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"Fish-in-Net" Encapsulation of Enzymes in Macroporous Cages as Stable, Reusable, and Active Heterogeneous Biocatalysts**

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There is currently great interest in enzyme immobilization to enhance enzyme stability and reusability, and to aid in separation from the reaction mixture,[1–17] but immobilized enzymes on commonly used inorganic and organic solid supports show low activities. This is a result of the leaching of the enzymes from the solid supports and the limited conformational transitions available to the enzymes for chemical interaction on the supports.[1–4] Enzymes encapsulated by a sol–gel/polymer[3–10] show good activity, but the wide pore-size distribution in sol–gel/polymers cannot be well controlled, and this adversely influences the diffusion of reactants and products during biocatalysis to the detriment of their practical application.[3,4,16] Recently, a number of successful examples of good enzyme activity resulting from enzyme immobilization in uniform mesopores of ordered mesostructured materials have been reported.[14–17] However, enzyme immobilization in mesopores is limited by the pore size of the mesostructured materials, so that bulky enzymes or enzyme aggregates larger than the mesopores cannot be immobilized.

A general and facile approach for the encapsulation of enzymes of various sizes in ordered mesoporous silica is reported here, where the enzymes are entrapped in *macroporous* cages connected by uniform mesoporous channels. These encapsulated enzymes show good activity, long-term stability, and excellent recycling characteristics. The concept of "fish-in-net" encapsulation of enzymes in ordered mesoporous silica under mild conditions is illustrated in Figure 1. Tetraethylorthosilicate (TEOS) was first assembled from a triblock ethylene oxide (EO)/propylene oxide (PO) copolymer surfactant ($EO_{20}PO_{70}EO_{20}$, P123) in ethanol. After evaporation of the ethanol and addition of glycerol, preformed precursors with ordered mesostructured silica particles were obtained in the glycerol solution, which is a non-denaturing solvent for enzymes. The preformed precursors were mixed with the enzyme solution under stirring at 4 °C. During the interaction between the enzymes and the preformed precursors, active enzymes (acting as the "fish") were gradually entrapped in the "net" formed by the polymerization and condensation of the ordered mesostructured silica particles. After removal of the glycerol and water by evacuation, the xerogels with encapsulated enzymes were washed with ethanol and water several times to remove polymer surfactants in the mesopores. In contrast to the "ship-in-a-bottle" technique,[18] the enzymes in this work were used as templates for the formation of the macroporous cages. The encapsulated enzymes in the mesoporous silica are typical nanoreactors, which combine the advantages of native enzymes with those of mesoporous channels. When water is introduced into the cages, the chemical environment of the enzymes in the cages is similar to that of native enzymes in aqueous solution. This is beneficial for protein rotation and conformational transitions, and provides for high biocatalytic activity.[3,16] Moreover, the ordered mesoporous channels play a very important role in protecting the enzymes from leaching and providing a path for the diffusion of reactants and products during the biocatalytic processes.

Table 1 presents the catalytic activities for various reactions of native and encapsulated enzymes of fumarase, trypsin, lipase, and porcine liver esterase (PLE). Fumarase is catalytically active for the chiral synthesis of L-malic acid, which is one of the most important food acids (Fig. 2a).[19] Trypsin is a hydrolytic biocatalyst for the characterization of enzyme activity (Fig. 2b).[20] Lipase is a typical biocatalyst for enantiomeric separations (Fig. 2c).[21] PLE is a representative biocatalyst for the conversion of cyclopropyl acetate into cyclopropanol (Fig. 2d).[22] All of the encapsulated enzymes retain over 90 % (±13 %) of the activities of the native enzymes (Table 1, Runs 1–16). The high efficiency of the encapsulated enzymes can be partly attributed to the mild conditions (neutral

