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Efficient retention of laccase by non-covalent immobilization on amino-functionalized ordered mesoporous silica

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ABSTRACT

The present work aims to be a step forward in the synthesis of siliceous ordered mesoporous materials (OMM) as tailor made matrices to optimize the immobilization and stabilization of enzymes. Based on a classic non-covalent adsorption by electrostatic interactions we have developed the syntheses of materials especially designed for this enzyme, in order to optimize the properties of the final biocatalyst. Siliceous materials with a hexagonal arrangement of parallel mesoporous channels (SBA-15 type of structure) have been synthesized, whose pore diameter has been tuned according to the molecular dimensions of laccase. The synthesis conditions used allowed to obtain pore sizes large enough to permit laccase entrance and diffusion through the pore channels. Diffusion of the enzyme is crucial to obtain high immobilization yield since most of the surface area of the particles is the internal surface of the pores. A poor diffusion would involve retention of enzyme molecules in the pore mouths preventing new ones to access the channel and leading to a low enzyme loading of the catalyst. A micelle swelling agent has been used to expand the supramolecular aggregates that generate the pore architecture of SBA-15 silica. The surfaces of the supports were functionalized with amino groups aiming to strengthen electrostatic interactions between support and enzyme at a suitable pH. Two strategies of surface functionalization of the large-pore ordered mesoporous silica materials were followed: (1) anchoring of an amino-functional alkoxysilane on mesoporous silica and (2) direct co-condensation of a silicon alkoxide and an amino-functional alkoxysilane to obtain the functionalized material in one step. The possibility to prepare carriers where each characteristic has been separately studied and optimized has allowed to obtain biocatalysts with optimal properties. Enzyme loading up to 187 mg/g of catalyst and high activities were achieved with the amino-functionalized large-pore supports. Furthermore, immobilization improved enzyme stability in ethanol. Strong binding forces were capable of housing and retaining the enzyme irreversibly, fully preventing leaching in aqueous medium. Through the careful design of the support material, the biocatalysts obtained share the advantages of enzyme-support covalent attachment regarding absence of leaching and stability, while avoiding drawbacks like loss of activity and enabling the reuse of the support.

1. Introduction

The field of ordered mesoporous materials (OMMs) has been progressively growing in the last two decades, and a wide variety of new different types of materials and structures have been obtained [1]. These mesostructured materials have rapidly acquired significant attention in the field of materials science and in many areas of chemistry and biotechnology [2–5]. Using cationic surfactants to template the pore architecture, mesoporous silica materials with well-ordered pore networks were first obtained by Mobil Corporation in 1991 [6,7]. The possibility to obtain silica with different pore network topologies and the ability to tailor pore size boosted the rapid growth of research activity in this field of materials science. Pore size control in OMMs can be achieved by modifying surfactant type and chain length or by adding a swelling agent [8]. Silica with a pore size of approximately 3 nm can be made using cationic surfactants, with reasonably short alkyl chains [6]. By replacing the cationic head group by a larger non-ionic group, the size of the surfactant micelles can be increased and materials with pore sizes in the range 5–6 nm are obtained [9]. Even larger surfactants like poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) triblock copolymers can produce materials with pore sizes of

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7 nm and higher such as SBA-15 silica, which possesses a two-dimensional hexagonal structure of cylindrical mesopores [10]. These pore diameters are however not sufficient for the immobilization of large enzymes with high molecular weight. On the other hand, the synthesis of mesoporous silica with large pore size tends to lead to less ordered structures. Making use of this approach, the size of mesopores is limited by the dimensions of the micelle templates, and well-ordered SBA-15 materials with pore size up to 10 nm have been obtained [11]. Other mesostructures, named foam-like (MCF) or worm-like mesoporous molecular sieves, have also been reported showing disordered mesoporous structures with a pore size up to 10 nm, (HMS (D_p = 2–10 nm) [12]; MSU-J (D_p = 2.5–10 nm) [13–15]).

The addition of swelling agents which are dissolved inside the hydrophobic regions of the surfactant micelles allows to further increase the pore diameter of the solid [9,16]. Hydrophobic swelling agents such as substituted aromatic hydrocarbons (1,3,5-trimethylbenzene, 1,3,5-triisopropylbenzene) or other organic compounds such as alcohols (butanol, pentanol, hexanol), aliphatic hydrocarbons (dodecane), tertiary amines, poly(propylene glycol), etc., have been used to expand the pore size of SBA-15 silica up to 12–15 nm [8,17,18]. However, the pore diameter of these materials has to be primarily controlled through the selection of the initial synthesis temperature, and further adjusted through the selection of the hydrothermal treatment temperature and time, as well as the type and amount of the swelling agent [18]. Otherwise, an increase in the amount of the swelling agent causes the formation of a different structure, called a mesocellular foam (foamlike), which is poorly ordered or disordered and features spherical mesopores of large diameter (20–40 nm) [19,20]. In most cases it results in disordered mesostructures with the loss of the 2D hexagonal ordering [4].

