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Covalent immobilization of glucose oxidase on mesocellular silica foams: Characterization and stability towards temperature and organic solvents

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ABSTRACT

Glucose oxidase (GOx) immobilization onto mesoporous SBA-15 silica and two mesocellular foams (MCF) characterized by similar surface area and pore volumes but different pore/cell dimensions was examined. The covalent grafting of the enzyme through amide bonds was evidenced by controlling pH conditions, thus preventing GOx leaching. The immobilized protein activity was found to be significantly higher for the mesocellular foam with both cells and windows size larger than the enzyme dimensions. The Michaelis–Menten parameter K_M for the immobilized GOx was similar to that of the free enzyme. GOx exhibited higher thermal stability when immobilized on the mesocellular foam compared to the free enzyme. The activity decay of GOx in presence of water soluble organic solvents, i.e., acetonitrile or methanol, was studied. At 50 °C, half of the immobilized GOx activity could be retained in 40 v/v% MeOH/acetate buffer.

1. Introduction

Current existing production processes for commodity chemicals have to be revised because of the increasing concern of the chemical industry toward sustainability issues which drives the impetus of green chemistry [1,2]. This includes the field of oxidation where peracids could be replaced by more environment-friendly reagents such as hydrogen peroxide, whose reduction produces water instead of carboxylic acids by-products [3]. In situ controlled production of hydrogen peroxide using palladium as a catalyst has been shown to limit H_2O_2 disproportionation that is detrimental to the oxidation yields [4–6]. Biocatalysis using glucose oxidase (GOx), an enzyme catalyzing the reduction of O_2 to H_2O_2, concomitantly to the oxidation of glucose to gluconic acid, is an alternative method for the in situ production of H_2O_2. Several homogeneous [7] or heterogeneous [8] chemo-enzymatic systems (also called tandem catalysts) combining biocatalytic hydrogen peroxide production and subsequent oxidation of alkene to epoxides using inorganic catalysts have been described. It is interesting to note that in such processes, the by-production of gluconic acid can be valorized as it is widely used in the food industry [9,10] as a preservative.

Enzyme immobilization on a solid support often prevails in biotransformation processes because it allows the recycling of the catalyst and enhances operational and storage stability, thus reducing the costs. Silica and alumina supports with high surface area and chemical and thermal stability [11] are of particular interest for tandem catalysis because they are suitable for hosting both the enzyme and the metal catalyst. Vennestrøm et al. have reported the immobilization of GOx onto TS-1, a redox-active molecular sieve with MFI framework [8], for the successful epoxidation of allyl alcohol to glycidol. However, as TS-1 micropores diameter is only 0.6 nm, GOx, active as a globular dimer with dimensions $6.0 \times 5.2 \times 7.7\ nm^3$, was likely to be localized outside the pores. Consequently, hydrogen peroxide production took place on the external surface of the material while most of the epoxidation occurred inside the pores, close to the titanium sites. An alternative method would be to locate both the enzyme and the metal catalyst inside the porosity, thus minimizing the distance between the H_2O_2 production and epoxidation sites with several advantages: (i) no diffusion limitation of H_2O_2, (ii) decrease of GOx denaturation by H_2O_2 as well as of H_2O_2 losses by dispropor-

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tionation because of the rapid consumption of hydrogen peroxide. Suitable supports for that purpose could be mesoporous silica such as MCM-type, SBA-type materials or mesocellular silica foams synthesized by surfactant templating techniques. These materials have been successfully used to immobilize proteins [12–24] for applications in analysis [25] or sensors [26–28], as recently reviewed by Zhou and Hartman [29]. The large pore volume, controllable and well-defined pore size of these mesoporous materials ensures the diffusion of both reagents and products inside the pores, even allowing the transformations of bulky reactants. Different immobilization methods including entrapment, adsorption and chemical binding are used to immobilize enzymes [30]. Adsorption is often favored because it is simple and is expected to induce limited modification of the protein structure [23,31]. However it is also associated with the problem of enzyme leaching in addition to non-specific protein-silicate binding through hydrophobic interactions [32]. Therefore, functionalization of mesoporous materials, usually amination through amino silane, is a widely used method to covalently immobilize proteins [17,32] and among them GOx [21,24]. In a recent approach, the crosslinking of adsorbed proteins in situ inside the pores of mesoporous materials was also found to be very effective in preventing enzymes, respectively lipase, invertase and choloroperoxidase/GOx, leaching [17,21,33,34]. In addition, several methods such as combination of differential interference contrast and fluorescence microscopy [35] or small-angle neutron scattering [36] demonstrated that the enzymes, respectively lipase and chloroperoxidase, are confined in the nanoscale pores of the mesoporous support.

So mesoporous siliceous material, with pores large enough to accommodate proteins and functional groups able to sequester active enzyme are available. However, no general answer can be derived regarding an optimized immobilization protocol for a given enzyme or a targeted biotransformation reaction remains an issue.