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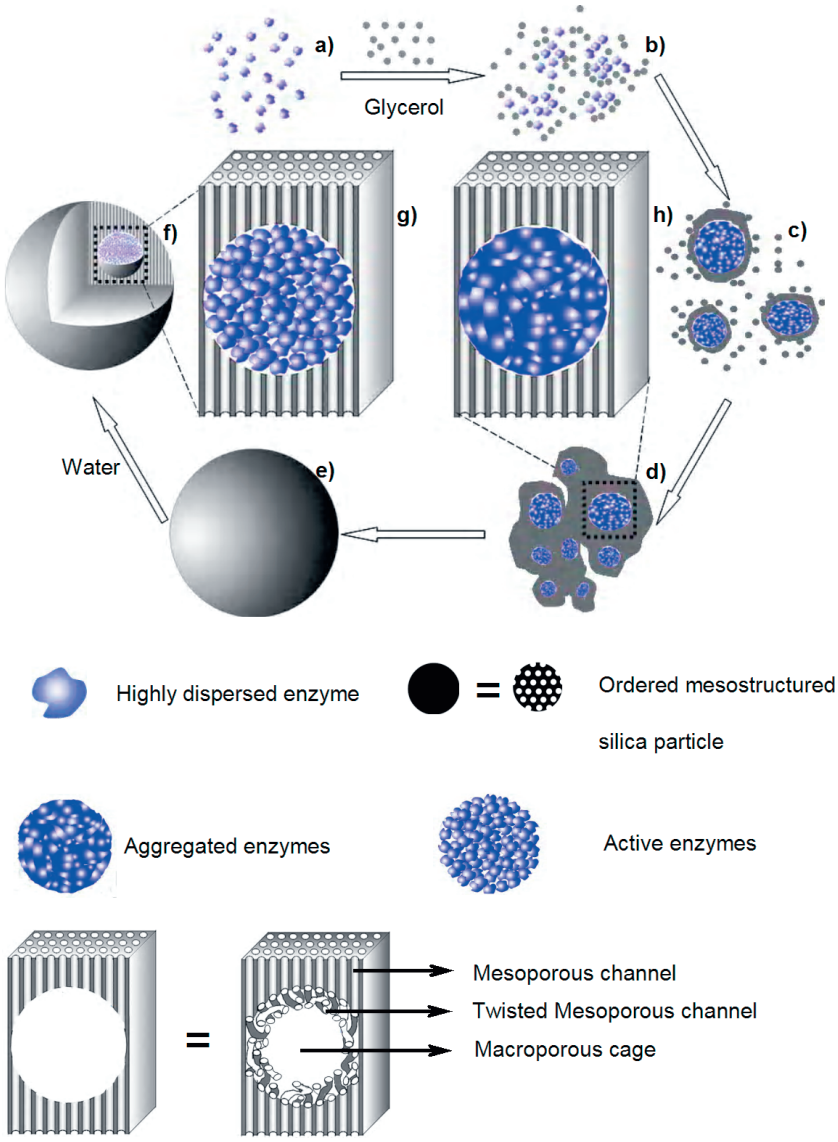

Figure 1. Schematic representation of "fish-in-net" encapsulation of enzymes: a) buffer solution containing highly dispersed enzymes; b) preformed precursors with ordered mesostructured silica particles mixed with the enzymes; c) assembly of enzymes with ordered mesostructured silica particles; d) inorganic polymerization and condensation; e) formation of silica spheres encapsulating enzymes in macroporous cages; and f) silica sphere with the encapsulated enzymes under biocatalytic conditions, with a chemical environment for the enzymes in the macroporous cages similar to that of native enzymes in solution. It is probable that the enzymes are dispersed; g) magnification of the enzyme-filled macroporous cages from (f); and h) magnification of the enzyme-filled macroporous cages from (d), where the enzymes are aggregated in the macroporous cages.

pH, non-denaturing solvent, and mild temperature and pressure), which minimize enzyme denaturation.[3–6] In addition, the recycled encapsulated enzymes still show comparable activities to those of the native enzymes (Runs 3–4, 7–8, 11–12, and 15–16), even though monodisperse trypsin is only slightly smaller in size than the mesopore (Table 1). For example, after being recycled 30 times, the encapsulated trypsin still exhibits 90 % (±10 %) of the initial activity (Fig. 3). In contrast, the corresponding enzymes immobilized on SBA-15 mesoporous silica (denoted SBA-15/enzymes) show relatively low stability, activity, and reusability. For example, after 6 recycles, all of the SBA-15/enzyme samples lose their activities. These results indicate that the enzymes encapsulated by the "fish-in-net" technique are stable, reusable, and active.

