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Synergetic Effects of Nanoporous Support and Urea on Enzyme Activity

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ABSTRACT

We report synergetic effects of functionalized mesoporous silica (FMS) and urea to promote favorable protein conformational changes. The specific activity of glucose isomerase (GI) entrapped in FMS in the presence of urea was approximately double that of GI in solution in the absence of urea. Rather than losing all activity in a denaturing solution of 8.0 M urea, the specific activity of GI entrapped in FMS remained higher than the highest specific activity of GI free in solution.

Mesoporous silica has been extensively investigated for industrial and environmental processes due to its open pore structure, well-defined pore size and shape, and large surface area.[1] In 1985, Mosbach and co-workers published a pioneering report of glucose oxidase entrapment in polysiloxane-coated porous silica; however, the enzyme activity retained after immobilization was no more than 5.0%.[2] In the last several years, both unfunctionalized and functionalized mesoporous silica (UMS and FMS) have been tested for enzyme immobilization.[3−11] Recently, we reported enhanced activity and stability for enzymes entrapped in appropriate FMS compared to unfunctionalized mesoporous silica or normal porous silica. The activity and stability enhancements were attributed to the rigid, uniform, and large open mesopore geometry of FMS and to the favorable environments introduced by the functional groups.[12,13] Here we use covalent and noncovalent immobilization procedures to show enhanced stability to denaturant and demonstrate that the combination of urea and FMS can induce favorable conformational changes that dramatically enhance enzyme activity.

Urea is commonly used as a chaotropic protein denaturant to monitor the conformational stability of a protein. In most cases, the protein is inactivated to some extent at urea concentrations lower than those required for the complete unfolding.[14,15] However, some cases have reported that urea can induce the favorable conformational changes of free enzymes in solution.[16,17] Low concentrations of urea can loosen up the structure of a partially misfolded protein, and the increased enzyme activity may be due to the increasing conformational flexibility at its active site.[16,17] In this work, we found that the specific activity of partially active glucose isomerase (GI) immobilized in FMS in the presence of urea was dramatically increased to more than double that of GI in solution in the absence of urea. And even in 8.0 M urea,[15] the specific activity of GI entrapped in FMS was still higher than the highest specific activity of GI free in solution, indicating strong resistance of GI in FMS to the denaturant. These findings are noteworthy because the specific activity of immobilized enzymes using conventional methods is usually far less than that of free enzymes in solution prior to immobilization,[18−22] and in 8.0 M urea, many proteins are completely denatured.[15]

In this work (see the experimental details in Supporting Information), the hexagonally ordered mesoporous silica (SBA-15) with pore size of 300 Å, and the corresponding 20% NH_2−FMS were from the same batch prepared for the previous work.[1,12,23] A coverage of 20% NH_2−FMS means 20% of the total available surface area of the mesoporous silica would be silanized with tris-(methoxy) aminopropylsilane with the functional group NH_2. We also used normal porous silica (NPS) for comparison experiments.[24] Figure 1 shows the transmission electron microscopy (TEM) images of NPS, UMS, and 20% NH_2−FMS. The TEM image shows NPS is made of silica particles partially sintered together (Figure 1A). In comparison, the TEM images of UMS and FMS reveals cagelike nanoporous structure (Figure 1B,C). Surface area and pore sizes were analyzed using Barrett−Joyner−Halenda method.[25] Unlike 30 and 100 Å mesoporous silica, the 300 Å mesoporous silica has a large degree of disordering,[23] but the pore sizes are more or less uniform.[26]

We define the protein amount (mg) of an enzyme immobilized with one milligram of FMS support as the protein loading density (P_{LD}), and the corresponding activity (units) as the activity loading density (A_{LD}). The immobilization efficiency (I_e) is defined as the ratio of the specific activity of the immobilized enzyme (A_{LD}/P_{LD}) to the specific activity of the free enzyme in stock solution. FMS was incubated in the enzyme stock solution, thereby avoiding

