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A Magnetically Separable, Highly Stable Enzyme System Based on Nanocomposites of Enzymes and Magnetic Nanoparticles Shipped in Hierarchically Ordered, Mesocellular, Mesoporous Silica**

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Enzymes are versatile nanoscale biocatalysts and find increasing applications in many areas, including organic synthesis[1–3] and bioremediation.[4–5] However, the application of enzymes is often hampered by the short catalytic lifetime of enzymes and by the difficulty in recovery and recycling.

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Recent advances in nanotechnology provide more diverse materials and approaches to solve these problems. For example, mesoporous materials offer potential advantages as a host of enzymes due to their well-controlled porosity and large surface area for the immobilization of enzymes.[6,7] On the other hand, it has been demonstrated that enzymes attached on magnetic iron oxide nanoparticles can be easily recovered using a magnet and recycled for iterative uses.[8a,b] The magnetic nanoparticle and enzyme were simultaneously entrapped in a sol–gel silica matrix, resulting in magnetically separable, heterogeneous biocatalysis.[8c] Sol–gel entrapped lipase and ruthenium complexes have been used for a one-pot esterification and hydrogenation reaction.[8d]

In this paper, we report the development of a magnetically separable and highly stable enzyme system by the combined use of two different kinds of nanostructured materials: magnetic nanoparticles and mesoporous silica. We immobilized nanometer-scale composites of enzyme molecules and magnetite nanoparticles in hierarchically ordered, mesocellular, mesoporous silica (HMMS) via a ship-in-a-bottle approach, which employs co-adsorption of enzymes and magnetite (Fe_3O_4) nanoparticles followed by crosslinking enzyme molecules via glutaraldehyde (GA) treatment. HMMS, prepared using a single structure-directing agent under near-neutral conditions, consists of 37-nm cellular mesopores and 13-nm-sized mesoporous channels around the mesocellular pores.[9] Under optimal conditions, the crosslinking of enzyme molecules via GA treatment can result in the effective entrapment of neighboring magnetite nanoparticles, and the composites of crosslinked enzyme aggregates (CLEAs) and magnetite nanoparticles in the large mesocellular pores will not leach out from the HMMS through the narrow mesoporous channels. This ship-in-a-bottle approach will result in effective enzyme immobilization that is magnetically separable, stable in enzyme activity, iteratively recyclable, and highly loaded with a large amount of enzymes. We name these composite aggregates magnetically separable, crosslinked enzyme aggregates (M-CLEAs).

To cause the effective entrapment of magnetite nanoparticles in CLEAs, it is important to prepare a homogeneous mixture of magnetite nanoparticles and enzyme molecules in solution. However, this task is challenging as magnetite nanoparticles are dispersable in organic solvents in which enzymes are generally not soluble.[10] On the other hand, the solubilization of each magnetite nanoparticle into water is still difficult without nanoparticle aggregation. Herein, we solubilized enzymes in an organic solvent by forming ion-pair complexes with surfactant.[11] This solubilization method can be used to prepare a high concentration of various enzymes in organic solvents[11] and has been previously used to incorporate enzymes into various composite materials.[12] In this paper, this technique was used for the preparation of a homogeneous mixture of enzymes and magnetite nanoparticles in organic solvents, such as hexane.

As an initial model case, we used α-chymotrypsin (CT) and 6-nm-sized, monodisperse magnetite nanoparticles for the preparation of of M-CLEAs in HMMS. We chose 6-nm magnetite nanoparticles to allow the easy adsorption of magnetite nanoparticles into HMMS. It is also important to use these superparamagnetic nanoparticles rather than ferromagnetic nanoparticles for practical applications, because the final magnetic nanocomposites should retain no residual magnetism after the magnetic field is removed (see Supporting Information).

The preparation of M-CLEA-CT in HMMS consists of a two-step process, as shown in Figure 1 a. The typical preparation procedure for M-CLEA-CT is as follows: 6-nm mag-

