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Immobilization of lipase in hierarchically ordered macroporous/mesoporous silica with improved catalytic performance

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ABSTRACT

Hierarchically ordered macroporous/mesoporous silica (3DOM/m-S) material was prepared through the dual templating method with polystyrene (PS) colloidal crystals as the hard template and amphiphilic triblock copolymers (P123) as the soft template. The achieved 3DOM/m-S possesses ordered macropores of 400 nm and mesopores of 5.1 nm, which provides a promising platform for enzyme immobilization. Lipase B from *Candida antarctica* (CALB) was employed as a model enzyme to verify the possibility and advantages of enzyme immobilized on 3DOM/m-S. The immobilized lipase shows excellent stability towards heat even at 80 °C and organic solvents for long-term incubation (288 h). Also, the immobilized CALB could be used for esterification reactions between acids and alcohols with different chain lengths, and 90% of conversion rate could be reached. In examining the reusability in esterification of oleic acid and ethanol, the conversion rate can retain 75% after 10 reaction cycles, indicating a remarkable reusability of the immobilized lipase.

1. Introduction

Three-dimensionally ordered macroporous (3DOM) materials are of interest for a wide range of applications due to their distinct structural features, ranging from photonic crystals to sensors, advanced adsorbents, and catalysts [1–4]. Amongst the applications of 3DOM materials, 3DOM heterogeneous catalysts have gained particular attention because of their high specific surface area, increased pore volume, limited tortuosity, and enhanced reactant throughput and diffusion. A variety of 3DOM catalyst systems relevant to environmental catalysis and chemical fuel catalysis have been developed and the catalytic performances of the catalysts were investigated [4]. For example, 3DOM $Ce_xZr_{1-x}O_2$ supported gold nanoparticle catalysts have been produced and high catalytic activities for soot combustion were observed [5]. 3DOM γ-Al_2O_3 [6], TiO_2 [7,8] and ZnO [9,10] materials were also synthesized and employed as supports for heterogeneous catalysts, where advantages of the 3DOM structure were realized.

The immobilization of enzymes on suitable support materials has been widely studied in biocatalysis as means to improve the catalytic properties of the enzyme and recognize the benefits of easy recovery and re-use. In recent years, 3DOM material has been proved to be a proper support for enzyme. For instance, glucose oxidase has been immobilized covalently on the surface of 3DOM silica. Compared to glucose oxidase bound to a planar silica surface or free in solution, the turn-over rate of glucose oxidase bound to 3DOM silica was enhanced, which can be attributed to the structural stabilization of the enzyme and geometric advantages associated with the 3DOM structure [11]. By combining the superiority of the 3DOM structure and the bioadhesion, Jiang and coauthors developed a simple and versatile method for immobilization of enzyme, where norepinephrine was utilized as a stable anchor and Penicillin G acylase was chosen as model enzyme. After immobilization, broader pH profile, better thermal, storage and operational stability were observed relative to the native form [12]. Additionally, lipase CLEAs were also prepared in the macropores of 3DOM silica by Jiang and coworkers, which exhibited enhanced thermal stability, mechanical stability in both aqueous and organic phases [13].

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While 3DOM materials provide certain advantages for enzyme immobilization as previously discussed, their lower surface area compared to mesoporous materials may hamper the broader applications. Fortunately, 3DOM materials with mesopores in the wall (3DOM/m material) which combines the advantages of both macropores and mesopores provide potential alternative to conventional 3DOM materials [1,2,14–17]. For example, 3DOM/m materials with various framework compositions, such as carbon [18], metal oxides [19–24] and silica [14,15,25], have provided substantial performance boosts in some applications including protein separation [15], photoelectrocatalysis [21,23], catalyst support [25], TNT detection [26] and liquid/gas-phase catalysis [24]. However, to the best of our knowledge, there is no report that uses 3DOM/m materials as supports for enzyme immobilization. The macropores of 3DOM/m materials can provide efficient mass transportation, and the mesopores can create additional surface area for enzyme loading and enzyme–substrate interactions, helping to improve the catalytic performance. Considering the above mentioned superiority, it is expected that the 3DOM/m materials hold great potential for immobilization of enzymes.

Thus, this study reports the preparation and characterization of hierarchically ordered 3DOM/m silica materials followed by enzyme immobilization. 3DOM/m silica materials were synthesized by using polystyrene (PS) colloidal crystals as the hard template and amphiphilic triblock copolymers (Pluronic P123) as the soft template. Lipase B from *C. antarctica* was chosen as model enzyme. The activity, thermal stability, organic solvents tolerance and reusability of the immobilized lipase were investigated in detail. Furthermore, applications in esterification between acids and alcohols with different chain length were also explored to confirm the versatility of immobilized lipase on 3DOM/m-S. Thus, the objective of this study is to address the possibility of immobilizing enzyme on 3DOM/m material and investigate the catalytic properties of immobilized enzyme. The lipase immobilized on 3DOM/m silica showed improved catalytic performance, which make this strategy potential for practical applications.

