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COMMUNICATIONS

Published on Web 08/28/2002

Entrapping Enzyme in a Functionalized Nanoporous Support

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Received May 9, 2002

There is a long history for enzyme (protein) immobilization using solid supports via adsorption, encapsulation, and covalently linking.[1] One of the most widely used methods for immobilizing enzymes is encapsulation inside sol−gel silica.[2] However, due to small pore size and non-open-pore structure, most studies showed lower specific activity than that of the free enzymes in solution.[3]

Unlike sol−gel silica, mesoporous silica provides a rigid, uniform open-pore structure. Functionalized mesoporous silica (FMS) has exhibited very high affinity for binding heavy metal ions with mercapto functional groups.[4] FMS could have great potential for high enzyme loading, provided that (1) the pore size is sufficiently large for the enzyme to be "comfortably" hosted and also for its substrate and product to access and diffuse easily through open pore channel and (2) appropriate functional groups provide high affinity for protein molecules. Recently, mesoporous silica has begun to attract attention for enzyme immobilization.[5,6] The best results showed that 84% of the initial specific activity remained after immobilization albeit with very low protein loading (0.2%, w/w), presumably due to the small pore size (<6 nm). To our knowledge, the great potential advantage of FMS for enzyme entrapment has not yet been realized.

In this work, we used mesoporous silica with large pores that were subsequently functionalized to thereby yield high protein loading and enhanced enzyme activity simultaneously. Surprisingly, by introducing only 2% coverage of HOOC-CH_2-CH_2- groups on the internal silanol wall, the specific activity of organophosphorus hydrolase (OPH) entrapped could reach more than 2 times as high as that of the free enzyme prior to immobilization. The original unfunctionalized mesoporous silica (UMS), prepared by using nonionic block copolymer surfactant as the template,[7] had a pore size of 30 nm by the Barrett−Joyner−Halenda method,[8] while the surface area was as high as 533 m^2/g with an average bead size of 12−15 μm. We also used normal porous silica (NPS) for comparison experiments throughout this work.[9] The N_2 adsorption isotherms suggest that NPS had very wide pore size distribution, while the surfactant templated mesoporous silica had very narrow pore size distribution. Figure 1a shows the transmission electron microscopy (TEM) image of NPS made of silica particles partially sintered together. In comparison, the TEM image of the mesoporous silica (Figure 1b) reveals a uniform cagelike porous structure.[7]

To prepare FMS, we mixed Tris-(methoxy)carboxylethylsilane (TMCES) or Tris-(methoxy)aminopropylsilane (TMAPS) with mesoporous silica in an appropriate solvent.[4,10] We selected TMCES and TMAPS as functionalization reagent mainly because HOOC- and NH_2- would offer electrostatic, H-bond, and hydrophilic interaction with the charged amino acid residues of the protein molecules.

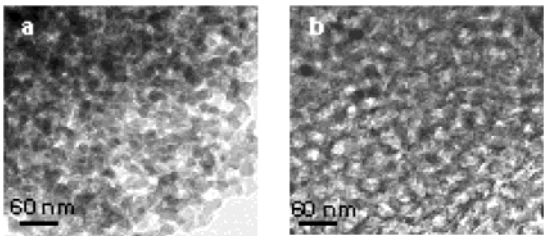

Figure 1. TEM images of (a) normal porous silica, (b) mesoporous silica.

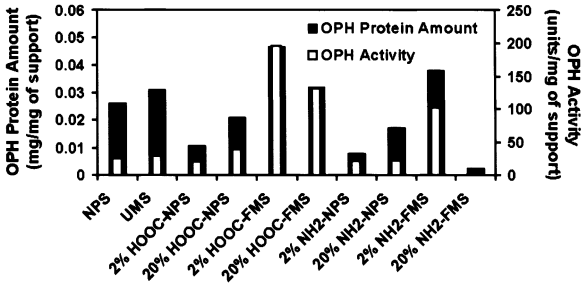

Figure 2. Comparison of different porous silica support for OPH immobilization in pH 7.5, 20 mM HEPES at 21 ± 1 °C.