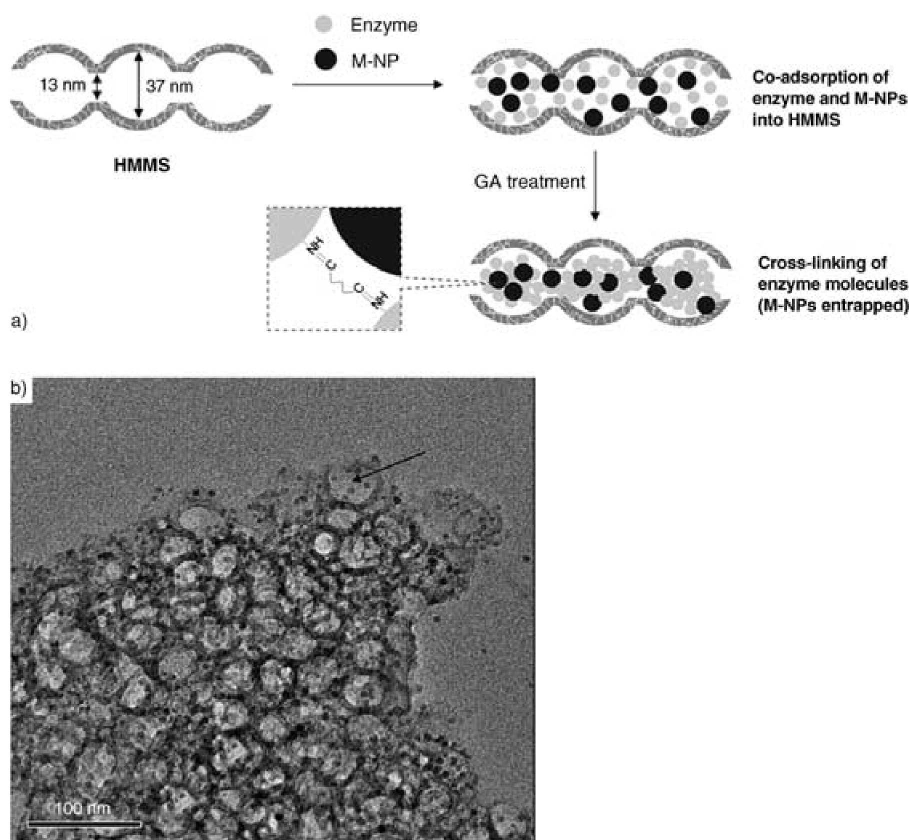

Figure 1. a) A schematic diagram for the preparation of M-CLEAs in HMMS (M-NPs: magnetic nanoparticles). b) Transmission electron microscopy image of M-CLEA-CT in HMMS. One of the mesocellular pores (40 nm) is arrowed to show the effective entrapment of the magnetic nanoparticles without any aggregation.

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netite nanoparticles[10b] in hexane (0.8 mL; 5 mg mL^{-1}) and CT in hexane (0.8 mL; 5 mg mL^{-1}) were mixed well, and HMMS (10 mg) was added for the co-adsorption of CT and magnetite nanoparticles. The mixture was incubated at room temperature by shaking (250 rpm). After 30 min incubation, the sample was dried at room temperature until the hexane was completely evaporated. Glutaraldehyde solution (0.1 %) treatment, followed by Tris-capping (Tris: Tris(hydroxymethyl)aminomethane) of unreacted aldehyde groups, generated M-CLEA-CT. A control sample without the glutaraldehyde treatment was also prepared.

After Tris-capping, M-CLEA-CT and the control sample were further washed under shaking at 250 rpm, followed by centrifugation or by application of a magnetic field. The supernatant of M-CLEA-CT after the centrifugation is colorless and transparent (Figure 1S a, Supporting Information) and contains no Fe, according to inductively coupled plasma (ICP) analysis. This indicates that magnetite nanoparticles do not leach out of HMMS under vigorous shaking (250 rpm). On the contrary, the supernatant of the control sample has a brownish color (Figure 1S a), representing the leaching of magnetite nanoparticles from HMMS. In addition, the supernatant of the control sample (no GA treatment) after applying a magnet is turbid, suggesting that HMMS powder cannot be captured any more by a magnet due to a rigorous leaching of magnetite nanoparticles from HMMS (Figure 1S b, Supporting Information). These results suggest that GA treatment results in effective entrapment of magnetite nanoparticles in HMMS.

M-CLEA-CT was characterized by transmission electron microscopy (TEM) (Figure 1 b). The magnetite nanoparticles are well distributed all over the pores in HMMS, and there is no aggregation of magnetite nanoparticles outside the pores of HMMS, demonstrating that magnetite nanoparticles are well entrapped inside the HMMS silica. CT cannot be observed in the TEM images due to the low contrast of the organic component in CT.

The loadings (w/w) of immobilized CT molecules and magnetite nanoparticles in M-CLEA-CT, measured using elemental analysis and ICP analysis, were 19.6 and 3.09 %, respectively, while those in the control sample were 8.87 and 3.53 %, respectively. This suggests that GA treatment improves the retention of initially adsorbed CT in HMMS, while the control sample without GA treatment results in a continuous leaching of CT. The yield of CT adsorption was 61 % for the preparation of M-CLEA-CT, showing that GA treatment entrapped 2.44 mg of CT out of the initially added 4 mg of CT. The CT-adsorption yield for the control sample was lowered to 24 % due to a rigorous leaching of CT.