Figure 4 shows scanning electron microscopy (SEM) and transmission electron microscopy (TEM) images of the encapsulated enzyme samples. Samples containing the encapsulated enzymes have a spherical morphology with a particle size of 10–20 μm (Figs. 4a,b). Many macroporous cages of sizes 100–2000 nm are observed inside these spheres (Fig. 4c). The macroporous cages provide enough space for rotation and the conformational transitions of the enzymes, which are essential for biocatalysis.[1–4] In contrast, these macroporous cages are not formed when the enzymes are absent during the preparation of the samples. This suggests that the macroporous cages are formed using the enzymes as templates. Taking into consideration that the sizes of the monodisperse enzymes are 3–10 nm depending on the enzyme,[3,16,23] it is proposed that the enzymes in the glycerol solution are aggregated.[3,23] Furthermore, the TEM images (Figs. 4d–f) show that all the macroporous cages are connected by ordered and uniform mesopores, which is helpful for the fast diffusion of reactants and products during the biocatalysis.

The ordered hexagonal mesopores around the macroporous cages are gradually twisted (Figs. 4d–f). This phenomenon might be related to an interaction between the charged enzymes and the surfactant micelles, which is in good agreement with salt effects in the synthesis of ordered mesoporous materials.[24] It is possible that the twisted mesopores are another factor that prevents leaching of the enzymes from the samples.

The high activity and stability, and the excellent recycling behavior of the encapsulated enzymes in the samples should be attributed directly to this unique "fish-in-net" encapsulation under mild conditions. Although this method was tested by using fumarase, trypsin, lipase, and PLE enzymes, it should be applicable to other enzymes and cells. In addition, other surfactants can also be used. For example, if surfactants such as F127 ($EO_{106}PO_{70}EO_{106}$) and Brij56 ($C_{16}EO_{10}$) are used in the synthesis, encapsulated enzyme samples with various mesostructures (cubic and hex-

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Table 1. Catalytic activities in various reactions over native and encapsulated enzymes.

Enzyme [a]	Biocatalytic conversion	Run	Sample	Conversion [%]	Relative activity [b]
Fumarase	From fumaric acid into	1	Native	81.5	1.00
M_w = 196 000 g mol^{-1}	L-malic acid [d]	2	Encapsulated (1) [c]	77.2	0.936
Spherical size = 7.8 nm		3	Encapsulated (2)	76.8	0.931
		4	Encapsulated (10)	77.3	0.937
Trypsin	From *N*-benzoyl-L-arginine-*p*-nitroanilide	5	Native	99.0	1.00
M_w = 23 400 g mol^{-1}	into benzoyl-L-arginine and *p*-nitroaniline [e]	6	Encapsulated (1)	92.1	0.930
Spherical size = 3.8 nm		7	Encapsulated (2)	93.1	0.941
		8	Encapsulated (30)	90.1	0.910
Lipase	From glycidyl butyrate into *R*(–)-glycidyl	9	Native	58.6	1.00
M_w = 33 000 g mol^{-1}	butyrate [f]	10	Encapsulated (1)	68.5	1.17
Spherical size = 4.3 nm		11	Encapsulated (2)	66.7	1.14
		12	Encapsulated (10)	65.5	1.12
PLE	From cyclopropyl acetate into	13	Native	91.0	1.00
M_w = 162 000 g mol^{-1}	cyclopropanol [g]	14	Encapsulated (1)	87.3	0.959
Spherical size = 7.3 nm		15	Encapsulated (2)	86.2	0.947
		16	Encapsulated (10)	85.9	0.944

[a] Spherical size: the spherical diameter calculated from the partial specific volume (approximated to 0.74) [23]. M_w: weight-average molecular weight. [b] Relative activity: ratio of the activity of the encapsulated enzyme to the native enzyme (the amount of enzyme is the same in the reaction). [c] The value in parentheses denotes the number of times the encapsulated enzyme reactor was recycled. [d] The reaction was measured at 25 °C in 50×10^{-3} M phosphate buffer (pH 7.3, 3 h) [19]. [e] The reaction was measured at 37 °C in 50×10^{-3} M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer and 20×10^{-3} M $CaCl_2$ (pH 7.4, 15 min) [20]. [f] The reaction was measured at 27 °C in 50×10^{-3} M phosphate and 50×10^{-3} M hexane buffer (pH 7.6, 1 h) [21]. [g] The reaction was measured at 20 °C in 5×10^{-3} M sodium phosphate buffer (pH 7.5, 4 h) [22].

