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Design of a lipase-nano particle biocatalysts and its use in the kinetic resolution of medicament precursors

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ABSTRACT

Superparamagnetic iron oxide nanoparticles (Fe_3O_4) were prepared by the co-precipitation method and functionalized with 3-amino-propyltriethoxysilane (APTES) or branched-polyethylenimine (PEI). After that, two parallels methods to immobilize the lipase from *Thermomyces lanuginosus* (TLL) were performed: the first one by ionic exchange and the second one by covalent attachment after the functionalization of the support with glutaraldehyde (GA). X-ray powder diffraction, magnetometry and infrared spectroscopy analysis were used to characterize the TLL preparations. These analyses showed that all samples presented superparamagnetic properties even after the immobilization procedure. The SPMN (superparamagnetic nanoparticle) @APTES covalent preparation had around 450 min of half-life time at pH 7.0 and 70 °C while that of the free enzyme was 46 min. These biocatalysts were evaluated in the kinetic resolution of *rac*-1-methyl-2-(2,6-dimethylphenoxy)ethyl acetate in different co-solvents (acetonitrile, isopropanol, ethyl ether and tetrahydrofuran). The best results were for the enzyme/substrate ratio of 2:1, in the presence of the ethyl ether or THF (20% v/v both) at 30 °C during 24 h with the SPMN@PEI-TLL biocatalyst. The conversion attained was 50% and the enantiomeric excess of the product was 99%. The new SPMN support are an excellent strategy to easy recovery of the biocatalyst by applying a magnetic field.

1. Introduction

Lipases are one of the most used enzymes in biocatalytic processes, with great potential in lipid technology and in the synthesis of enantiomerically pure intermediates [1–3], at academic and industrial level [4]. These enzymes are used in a wide diversity of reaction medium, exhibiting excellent stability and activity in the presence of organic solvents, that may be used to make easier the solubility of the hydrophobic substrate to be modified [2,3].

Microbial lipases have high production costs and when used in its soluble form, it might be unstable in certain reaction conditions [5]. These problems might be overcome by enzyme immobilization before its industrial implementation, which is a simple way to separate the enzyme from the reaction media and to reuse it [5]. If properly performed, immobilization may tuning lipase properties, as activity, selectivity, specificity, resistance to inhibitors and stabilization [6].

One interesting support that may be used to immobilize enzymes are magnetic nanoparticles, which have suitable properties that allow effectively immobilization, because of their high

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specific surface area, besides being easily separated from reaction medium by the use of a magnet [7]. Moreover, the magnetic nanoparticles immobilize enzyme molecules on the surface, which prevents internal diffusional limitations [7]. In addition, other advantages of nanoparticles as supports for the immobilization of enzymes are biocompatibility, non-toxicity and possibility of superparamagnetism (SPM) [7]. When magnetic nanoparticles are synthesized below the critical volume, they can present SPM properties [8]. In this sense, the superparamagnetic nanoparticles (SPMN) are of great interest, since these nanoparticles present magnetic response only when they are in presence of an external magnetic field, ceasing the magnetism as soon as the magnetic field is removed [8] (see Fig. S1). However, they may have some drawbacks, as the lack of protective effects of the immobilization inside a porous system to interaction with external interfaces, but this issue may be solved via appropriate strategies [9].

In this context, the immobilization of *Thermomyces lanuginosus* lipase (TLL) on superparamagnetic iron oxide nanoparticles (Fe_3O_4) coated with polymer functionalizing branched-polyethylenimine (PEI) or 3-amino-propyltriethoxysilane (APTES) was investigated. TLL is thermo stable with high catalytic efficiency, strict enantioselectivity and enantiospecifcity, broad specificity, single chain protein consisting of 269 amino acids with a molecular weight of 31.7 kDa, isoelectric point of 4.4 and a size of 35 Å × 45 Å × 50 Å [10]. This enzyme has been applied in many different industrial areas, such as in the production of biodiesel, detergent, cosmetic, and other organic chemicals [10].

TLL has an isoelectric point of 4.4, therefore, at pH 9 the protein surface will have a strong anionic character. However, it is important to remark that ion-exchange adsorption of proteins is favored if the protein and the support have an opposite net charge but this is not compulsory. Some authors report [11,12] that the positive or negative regions that are available on protein surface are capable of adsorb on the support via multipoint interaction. For example, more than 80% of the proteins contained in crude extracts could be immobilized on PEI [11] coated agarose. Moreover, a large percentage of proteins could be immobilized on mixed cation/anion exchangers, in fact, it was possible to immobilize proteins that were not immobilized in any of the "pure" ion exchangers [12].