The specific activities of the control sample, M-CLEA-CT, and free CT were 13, 1041, and 25 200 μM min^{-1} per milligram of CT, respectively. The specific activity of M-CLEA-CT is 24 times lower than that of free CT, but 80 times higher than that of the control sample. The huge difference in the specific activities between the control sample and M-CLEA-CT can be explained by the denaturation and/or autolysis of CT molecules in the control sample during the immobilization process. CT is well known to be autolyzed,[13] while GA crosslinking is also known to inhibit this autolysis.[14]

We also prepared M-CLEA with *Mucor javanicus* lipase (LP); these molecules do not inactivate via autolysis. The specific activities of the control sample and M-CLEA-LP were 0.67 and 0.73 μM min^{-1} per milligram of LP, respectively. These values of specific activities are comparable to that of free LP (0.75 μM min^{-1} per milligram of LP), suggesting that LP was marginally inactivated during adsorption and the subsequent GA treatment. This marginal inactivation of M-CLEA-LP is well supported by a recent report on the preparation of CLEAs with various enzymes[15] and also suggests that the lowered activities of M-CLEA-CT and its control sample are due to the autolysis of CT molecules during the immobilization process (see Supporting Information for a detailed explanation).

Figure 2 shows the stability of the M-CLEA-CT in aqueous buffer (10 mM sodium phosphate, pH 7.8) at room temperature. The samples were shaken at 250 rpm for at least 30 min and separated by using a magnet. For each cycle, buffer was decanted after separation, and the same amount of new buffer was added (Figure 2 a). The control sample showed a continuous loss of CT activity, which is derived from continuous leaching of CT and magnetite nanoparticles from HMMS. On the other hand, M-CLEA-CT clearly stabilized the CT activity for many iterative cycles of enzymatic reaction and separation using a magnet. For example, 89 % of initial activity was preserved after 29 iterative cycles of enzyme reaction and separation. The crosslinking between CT molecules by GA treatment not only prevented the leaching of CT and magnetite nanoparticles but also stabilized the CT activity during the iterative recycling of M-CLEA-CT. This suggests that magnetite nanoparticles are well entrapped in CLEA-CT, and the resulting M-CLEA-CT stabilized the CT activity due to both no leaching of M-CLEA-CT from HMMS and prevention of CT autolysis. We also observed a similar stabilization of *Mucor javanicus* lipase (LP) in another form of M-CLEAs (see the Supporting Information).

Figure 2 c shows the stability of the M-CLEA-CT under shaking (200 rpm). Free CT was completely inactivated after six days due to autolysis. The control sample showed a continuous decrease of the CT activity. These results suggest that physically adsorbed CT molecules in the control sample continuously leach out from HMMS under shaking. Leached CT would be quickly inactivated due to autolysis and/or irreversible denaturation. On the other hand, the initial activity of M-CLEA-CT was maintained for 14 days under harsh shaking. This result suggests that M-CLEA-CT in HMMS is too large to be leached out through the smaller mesoporous channels of HMMS, resulting in the impressive stabilization of CT activity even under rigorous shaking.

Finally, we investigated the stability of M-CLEA-LP in the presence of two proteases (Figure 3). Since some applications of enzyme reactions occur in the presence of various proteases,[16] the proteolytic resistance of immobilized enzymes is a benefit in their practical application. CT and trypsin (TR) were used as model proteases.[17] Figure 3 shows the stability of free LP and M-CLEA-LP in the pres-

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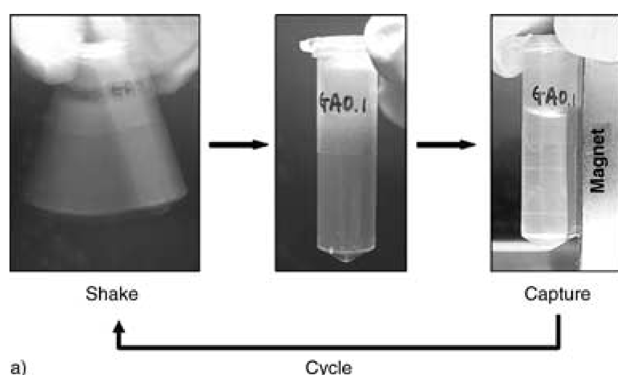

