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Roles of nanostructures and carboxylic acid functionalization of ordered cubic mesoporous silicas in lysozyme immobilization

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ABSTRACT

Cage-type cubic mesoporous silicas FDU-12 functionalized with a tunable content of carboxylic acid (−COOH) groups, ranging from 0 to 50 mol% based on silica, were used for immobilization of lysozyme. The particle size of −COOH functionalized FDU-12 was highly dependent on the relative amount of carboxyethylsilanetriol sodium salt (CES) and tetraethoxysilane (TEOS) in the reaction mixture. The particle size was around 200 nm for the materials prepared with the CES/(TEOS + CES) molar ratio of 20% −30%. The combination of −COOH functionalization and nanoscale dimension significantly enhanced the lysozyme adsorption capacity of the prepared materials. A remarkably high adsorption capacity of 895 mg g^{-1} at 37 °C (pH 9.6) was attained with the FDU-12 nanostructure functionalized with 30 mol% of −COOH groups. Meanwhile, the structural stability of lysozyme was unaltered after immobilization. A very low leaching rate was observed, indicating that protein molecules were tightly adsorbed onto the −COOH functionalized FDU-12 nanostructures due to the strong electrostatic interactions between the adsorbent and the lysozyme molecule. The zeta potentials of the materials also correspond well to their adsorption capacities observed. The enzymatic activity of the immobilized lysozyme was also examined as a function of the −COOH contents present on the mesopore surface of the materials.

1. Introduction

The immobilization of biomolecules onto ordered mesoporous silica materials to improve their stability has received much attention in the last two decades. Since most native biomolecules exhibit their catalytic activities only under normal environmental conditions, they might be easily inactivated due to denaturation under harsh conditions, e.g., the presence of organic solvents and at extreme temperatures or pH. To pursuit an ideal host to maintain the activity and stability of biomolecules, ordered mesoporous materials turn out to be promising candidates, since they exhibit tunable pore size in the nanometer scale, high surface area, and large pore volume that are beneficial for adsorption of biomolecules such as enzymes and proteins. Moreover, the pore size of mesoporous silica materials can be tuned to entrap large biomolecules. These features make them particularly useful for biomedical applications such as immobilization of biomolecules [1,2], drug delivery and controlled release systems [3–8], separation, biosensors, and biocatalysis [2,9–11]. In the case of pure silica materials, another key factor for adsorption of biomolecules is the electrostatic interactions between the surface silanol groups of mesoporous silicas and the surface charge of biomolecules. The immobilization of several enzymes, such as cytochrome *c*, papain, and trypsin, on mesoporous silica MCM-41 via physical adsorption has been first reported by Diaz and Balkus [12]. Then onwards, the progress in the use of mesoporous silica materials for enzyme immobilization has been made in parallel to the development in the synthesis of various ordered mesoporous materials [13]. It is generally accepted that the structural properties of the adsorbent play an important role during immobilization. However, it still remains a challenge to develop adsorbents suitable for biomedical applications. For example, the diffusion of biomolecules somehow was limited by small pores, whereas the possibility for immobilization was increased by large pores [14–17]. Moreover, mesoporous silicas with 3D cubic pore channels are more favorable for biomolecule adsorption than those with 1D arrays of pores, mainly because the former can avoid potential pore blockage problems and thus lead to a better mass transfer in the pore channels. However,

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only a few literature reports the use of cubic mesoporous silica materials to immobilize biomolecules. Balkus et al. have demonstrated that the loading of cytochrome *c* is influenced by the structure of the support based on a comparative study between MCM-41 [10], MCM-48, and SBA-15 [18]. A higher adsorption capacity was achieved by MCM-48 with 3D interconnected pores and the highest surface area. Fan et al. have successfully synthesized a cage-type mesoporous silica FDU-12 and found that the adsorption capacity of lysozyme was increased with the increase in the entrance size of the mesopores [16]. Vinu et al. used another cage-type mesoporous silica KIT-5 to study the adsorption capacity of lysozyme, but showed a very poor adsorption capacity due to its small pore volume [19]. Highly ordered cubic mesoporous silica KIT-6 with the *Ia3d* symmetry was also used as adsorbents for the immobilization of lysozyme, and showed high adsorption capacity due to their larger pore volume and pore diameter [20]. However, some studies revealed that the materials with a pore size larger than the enzyme sizes had adverse effects on enzyme activity [21]. By matching the pore diameters with the enzyme sizes, not only the enzyme leaching problems can be reduced, but also the stability and activity of the immobilized enzyme can be enhanced [21,22]. Moreover, the native conformation of protein remains unaltered when its molecular dimension is close to the pore size of the adsorbents.