Mesostructured materials offer versatile features, such as high specific surface area, large specific pore volume, narrow pore size distribution, mesopores interconnected by micropores, tunable chemical properties and high chemical and mechanical stability. These unique properties have been previously reported in our group for lipases [21–23]. The aim on this work is to go further in the synthesis of OMM with larger pore sizes which open new possibilities for immobilization of molecules too large to fit into the pores of standard OMM. This is the case of laccase from *Myceliophthora thermophila* (*Mt*L), an enzyme with larger dimensions than lipase.

Laccases (benzenediol:oxygen oxidoreductase, E.C. 1.10.3.2) belong to the group of blue multicopper oxidases, an important class of enzymes found in many organisms, including plants, insects, fungi and bacteria [24–27]. These enzymes catalyze the reduction of molecular oxygen to water by oxidation of various phenolic compounds. The fact that they only require molecular oxygen for catalysis makes them suitable for different applications [26].

In last years, laccases have been used for many different biotechnological processes, such as pulp delignification, oxidation of organic pollutants, textile and petrochemical industries [28], development of biosensors [29], or wine and beverage stabilization [30–34] among other uses [33,35–37]. Some attempts to use laccase in wine and beer industries have been done [30,33,34,38] and this seems to be an interesting application to study. However, the use of soluble laccase is not allowed in these industries, which highlights the relevance of immobilized enzyme with optimized properties.

Furthermore, the industrial application of free laccases is limited since their stability and catalytic activity are considerably affected by a variety of environmental conditions [35]. In fact, immobilization allows easy separation from the reaction medium by simple filtration, potential reuse of the biocatalyst and sometimes more resistance of the enzyme to a thermal or chemical inactivation [39].

In this work, a classic enzyme immobilization method on amino-containing surfaces is used. Particularly, the design of ordered mesoporous materials with the exact pore size required by laccase, and the synthesis conditions of this kind of materials also containing amino groups are studied and presented. Pore size is maybe the most relevant parameter; it must be wide enough to enable the enzyme to enter and diffuse along the channels, leaving room for new molecules to enter as well. In the opposite situation, if the enzyme does not diffuse, then the molecules would be stacked in the pore mouths, acting as a stopper and preventing new laccase molecules to enter the pore. As a consequence, most of the inner pore surface would not be occupied by enzyme and the immobilization yield would be low.

Immobilization of laccases on OMM have been reported in the literature with varying degrees of success, mainly due to denaturation processes and restricted diffusion of the substrate [40–42]. We have chosen electrostatic interactions as the driving forces for laccase immobilization. *Mt*L has a low isoelectric point, so we propose in this work the functionalization of the siliceous supports with high pK_a groups (amine) [4,43] to interact with negative charges of the enzyme [44].

Many research efforts have focused on surface functionalization of mesoporous silica materials with organic groups (such as amine) by the direct incorporation of these functionalities through co-condensation of siloxane and organosiloxane precursors [45,46] or by post-synthesis grafting of organic groups onto the surface of the mesoporous silica [2,47]. The presence of these groups in mesoporous materials additionally decreases pore sizes which again makes necessary wider channels [48,49].

In the present study, we extend the use of the swelling agent 1,3,5-triisopropylbenzene (TIPB) to synthesize large pore ordered materials bearing amine groups. We use the principle of large pore sizes in combination with surface functionalization of mesoporous silicas with amino groups for improving the non-covalent immobilization of laccase.

2. Experimental

2.1. Materials and reagents

2.1.1. Chemicals

Triblock co-polymer poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) Pluronic P123 ($PEO_{20}PPO_{70}PEO_{20}$), from Aldrich (USA), was used as structure directing agent. Tetraethoxysilane (TEOS) (Merck, Germany) and 3-aminopropyltriethoxysilane (APTS) were provided by TCI (Belgium). Ammonium fluoride (NH_4F) and 1,3,5-triisopropylbenzene (TIPB) were from Alfa Aesar (Germany). 2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was from Sigma (USA). Amorphous silica MS-3030 was kindly donated by Silica PQ Corporation (USA).

The extract of soluble laccase (Suberase) from *M. thermophila* expressed in *Aspergillus oryzae* was kindly donated by Novozymes (Denmark). Bovine serum albumin (BSA, Sigma–Aldrich, USA) was used as protein standard for protein content determination by the Bradford method [50]. The reagents for electrophoresis (SDS-PAGE) and broad molecular weight standards were from Bio-Rad (USA).

Potassium chloride, phosphoric acid, citric acid and toluene were purchased from Sigma–Aldrich (USA). Sodium acetate was purchased from Scharlau (Spain). Sodium di-hydrogen phosphate 1-hydrate, hydrochloric acid, ethanol, acetic acid and acetone were purchased from Panreac (Spain). Tri-sodium citrate dehydrate was from Analyticals Carlo Erba (Italy). Solvents were all analytical or HPLC grade and salts were of high purity. All materials were used as obtained without further purification. Water was grade Milli-Q.