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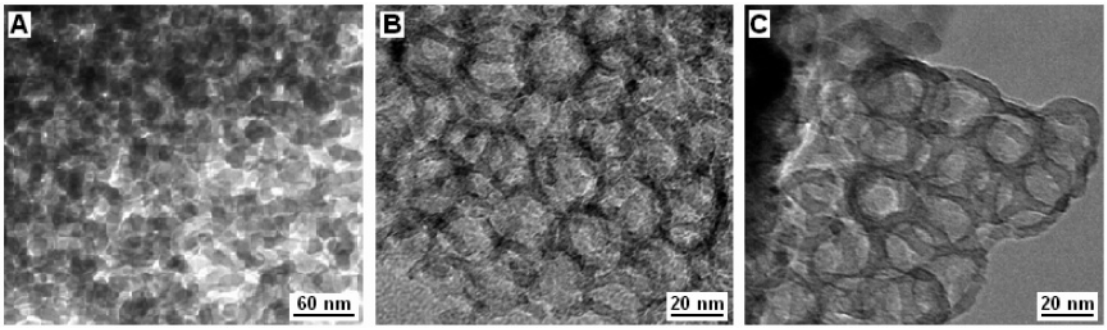

Figure 1. TEM images of NPS (A), 300 Å UMS (B), and the corresponding 20% NH_2−FMS (C).

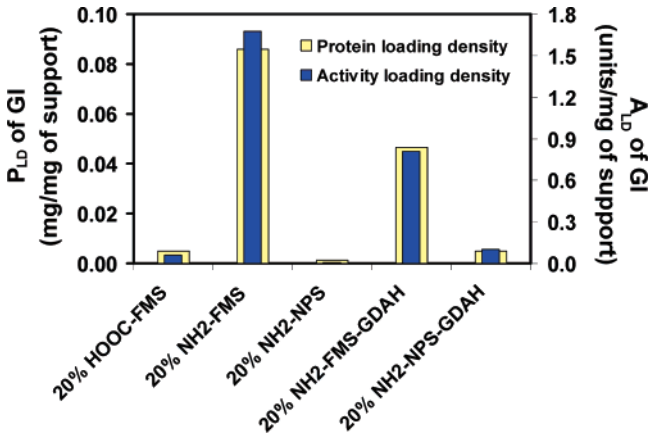

Figure 2. Outstanding protein amount and enzyme activity of immobilized GI in NH_2−FMS by spontaneous entrapping or covalent linking in pH 7.5, 20 mM sodium phosphate/0.15 M NaCl/5 mM $MgSO_4$. Glutaric dialdehyde (GDAH) = the covalent linker.

harsh immobilization conditions that destroy enzymatic activity. Glucose isomerase was selelected for this work because of its known crystal structure[27] and extensive applications in the high-fructose syrup industry.[28,29] GI from *Streptomyces rubiginosus* is a tetramer (MW = 173 kDa) comprising four identical subunits.[27]

In our previous work, unlike organophosphorus hydrolase (OPH) and glucose oxidase in FMS,[12,13] GI did not display a significantly increased specific activity when immobilized in FMS. The specific activity of the immobilized GI was well maintained in FMS and slightly higher than that prior to the immobilization, showing great advantages over conventional immobilization approaches that yield sharply lower specific activity. As an approach to further explore strategies to enhance GI activity in FMS, we deliberately chose a GI sample whose specific activity had significantly declined after 4 years of storage at −80 °C. The specific activity of this GI sample had decreased from an initial value of 43.9 to 17.7 units/mg, indicating that the stored GI was partially denatured (see Supporting Information).

When pH is above the isoelectric point (pI) of GI (4.0), we expected that the negatively charged GI (with a surplus of HOOC− residues) would prefer NH_2−FMS. Figure 2 shows that, in pH 7.5, 20 mM sodium phosphate/0.15 M NaCl/5 mM $MgSO_4$, P_{LD} of GI spontaneously entrapped in 20% NH_2−FMS was 86.0 mg/g FMS, with the specific activity of 19.5 units/mg, resulting in an I_e of 110.2% (the specific activity of GI stock was 17.7 units/mg). In contrast with 20% NH_2−FMS, A_{LD} and P_{LD} of GI with the negatively charged 20% HOOC−FMS were much lower due to electrostatic repulsion (Figure 2) but still higher than 20% NH_2−NPS due to potential physical adsorption effects (Figure 2).