The present work focused on the immobilization of GOx on a functionalized siliceous support and compared both adsorption and covalent immobilization methods. The main goal of this study is to maximize the covalent coupling at the expense of adsorption and the operational stability thanks to the minimization of enzyme leaching. Indeed, both covalent and adsorption immobilization processes take place when mixing the support and the enzyme but the issue of minimizing the remaining non-covalently bound protein on the surface is seldom addressed in the literature. Two MCFs materials were tested as models of ultra large open porosity with connected cells large enough to host glucose oxidase dimers. These materials were compared to SBA-15 type support, whose pores are smaller than the GOx dimensions. In addition, as one of the targeted application of the so-produced hydrogen peroxide is alkene epoxidation that usually takes place at temperatures higher than room temperature and often requires an apolar environment in case of hydrophobic alkenes, thermal stability of the immobilized GOx as well as its efficiency in water/acetonitrile and water/methanol mixtures will be studied by measuring GOx activity, i.e., its ability to produce hydrogen peroxide.

2. Experimental

2.1. Chemicals

Tetraethyl orthosilicate (TEOS, 99%, Aldrich), triblock copolymer $EO_{20}PO_{70}EO_{20}$ (Pluronic P123, Aldrich), 1,3,5-trimethylbenzene (TMB, 97%, Acros), ammonium fluoride (NH_4F, 98%, Fluka), hydrochloric acid (HCl, 37%, VWR), 3-aminopropyltriethoxysilane (APTES, 99%, Aldrich), anhydrous toluene (VWR), acetone (VWR), ethanol (VWR), methanol (Sigma Aldrich), acetonitrile (Sigma Aldrich), D(+)-glucose (99.5%, Sigma), sodium acetate (anhydrous, 99%, Alfa Aesar), potassium dihydrogen phosphate (KH_2PO_4, 99.5%, Riedel-de-Haën), di-potassium hydrogen phosphate (K_2HPO_4, anhydrous, Merck), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, 98%, Sigma), *N*-hydroxysuccinimid (NHS, 97%, Aldrich), 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide hydrochloride (EDC, Sigma), peroxidase from horseradish (HRP type VI, 253 units/mg solid, Sigma), glucose oxidase from *Aspergillus niger* (type X-S, 120 units/mg solid, Aldrich) were used as received.

2.2. Synthesis of SBA-15

SBA-15 silica was synthesized according to the method described by Zhao [37]. Briefly, 4 g of P123 were stirred for 2 h at 40 °C in 150 mL of 1.6 M aqueous HCl. Then, 9 mL (40 mmol) of TEOS were slowly added under stirring. The resulting gel was aged at 40 °C for 24 h, and then hydrothermally treated at 100 °C for 24 h in a FEP® bottle. The resulting material was filtered, washed thoroughly with deionized water and dried at 100 °C for 24 h. Finally, the surfactant was removed by calcination at 550 °C in air for 6 h after using a 24 °C per h ramp.

2.3. Synthesis of MCFs (mesocellular foams)

Two samples of pure silica MCFs (MCF1 and MCF2) were prepared by a hydrothermal method developed by Schmidt-Winkel et al. [38]. Hence, 4 g of P123 were dissolved in 150 mL of 1.6 M aqueous HCl at 40 °C. TMB was then slowly added and the resulting solution stirred vigorously (1000 rpm) at 40 °C for 2 h. Then 9 mL (40 mmol) of TEOS were slowly introduced while stirring. The resulting gel was aged in a water bath at 40 °C for 24 h. In the case of MCF2, 48 mg of NH_4F were also added. Then the mixture was hydrothermally treated for 24 h at 100 °C in a FEP® bottle. The resulting material was filtered, washed thoroughly with deionized water and dried at 60 °C for 24 h. Finally, the surfactant was removed by calcination at 550 °C in air for 6 h after using a 24 °C per h ramp.

2.4. Functionalization of SBA-15, MCF1 and MCF2 silicas with APTES

Prior to the grafting step, 1 g of siliceous material was activated at 350 °C for 3 h in air. The material was then transferred into a dried round-bottom flask equipped with a septum and a condenser. Dry toluene (50 mL) was rapidly introduced and the resulting suspension was vigorously stirred with a glass coated magnetic stirrer under inert gas. APTES (1.0 mL, 4 mmol) was added dropwise and the mixture was stirred for 1 h and then refluxed for 24 h still under inert gas. After cooling, the resulting material was recovered by suction filtration and washed with toluene (30 mL), acetonitrile (30 mL) and ethanol (30 mL). The white solid was then dried at 60 °C under air. Finally, the excess of APTES was extracted by dichloromethane (Soxhlet) for 24 h and then dried at 60 °C under air.

2.5. Characterization of mesoporous materials

N_2 sorption isotherms were measured on a Micromeritics ASAP 2020 instrument at −196 °C. Before, samples were degassed overnight at 120 °C. The total pore volume was estimated from the amount of N_2 adsorbed at $P/P_0 = 0.99$. The BET specific surface area, S_{BET} was obtained from the adsorption data in the relative pressure range of $P/P_0 = 0.05–0.25$. The mean pore diameter of SBA-15 was determined using the BJH model [39]. MCFs pores are considered to be of the ink-bottle-type interconnected by narrow windows.