We selected OPH for this work since it has been widely investigated for biosensing and decontamination of poisonous agents.[11] OPH has a well-known crystal structure (a dimer with two identical monomers) and dimensions of about 92 Å × 56 Å × 40 Å.[12] By incubating FMS in OPH stock solution, the enzyme was spontaneously sequestered into the functionalized mesopores.[13]

Figure 2 shows the protein amount and activity of OPH immobilized with 1 mg of each silica support. The isoelectric point (pI) of OPH is 8.3; thus, the overall charge of the protein is positive at pH 7.5.[13] As shown, the positively charged OPH could be entrapped in UMS with a protein loading of 3.1% (w/w) due to electrostatic interaction with the negatively charged silanol surface. However, it displayed a low specific activity of 935 units/mg of entrapped protein (calculated from Figure 2, see relative heights). In contrast, higher protein loading and much higher specific activity were obtained with the negatively charged HOOC-FMS. When a 2% surface coverage of carboxylic groups was introduced to the mesoporous silica, a higher protein loading of 4.7% (w/w) with significantly enhanced specific activity of 4182 units/mg was obtained, showing an exceptional high immobilization efficiency of more than 200% (the ratio of the specific activity of the entrapped OPH with that of the free OPH in solution).[12] These results demonstrate that the organic functionalization (-CH_2-CH_2-COOH) provided the enzyme with a benign surrounding microenvironment, showing higher affinity for the protein and inducing a dramatic change of specific activity. With 20% coverage of HOOC-FMS, a high specific activity of 4109 units/mg was also achieved, but the total protein loading and activity were reduced. We speculate that

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10.1021/ja026855o CCC: $22.00

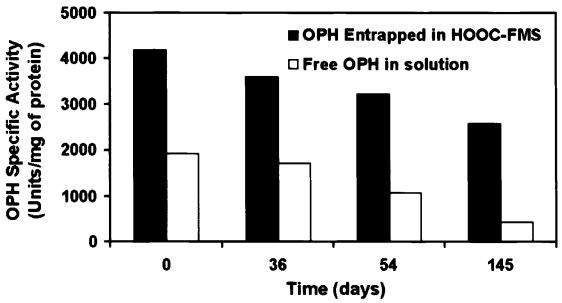

Figure 3. Stability of OPH entrapped in 2% HOOC-FMS and free OPH in pH 7.5, 20 mM HEPES. The first 47 days of storage was carried out at 4 ± 0.2 °C, and the following 98 days was at 21 ± 1 °C.

the reduction of the loading amount might be attributed to the decrease in the effective surface area by more close-packed functional groups.

In contrast, positively charged OPH was almost completely repelled from the positively charged 20% NH_2-FMS, with a very low protein loading (0.25%, w/w) and nearly zero activity. Therefore, selective entrapment could be achieved by rational choice and displacement of the functional groups in relation to the overall charge of the protein. Unexpectedly, the specific activity with 2% NH_2-FMS was as high as 2691 units/mg with a protein loading rate of 3.8% (w/w). This phenomenon could be explained by the fact that the small coverage of amino groups is less closely packed[4] and there are still plenty of local, negatively charged silanol groups. Its overall environment still favored the entrapment of positively charged protein. Some negatively charged amino acid residues on the overall positively charged protein could interact with the small coverage of the positively charged functional group as well. The fact that even 2% NH_2-FMS entrapped OPH more effectively than negatively charged UMS demonstrated again that the organic functionalization ($-CH_2-CH_2-CH_2-NH_2$) of mesoporous silica enhanced the affinity of the nanopores to the enzyme and induced an increase of the specific activity. From these results, we can conclude that fuctionalization of mesoporous silica and the type and coverage of functional group are paramount for both high loading amount and immobilization efficiency.

For comparison, the simple adsorption of OPH on NPS with or without functionalization displayed lower protein loading and less specific activity (Figure 2), demonstrating the great advantage of rigid and uniformly open porous FMS over NPS with the non-open pores and low surface area. The 2% HOOC-NPS displayed only about 0.1 of OPH activity entrapped with the same amount of 2% HOOC-FMS.