For efficient immobilization of protein on mesoporous silica materials, it is important that the biomolecules have strong interactions with the surface of the adsorbent. The use of pure mesoporous silica materials for this purpose is inadequate as the attraction between the mesopore surface and biomolecules is relatively weak. As a result, significant leaching of biomolecules from pure mesoporous silica materials may occur. It is therefore desirable to modify the surface properties of pure mesoporous silica materials by incorporating appropriate organic functionalities. For example, Yiu et al. have found that SBA-15 functionalized with thiol groups could adsorb a variety of proteins and exhibited enhanced protein adsorption capacity due to the presence of thiol groups [14]. Penicillin acylase has been immobilized on amino-functionalized SBA-15, which leads to a higher adsorption capacity as compared to pure silica SBA-15 [23]. The lysozyme adsorption capacity of rod-like SBA-15 functionalized with a small amount (5%) of carboxylate group was two times higher than that of pure SBA-15 [24]. Hartono et al. observed 8-fold higher bovine serum albumin (BSA) adsorption capacity of amine functionalized FDU-12 than that of pure silica FDU-12 due to the strong electrostatic interactions [25]. Therefore, for the mesoporous silica material being an effective adsorbent, an essential requirement is to tune its surface chemistry with an appropriate functional group.

The morphology and particle size of mesoporous silica materials could also have a pronounced effect on the protein and enzyme immobilization. The lysozyme adsorption capacity of rod-like SBA-15 was found to be 7 times higher than the conventional SBA-15 due to the short pore channel present in the former [26]. Compared with mesoporous silica materials, whose particle size is often larger than a micrometer (or sub-micrometer), nanometer-sized mesoporous silica particles (size from 30 to 500 nm) offer additional interesting properties, e.g., fast mass transport. However, one drawback of mesoporous silica nanoparticles to be used as the protein adsorbent is the limited size of the mesopores, which usually ranges from 2 to 5 nm [27–29].

The use of the material with a smaller particle size minimizes the risk of enzyme denaturation due to the fast adsorption rate during the immobilization process [30]. The pore diameter of mesoporous silicas can be controlled by varying the synthesis conditions, e.g., acid concentration, surfactants, cosolvents, and swelling agents [31]. However, the synthesis of mesoporous silica nanoparticles in acidic media was found to be difficult. To date, the smallest size of the SBA-15 nanoparticles reported in the literature is around 500 nm [32,33]. It still remains a challenge to synthesize mesoporous silica nanoparticles with diameters smaller than 200 nm in an acidic medium.

To address the above issues, cubic mesoporous silica materials functionalized with a tunable content of carboxylic acid groups were chosen as supports for immobilization of proteins in the present study. The cubic mesostructure can allow the protein molecules more easily diffuse into mesopores. The carboxylic acid groups, on the other hand, can provide the negative surface charges and good cation-exchange capacities under appropriate pH conditions, which could serve as anchor sites for biomolecules [34]. In this work, a tunable content of carboxylic acid groups (−COOH) was successfully incorporated into cage-type cubic mesoporous silica FDU-12 nanostructures (denoted as FTC-x, where x represents the −COOH functionalization level) with the particle size in the range of 200–400 nm. Interestingly, the particle size of the prepared material was highly dependent on the content of −COOH groups incorporated. In this work, the adsorption of lysozyme, a protein from hen egg white, was chosen as a model protein adsorption system by using the FTC-x materials having various extents of acidic functionalization (0–50 mol% based on silica) and controlled particle sizes. In particular, we emphasize the possibility to obtain the highest plausible adsorption capacity. Lysozyme is an antimicrobial protein with molecular dimensions of $4.5 \times 3.0 \times 3.0$ nm^3 and a molecular mass of 14.4 kDa [35]. It is an important enzyme, which holds a great potential in food preservation due to its preventive nature for many bacterial growths, and lack of toxicity to humans. Lysozyme is preferred to immobilized on mesoporous nanostructure as its dimension is comparable to the pore diameters of synthesized mesoporous nanostructures which may help to enhance the activity and stability of the protein as stated above [21,22]. The cubic pore connectivity of FTC-x is expected to allow a superior mass transfer, compared to the one-dimensional channel-like structure, and thus is more resistant to pore blocking. It is of great interest to explore the ability of FTC-x for immobilization of lysozyme to evaluate the important factors that govern the protein adsorption. The influences of the different amount of −COOH groups, solution pH, contact time, and particle size on protein adsorption were systematically investigated.

2. Experimental section

2.1. Materials

Tetraethoxysilane (TEOS), Pluronic F127 triblock copolymer, 1,3,5-trimethylbenzene (TMB) and buffer components such as disodium phosphate, sodium dihydrogen phosphate, sodium bicarbonate and sodium carbonate were received from Sigma–Aldrich. Carboxyethylsilanetriol sodium salt (CES, 25 wt.% in water) was purchased from Gelest. Lysozyme from hen egg white (70,000 U/mg) was purchased from Fluka. Micrococcus lysodeikticus cells were obtained from Sigma–Aldrich.

2.2. Synthesis of −COOH functionalized FDU-12

The synthesis of −COOH acid functionalized FDU-12 type mesoporous silica nanostructures followed the synthesis procedures that we previously reported [36]. A one-pot synthesis route was employed based on the co-condensation of TEOS and CES using Pluronic F127 as template under acidic conditions. In the typical synthesis of −COOH functionalized FDU-12, 1,3,5-trimethylbenzene (TMB) (1.0 g) and F127 (1.0 g) were first dissolved in HCl (60.0 mL, 2 M) and then mixed with KCl (2.5 g). The solution was kept stirring at room temperature for 24 h. TEOS and CES were pre-mixed and their