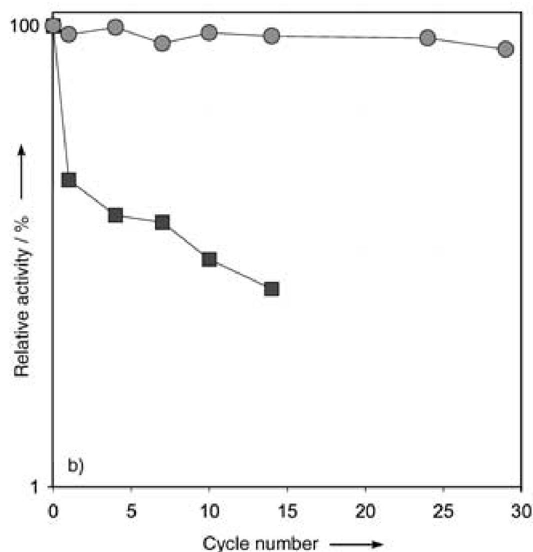

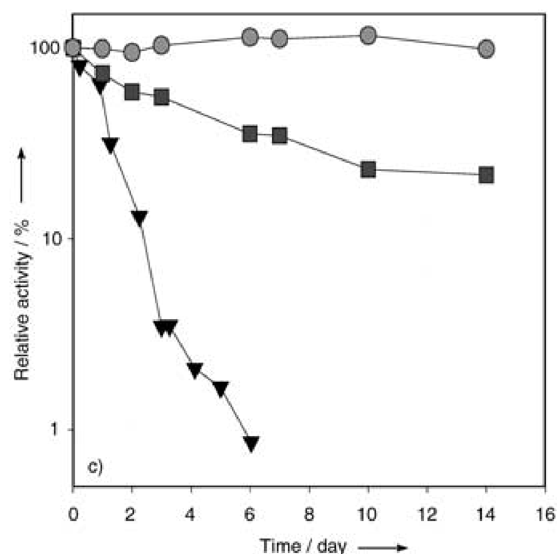

Figure 2. a) Iterative cycles of enzyme reaction and magnetic separation using a magnet. b) Stability of M-CLEA-CT (●) and the control sample (■, no GA treatment). The relative activity (%) represents the ratio of residual activity to initial activity of each sample. c) Stability of M-CLEA-CT (●) and the control sample (■) under rigorous shaking (200 rpm). ▼: free CT.

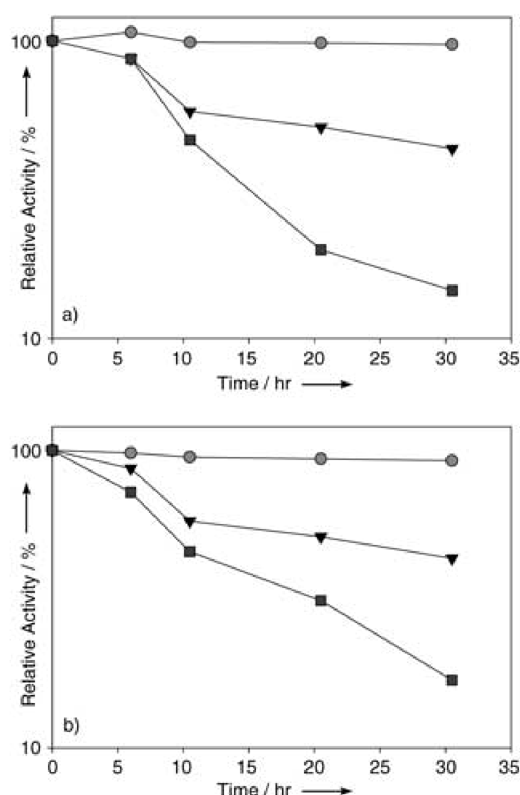

Figure 3. The stabilities of free LP (■) and M-CLEA-LP (●) in the presence of two different proteases: a) CT and b) TR (trypsin), under rigorous shaking. ▼: free LP in the absence of the proteases. M-CLEA-LP showed no decrease of LP activity at all in the presence of proteases.

ence of proteases under shaking. In the absence of protease, free LP resulted in 40% relative activity after 30 h of incubation under shaking. The addition of CT and TR reduced the relative activities of free LP to 14 and 17%, respectively. These results demonstrate that free LP is digested by these two proteases. However, M-CLEA-LP showed no decrease of LP activity at all in the presence of proteases. We speculate that most of the enzyme molecules in M-CLEAs are not accessible to the proteolytic digestion due to the steric hindrance against the penetration of proteases into the multipoint-attached enzyme aggregates. This proteolytic resistance of M-CLEAs will have a great impact on the applications of enzymes in the presence of proteases.

In summary, we have developed a unique approach for multifunctional nanocomposites of enzymes and magnetic nanoparticles in HMMS; this approach employs a simple, two-step process that involves the co-adsorption of enzyme and magnetic nanoparticles into HMMS followed by GA treatment. These nanocomposites, termed M-CLEAs, are proven to be magnetically separable, highly loaded with enzymes, stable under harsh shaking conditions, resistant to proteolytic digestion, and recyclable for iterative use with negligible loss of enzyme activity. This doubly effective, ship-in-a-bottle approach for a magnetically separable and stable form of enzyme immobilization can be easily expanded to many other enzymes and has great potential in many enzyme applications, such as bioremediation and bioconversion.

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enzyme catalysis · magnetic nanoparticles · magnetic separation · mesoporous materials · silica

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