As shown in Figure 3, the specific activity of the free OPH in solution was reduced 77% after 145 days of storage. In contrast, the enhanced specific activity of OPH entrapped in 2% HOOC-FMS decreased only 38% after the same storage, which was still 134% of the initial specific activity of the free OPH. In fact, after such long-term storage, the activity and protein amount in the supernatant was still undetectable.[13] This suggests that the decrease of the enzyme activity was attributed to protein denaturation, rather than the enzyme leaching from the FMS.

In conclusion, for the first time, we have demonstrated that as a result of the unique mesoporous structure and surface chemistry, the organically functionalized nanoporosity of HOOC-FMS provided both high affinity for the charged protein molecules and a favored microenvironment that resulted in exceptionally high immobilization efficiency (>200%) with enhanced stability, while conventional approaches yield far lower immobilization efficiency.[1,3,11] It has been reported that confinement from molecular crowding in biological cells can both stabilize and induce order-of-magnitude enhancements in catalytic reaction rates compared to enzymes in solution.[14] We hope that rational design of organically functionalized nanoporosity will lead to new approaches to entrap and stabilize biomolecules for a wide range of applications.

Acknowledgment. We thank L. Koriazova for help making the OPH expression vector. This work was supported by the U.S. Department of Energy under Contract DE-AC06-RLO1830.

Supporting Information Available: OPH expression and purification, charged surface amino acid distributions, and structural dimensions (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(9) Normal porous silica is uncoated silica bulk material with a pore size of 30 nm with a 12-μm bead size from PolyLC Inc., USA, item no. BMSI 1203.
(10) A controlled hydration and condensation reaction was used to introduce functional groups into UMS and NPS.[4] The amount of trimethoxy silanes needed was calculated on the basis of the fraction of the surface area to be occupied by silane molecules; e.g., a coverage of 2% HOOC-FMS or NH_2-FMS means 2% of the total available surface area of the mesoporous silica would be silanized with the trimethoxy silane with the functional group HOOC- or NH_2-. For UMS (pore diameter, 30 nm), the coverage of 2% corresponds to ~0.09 mmol silane/g assuming 5×10^{18} molecules/m^2 in a fully dense monolayer coverage, as indicated by previous solid-state NMR studies.[4]
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(12) OPH was cloned, expressed, purified, and stored at −80 °C. It was thawed at 4 °C before use and dialysed in pH 7.2, 0.1 M HEPES. The activity of OPH was measured in 1 mM paraoxon in pH 9.0, 0.15 M CHES buffer at 25 °C, in which one OPH unit is the active amount allowing 1.0 μmol paraoxon to be hydrolyzed per minute. Protein amounts were measured using Pierce BCA Assay Kit (Pierce, product no. 23227). OPH stock solution, which was diluted 20−50 times by pH 7.5, 20 mM HEPES for activity measurements, had an initial specific activity of 1928 units/mg of protein.
(13) Generally, an aliquot of 2−4 mg of FMS or other silica support in a 1.8-mL tube was added for incubation with 0.2−1.2 mL of the OPH stock solution, shaking at a speed of 1400 min^{-1} on an Eppendorf Thermomixer 5436 at 25 °C for 2 h, and then centrifuging at 12000 rpm for 10 min. Repeatedly, the resulting OPH−FMS deposit was shaken vigorously and washed in pH 7.5, 20 mM HEPES (≥10 × 1.0 mL) at room temperature (21 ± 1 °C) and centrifuged to remove any nonfirmly immobilized enzyme, which was monitored in later cycles until both the protein and activity were undetectable in the supernatant. Finally, the OPH−FMS composite was thoroughly resuspended in the same washing buffer with 100 μL of the buffer per mg of FMS for further protein and activity measurement. To avoid any scattering during spectrophotometric measurement of protein amounts, a centrifugation step was taken to remove the silica particles after incubation of the OPH−FMS composite with the working reagent. The errors for both the protein amount and activity measurements were less than 5%.
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JA026855O