In our covalent linking approach, NH_2−FMS was first reacted with a bifunctional cross-linking agent, glutaric dialdehyde (GDAH), and then the excess GDAH was washed out (see Supporting Information). This resulted in one aldehyde end covalently linked with the internal wall of FMS by forming a Schiff base, leaving the other aldehyde end available for the subsequent covalent linkage with GI. P_{LD} of GI covalently linked in this way was 46.6 mg/g FMS, with the specific activity of 17.3 units/mg, resulting in an I_e of 97.7%, indicating that our procedure reduced or eliminated the issue of the intermolecular or intramolecular cross-linking of the protein by the cross-linking agent. Introduction of the covalent linker results in a decrease of the pore surface area and pore size, so P_{LD} of covalently linked GI decreased in comparison with that spontaneously entrapped in NH_2−FMS (Figure 2).

Compared to FMS, NPS exhibited substantially lower A_{LD} and P_{LD} (Figure 2). These results, consistent with our earlier reports,[12,13] demonstrate that both appropriate functionalization and mesoporous nanostructures are paramount for high A_{LD} and P_{LD}.

Unlike our earlier studies with partially active OPH,[12,13] Figure 2 shows that there was no substantial activity enhancement with our partially active GI. We therefore asked whether intentionally induced conformational changes to GI in FMS might be required to significantly enhance its activity in FMS. Accordingly, different concentrations of urea were used to induce conformational changes in GI. We found that strong synergetic effects of urea and FMS can dramatically enhance GI activity, and once GI became entrapped in FMS, it displayed remarkable resistance even to very high concentrations of urea.

Figure 3 shows fluorescence emission spectra of urea-induced unfolding transitions of free GI and entrapped GI in FMS in the presence of increasing urea concentrations. Fluorescence emission was monitored at the excitation wavelength of 278 nm, allowing excitation of both tyrosinyl and tryptophanyl residues. In the presence of 0.4−6.8 M urea,

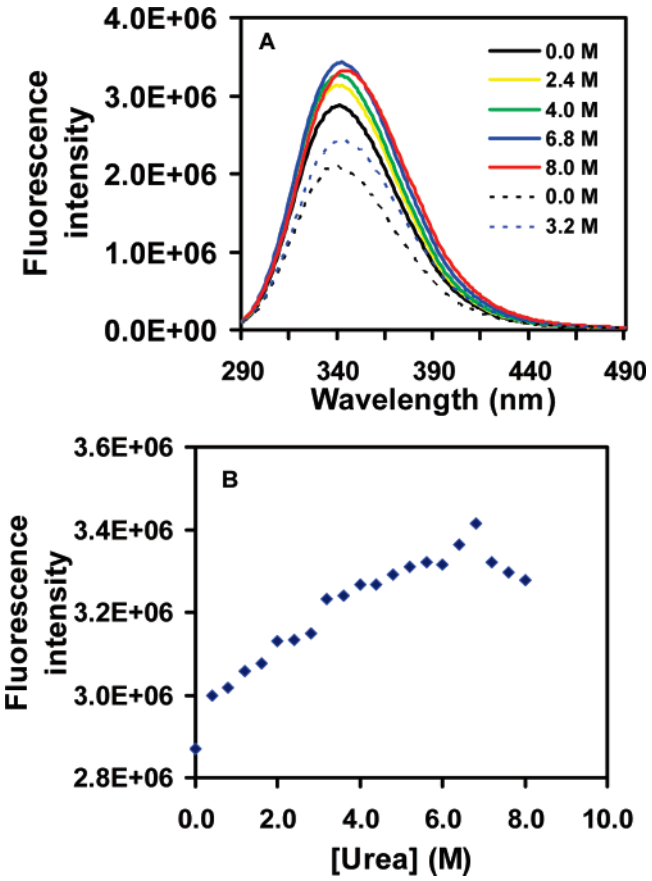

Figure 3. (A) Fluorescence emission spectra urea-induced conformational transitions of free GI (solid lines) and entrapped GI in FMS (dotted lines) with different urea concentrations in pH 7.5, 20 mM sodium phosphate/0.15 M NaCl/5 mM $MgSO_4$ with excitation at 278 nm at 21 ± 1 °C. The final protein concentrations of the free GI and the GI-FMS samples in the urea solutions were 0.10 and 0.05 mg/mL, respectively. (B) The fluorescence emission intensity at 340 nm of urea-induced conformational transitions of the free GI with the different urea concentrations.

the peak emissions of free GI were gradually red-shifted, accompanying the increasing emission intensity from more exposed tyrosinyl and tryptophanyl residues (Figure 3A,B). In the presence of >6.8 M urea, the peak emissions of free GI continued their red-shifts, where the peak wavelengths at 0.0 and 8.0 M urea are centered at 340 and 346 nm, respectively (Figure 3A), but the emission intensity decreased with decreasing exposure of the residues (Figure 3A,B). The entrapped GI in FMS shows the similar changing trend of the peak emissions. These observations confirmed conformational changes of GI induced by urea.