2. Material and methods

2.1. Materials

Lipase B from *Candida antarctica* in liquid form and Novozym 435 (N435) were purchased from Novozymes. Triblock copolymer P123 was purchased from Sigma-Aldrich. 4-nitrophenyl palmitate (p-NPP) was purchased from Alfa Aesar Chemical Co. Ltd. (Tianjin, China). Tetramethoxysilane (TMOS), HCl (37%), lauric acid, n-octyl alcohol, isooctane, ethanol, and acetone were purchased from Fengchuan Chemical Reagent Co. Ltd. (Tianjin, China). All other chemicals and reagents were analytical grade.

2.2. Preparation of PS colloid crystals and 3DOM/m silica

Noncrosslinked PS spheres with diameter of 450 nm were prepared as the protocol in previous works reported [27,28]. Then PS spheres were assembled by centrifugation at 1000 rpm for 2 d and dried in air at room temperature for 3 weeks. Then, PS colloid crystals were obtained.

The 3DOM/m silica materials (denoted as 3DOM/m-S) were prepared via a dual-templating method with close-packed ordered PS colloid crystals as hard templates and P123 as a soft template. Firstly, 2 g of P123 was dissolved in 4 g of TMOS under magnetic stirring at 50 °C. Then, 2 mL of HCl solution (2 M) was added into the solution with vigorous stirring for 1 min. Thus a homogeneous phase was observed. Several pieces of PS colloid crystals were immersed into the above solution to fill the voids of templates. After removal of the extra sol on the surface of the colloid crystals, the composites were transferred into a drying oven under vacuum at 60 °C overnight. Finally, the composites were calcined in air at 550 °C for 5 h at a heat rate of 3 °C/min to remove the templates and then the 3DOM/m-S was obtained.

For comparison, 3DOM silica (denoted as 3DOM-S) was fabricated as the procedure mentioned above without adding P123.

2.3. Immobilization of lipase in 3DOM/m-S and 3DOM-S and activity assay

Lipase solutions of different concentrations were prepared by diluting the commercial lipase solution with PBS (50 mM, pH 7.0) to carry out immobilization at 25 °C. 20 mg of 3DOM/m-S was immersed into 2 mL of lipase solution under magnetically stirring for predetermined time period. The immobilized lipases (denoted as CALB@3DOM/m-S) were separated by centrifugation, washed three times with PBS, and one time with acetone. After air-dried overnight, CALB@3DOM/m-S was stored at 4 °C until use. Before and after adsorption, protein contents of the solutions were measured through standard Bradford's method to obtain the adsorption equilibrium curve. After optimal lipase concentration determined, the same concentration of lipase was used to prepare CALB@3DOM-S. The protein amount adsorbed on the surface and the specific activity were measured.

Hydrolytic activity of immobilized lipase was measured by the hydrolysis of 4-nitrophenyl palmitate (p-NPP). The reaction mixture consisted of 20 mg of CALB@3DOM/m-S, 5 mL of PBS (50 mM, pH 7.0), and 200 μL of p-NPP solution (5 mg/mL in ethanol). After reacted 1 min at room temperature, the reaction mixture was filtered, and the content of 4-nitrophenol (p-NP) in supernatant was measured spectrophotometrically at 410 nm. One unit of enzyme activity was defined as the amount of enzyme needed to liberate 1 nmol of p-NP per minute under assay conditions.

2.4. Stabilities and activity assay

The thermal stability tests were carried out by pre-incubation of the same activity of CALB@3DOM/m-S and CALB@3DOM-S in isooctane and acetone, respectively, at different temperatures (30–70 °C) for 3 h. The residual esterification activities were measured. The activity of each sample kept at 30 °C was defined 100%, and the thermal stabilities were explained by relative activity (%).

Long-term storage stabilities of CALB@3DOM/m-S and CALB@3DOM-S in organic solvents were evaluated by incubating the samples with same activity into 5 mL of isooctane and acetone at 40 °C, respectively. At designated time intervals, the residual esterification activities were measured. The activity of each sample without solvent treatment was used as control and defined as 100%.

Esterification activity of CALB@3DOM/m-S was measured by the detection of free fatty acid concentration before and after the esterification. Typically, 50 mg of CALB@3DOM/m-S was added into the reaction mixture consisting of lauric acid (0.5 mmol), n-octyl alcohol (1 mmol), and isooctane (5 mL) at 40 °C under stirring. After 30 min, the residual content of lauric acid in the supernatant was measured by titration with 20 mM of NaOH solution in the presence of phenolphthalein. One unit of esterification activity was defined as the amount of enzyme that consumed 1 μmol of lauric acid per hour under assay conditions.

2.5. Esterification

Esterification reactions between different alcohols and acids were conducted by incubating 5 mL of solution of the alcohol (0.5 M) and the acid (0.5 M) in cyclohexane at 40 °C under magnetic