PEI has ionizable groups, mainly on its main chain [13]. This cationic polymer has several applications in biological, industrial and pharmaceutical fields [10,13]. PEI has been ascribed to be able to stabilize proteins via different factors, in addition has been observed that the increased lengthened polymer chain results in increasing the immobilization amount of enzyme [11]. APTES is a silane coupling reagent that is extensively employed for biomolecule immobilization to develop biosensors [14]. This reagent is used to create an amino layer on the support, that may be used for biomolecule immobilization [15]. Both structures are quite different, while PEI coating forms an ionic bed where the enzyme may be included [11], APTES only promotes the formation of a flat surface where the enzyme may interact.

In order to introduce a function able to covalently react with the enzyme [14], the resultant surface was next treated with glutaraldehyde [16]. Those aldehyde groups in the support may react with amine groups of the protein to immobilize the enzyme [16,17]. Furthermore, this reagent has a great potential in preparing biocatalysts with an intense multipoint covalent attachment, and may also introduce inter or intra crosslinking that can produce positive effects on enzyme stability [16,18].

Being aware of the fact that biocatalysis offers an alternative to improve the conventional chemical synthesis of chiral enantiomerically pure drugs [1], we investigated the new TLL biocatalysts in the resolution of racemic Mexiletine[1-(2,6-dimethylphenoxy)propan-2-amine] via hydrolytic reactions. An elegant strategy is to combine a biocatalytic step with a conventional chemical route, known as chemoenzymatic synthetic routes [2,3,9]. This enables the production of enantiomerically enriched chiral alcohols, which have high added value and are used as building blocks in the synthesis of various biologically active compounds. Specifically, the chiral alcohol 1-(2,6-dimethylphenoxy)propan-2-ol, when submitted to other reactions with exchange of the hydroxyl group by an amino group, leads to the formation of enantiomerically pure drug (*R*)-Mexiletine[(2*R*)-1-(2,6-dimethylphenoxy)propan-2-amine]. Mexiletine in its racemic form is an antiarrhythmic agent, but in enantiomerically (*R*)-enriched form is a compound able to block the sodium channels and has its activity enhanced [19].

2. Materials and methods

2.1. Materials

The commercial TLL extract (15.83 mg of protein per mL) was obtained from Novozymes (Spain). 6 BCL agarose, 25% (v/v) glutaraldehyde solution, cetyl trimethyl ammonium bromide (CTAB) and *p*-nitrophenyl butyrate (*p*-NPB) were purchased from Sigma Chemical Co (St. Louis, MO, USA). The commercial preparation of TLL covalently immobilized on immobead-150 (TLL, 250 U/g), branched-polyethylenimine (MW 10,000) and 3-amino-propyltriethoxysilane (>98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). $FeCl_3{\cdot}6H_2O$ and $FeSO_4{\cdot}7H_2O$ were supplied by Sigma-Aldrich and Vetec Química, respectively. All others reagents and solvents used were of analytical grade.

2.2. Synthesis of Fe_3O_4 and functionalization with APTES

In the co-precipitation route assisted by ultrasound, metallic salts containing Fe^{2+}/Fe^{3+} were dissolved and mixed in Milli-Q water in the molar ratio of 1:2 to form the spinel phase Fe_3O_4. Briefly, 5.82 mmol of $FeCl_2{\cdot}4H_2O$ and 10.57 mmol of $FeCl_3{\cdot}6H_2O$ were dissolved in a 50 mL of Milli-Q water. The aqueous mixture remained under vigorous ultrasound stirring when 10 mL of a solution of NH_4OH was added drop wise to form the precipitate, according with the proportion 1: 8 (Fe^{n+}/ammonium). The precipitate was washed several times with Milli-Q water until the residual solution became neutral, followed by drying the magnetic nanoparticles.

In the functionalization procedure, 100 mg of magnetite Fe_3O_4 were suspended in a beaker using 20 mL of ethanol and 20 mL of toluene. After that, 100 μL of APTES was added. The reaction system was subjected to vigorous ultrasonic stirring for 15 min at 50 °C. Finally, the nanocomposite formed was washed several times, dried and stored in a desiccator.

2.3. Synthesis of Fe_3O_4 and functionalization with PEI

Synthesis and functionalization of Fe_3O_4 SPMNs were performed in two-steps, using a probe ultrasound (Ultrasonique Desruptor) with frequency of 20 kHz and power of 750 W. Initially, two solutions were prepared. The first was an iron salts solution (Solution A) and the second one, a PEI aqueous solution (Solution B). Solution A was composed of 1.16 g of $FeSO_4{\cdot}7H_2O$ and 1.85 g of $FeCl_3{\cdot}6H_2O$ dissolved in 15 mL of deionized water, whereas, solution B was consisted of 1.0 g of PEI in 4.0 mL of deionized water.

Firstly, the solution A was sonicated for 4 min, until it reaches the temperature of 60 °C. Then, 7.0 mL of concentrated NH_4OH were added, under sonication, using a burette. Thereafter, the color of solution A changed from orange to black, evidencing the formation of Fe_3O_4 SPMNs. After 4 min, solution B was added to the reaction medium, which remained for more 4 min under sonication.