Figure 4 shows the specific activity of GI and GI−FMS as a function of urea concentrations. When urea was added to the free GI solution between 0.4−5.2 M, the GI conformation changed (Figure 3), but interestingly, the specific activity of the GI solution increased by as much as 43% compared to that in the absence of urea (Figure 4). These results show that the favorable conformational changes occurred and this trend was maintained regardless of the emission red-shifting until urea reached 5.2 M. At higher concentrations of urea, specific activity of free GI in solution decreased (Figure 4).

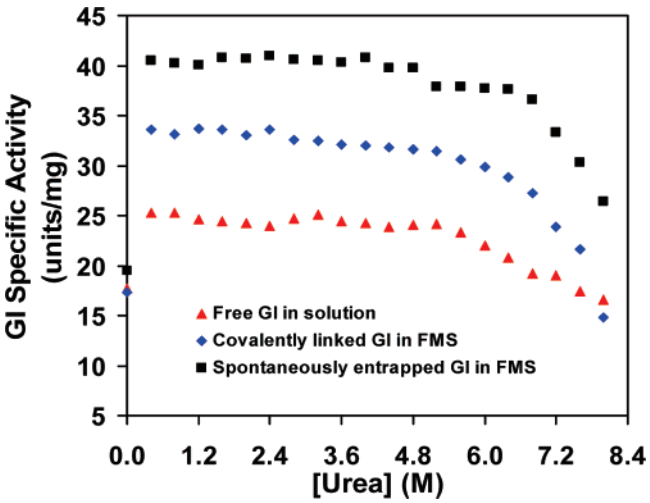

Figure 4. Urea-induced activity enhancement of GI and GI-FMS in pH 7.5, 20 mM sodium phosphate/0.15 M NaCl/5 mM $MgSO_4$, GI specific activity as a function of urea concentrations.

More importantly, specific activity of GI was enhanced as much as 128% and 90% when spontaneously entrapped and covalently linked in FMS, respectively, at the same concentrations of urea (Figure 4). The actual specific activity of GI−FMS (~41.0 units/mg) reach as high as more than double that of GI in solution in the absence of urea, almost completely returning to the specific activity level (43.9 units/mg) before freezing. These results demonstrate that, in the presence of urea, a much more favorable conformational change of GI confined in FMS occurred in comparison with free GI in solution. The specific activity of GI in FMS was relatively stable over the wide range of 0.4−6.4 M urea when GI spontaneously entrapped, and 0.4−5.2 M when GI was covalently linked in FMS. The spontaneously entrapped GI in FMS showed even more enhanced activity and stability than the covalently linked GI (Figure 4), demonstrating that the spontaneously entrapped GI might exploit its greater freedom toward more favorable conformational changes than the covalently linked GI. Even in 8.0 M urea, the specific activity of GI spontaneously entrapped in FMS was still higher than the highest specific activity of GI free in solution (Figure 4), indicating that the synergetic effect of urea and FMS played critical roles for the GI activity enhancement and the strong tolerance of GI in FMS to the high concentration of urea.

Our fluorescent measurements (Figure 3) confirmed urea-induced conformational changes of GI, and the increased specific activities (Figure 4) demonstrated these conformational changes were favorable. But at very high concentrations of urea, the free proteins in solution lost their activity rapidly toward the complete unfolding of the proteins as expected.[14−17] Molecular crowding/confinement theory and modeling efforts have predicted that a protein inside a confined space would be stabilized by some folding forces not present for proteins in bulk solutions.[30−33] The free GI in solution from *Streptomyces rubiginosus* used in this work may have become misfolded during the long-term frozen storage, but urea induced favorable conformational changes in GI to some extent by loosening up of the misfolded

protein. FMS might provide a beneficial confined space and thereby reinforce the trend toward favorable conformational changes, yielding much enhanced enzyme activity and stronger resistance to high concentrations of urea. The detailed mechanisms for these synergetic effects of FMS and urea needs to be further elucidated.

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Supporting Information Available: Material preparation and measurement details. This material is available free of charge via the Internet at http://pubs.acs.org.

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