a new material that is easily handled and effectively replicates peroxidase activity in the oxidation of the biomarker.

2. Experimental section

2.1. Chemicals

Iron (II) sulfateheptahydrate (FeSO_4) ($\geq 99\%$), tetraethyl orthosilicate (TEOS) ($\geq 99.0\%$), toluene (99.9%), 3-(trichlorosilyl)propyl methacrylate (TPM) ($\geq 90\%$), triethylamine ($\geq 99\%$), dimethyl sulfoxide (DMSO) ($\geq 99.9\%$), 4-vinylpyridine (VPY) (95%), azobisisobutyronitrile (AIBN) (98%), 3-indoleacetic acid (IAA) (98%), 5-hydroxyindole (5-HI) (97%), 5-hydroxyindole-3-acetic acid (5-HIAA) ($\geq 98\%$), N-acetyl-5-hydroxytryptamine (NA5-HT) ($\geq 99\%$), 5-hydroxytryptamine (serotonin hydrochloride) (5-HT) ($\geq 98\%$), 5-hydroxy-L-tryptophan (5-HTTP), 5-fluoroindole-3-acetic acid (5-FIAA) ($\geq 97\%$) were purchased from Sigma–Aldrich (USA). 5-hydroxyindole-3-ethanol (5-HTOL), was purchased from Wako (JPN). 5-hydroxyindole-3-acetamide (5-HIA), was purchased from International Laboratory (USA). Potassium nitrate (KNO_3) (99%), potassium hydroxide (KOH), ethylene glycol dimethylacrylate (EGDMA) (98%), ammonium hydroxide (25%), acetic acid (glacial) ($\geq 99.8\%$), acetonitrile (HPLC grade) and perchloric acid (70%) were purchased from Merck. Orthophosphoric acid (85%) was purchased from Fluka. Ethanol (reagent grade) and methanol (99.8%) were purchased from J.T. Baker (USA). Chlorohemin (iron(III)-Protoporphyrin IX) was purchased from Frontier Scientific (USA). Hydrogen peroxide was purchased from VWR International (USA). All solutions were prepared with high purity water produced by a Direct-Q 5 purification system (Millipore). Magnetic separations were carried on with the help of a neodymium–iron–boron (NdFeB) permanent magnet with an axial magnetization of 6200 Gauss (30 kg in each magnet face) (La boutique del imán, Spain). All reagents were used as received with no previous inhibitor removal step.

2.2. Instrumentation

A Genesys 10S UV/Vis Spectrophotometer (Thermo Scientific) was used to monitor the removal of the template and the non-polymerized chlorohemin disappearance from the catalytic polymers. A JEOL-2000 EX-II transmission electron microscope (TEM) and a JEOL-6610LV II scanning electron microscope (SEM) were used to obtain the structural and morphological characterization of the core/shell materials in each functionalization step. Varian 670-IR FT-IR spectrometer (Agilent Technologies) with a diamond ATR

accessory (Golden Gate, Specac) was used to obtain the attenuated total reflectance infrared spectra (ATR-IR) of the synthesized MPs. A Mettler Toledo TGA/SDTA 851E thermogravimetric analyzer (TGA) was used to obtain TGA curves. X-ray powder diffraction analysis (XRD) was carried out using $\text{Cu K}\alpha$ radiation ($\lambda = 1.5418 \text{ \AA}$) at 45 kV on a Philips X'Pert Pro X-ray diffractometer in the 2θ range $10^\circ < 2\theta < 115^\circ$ with 0.03° steps at 298 K. Magnetic measurements were obtained using a Vibrating Sample Magnetometer (VSM), MicroSense EV9 with a maximum magnetic field of 2.2 T (1751 kA/m). High performance liquid chromatography (HPLC) to characterize the catalytic oxidation products was performed by Shimadzu LC-20AD series HPLC, (Kyoto, Japan) equipped with a SPD-M20A UV–VIS photodiode array detector (PDA) and HP 1100 series LC/MS System. A reverse phase Pinnacle DB C18 column (150 mm \times 4.6 mm i.d., 5 μm) supplied by Teknokroma (Spain) was used for separation of all the compounds. Amperometric measurements were performed with an EG&G Princeton Applied Research 400 Electrochemical Detector coupled post-PDA detection with the HPLC system through a NI myDAQ interface connected via USB directly to the computer.

2.3. Polymer synthesis

2.3.1. Synthesis of octahedral Fe_3O_4 magnetic particles (MPs)

8 g FeSO_4 were dissolved in 56 mL highly purified oxygen-free water and heated at 90°C under mechanical stirring in a water bath. Then, 24 mL of an oxygen-free solution containing KNO_3 (646 mg) and KOH (4.49 g) was added dropwise over approximately 30 s. After addition, the mixture was heated for 30 min under N_2 atmosphere until reaction was completed and cooled 1 h in a refrigerator. Finally, the black precipitate was collected with a permanent magnet, washed with highly purified water ($7 \times 50 \text{ mL}$) and ethanol ($4 \times 50 \text{ mL}$) before drying under vacuum. No protection against air oxidation was needed once the reaction occurred.

2.3.2. Preparation of a protective silica layer (MPs/ SiO_2)

Typically, 1 g of Fe_3O_4 was dispersed in ethanol (60 mL) and highly purified water (10 mL) by sonication for 15 min. Ammonium hydroxide (3 mL, 25%) and tetraethyl orthosilicate (TEOS) (6 mL) were added sequentially over the stirred mixture. After reaction in air for 12 h at room temperature, the black product was collected by an external magnet and washed with highly purified water ($6 \times 20 \text{ mL}$) and ethanol ($3 \times 20 \text{ mL}$) by repeated magnetic separation and finally dried under vacuum.

2.3.3. Synthesis of methacrylic functionalized silica layer (MPs/ SiO_2 /TPM)

2 g of MPs/ SiO_2 were dispersed with ultrasonication in a solution of 3-(trichlorosilyl)propyl methacrylate (TPM) (4 mL) in anhydrous toluene (55 mL) for 15 min. Then, a solution of triethylamine (2 mL) in anhydrous toluene (5 mL) was slowly added to the former mixture and stirred for 12 h at room temperature. The resultant black precipitate was collected with a permanent magnet, washed with methanol ($10 \times 20 \text{ mL}$) and dried under vacuum.

2.3.4. Synthesis of a molecularly imprinted catalytic polymer layer (MPs/ SiO_2 /TPM/MIP)

MPs/ SiO_2 /TPM (500 mg), chlorohemin (32.6 mg, 0.05 mmol) as catalytic center, 4-Vpy (26.7 μL , 0.25 mmol) as functional monomer, ethylene glycol dimethylacrylate (EGDMA) (470 μL , 2.5 mmol) as crosslinker, azobisisobutyronitrile (AIBN) as radical initiator (250 mg, 1.52 mmol) and 5-HIAA as template (9.6 mg, 0.05 mmol) were dispersed into a solution of DMSO as porogen (20 mL, 281.6 mmol) in a sealed vial under ultrasonication for 15 min. Nitrogen was bubbled through the prepolymerization mixture for

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