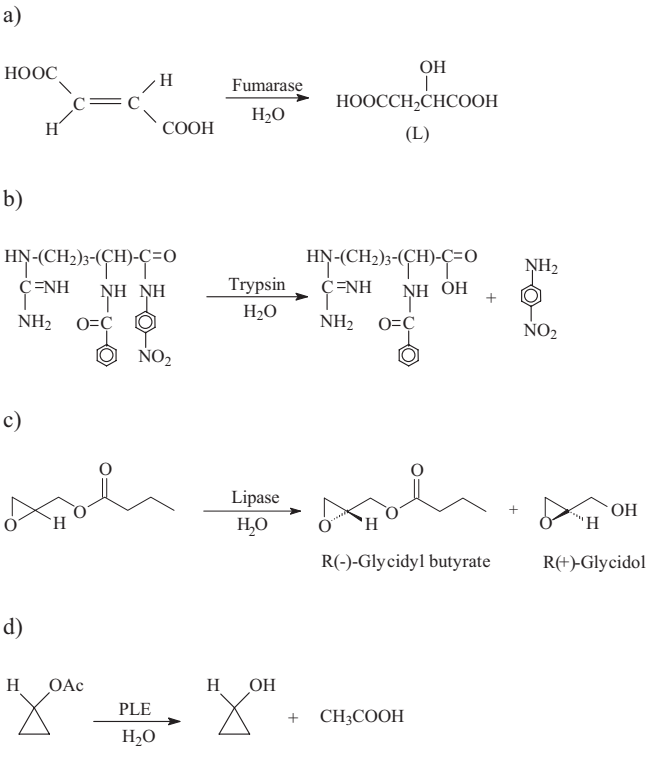

Figure 2. Reactions catalyzed by enzymes in this study.

agonal) and sizes (2–30 nm) can be obtained.[25–30] The strategy developed here therefore provides a general, facile, and unique approach for the encapsulation of enzymes with long-term and extraordinary recycling stability and high activity.

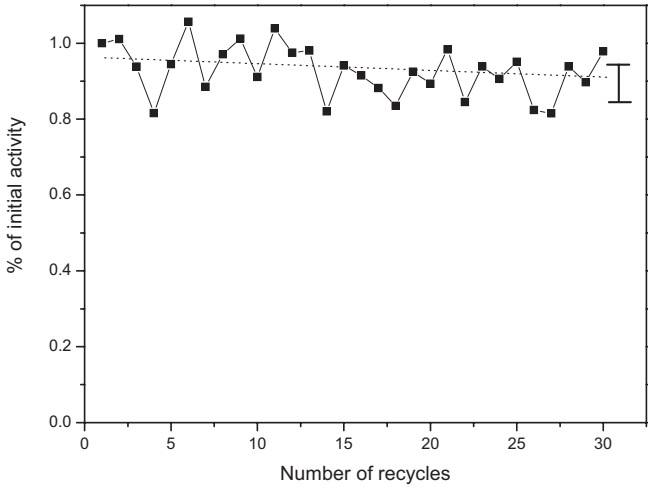

Figure 3. Stability of encapsulated trypsin activity with the number of recycles.

Experimental

A typical encapsulation of enzymes was prepared in the following way: 10 g of TEOS was mixed with 25 g of ethanol and 4.5 g of HCl (0.1 M aqueous solution) under stirring for 1–2 h, followed by the addition of P123 solution (4.2 g of P123 dissolved in 15 g of ethanol). After stirring at room temperature for 24–48 h to evaporate the ethanol in the solution, leaving 12.5 g of residue, 10 g of glycerol was added. After the removal of ethanol by evacuation under vacuum, preformed precursors with ordered mesostructured silica particles (14–15 g) were assembled in the glycerol solution. 5 g of the preformed precursors was added into 5 mL of a buffer solution containing the enzymes, followed by evacuation under vacuum at 4 °C until xerogels were formed. After removal of the polymer surfactant

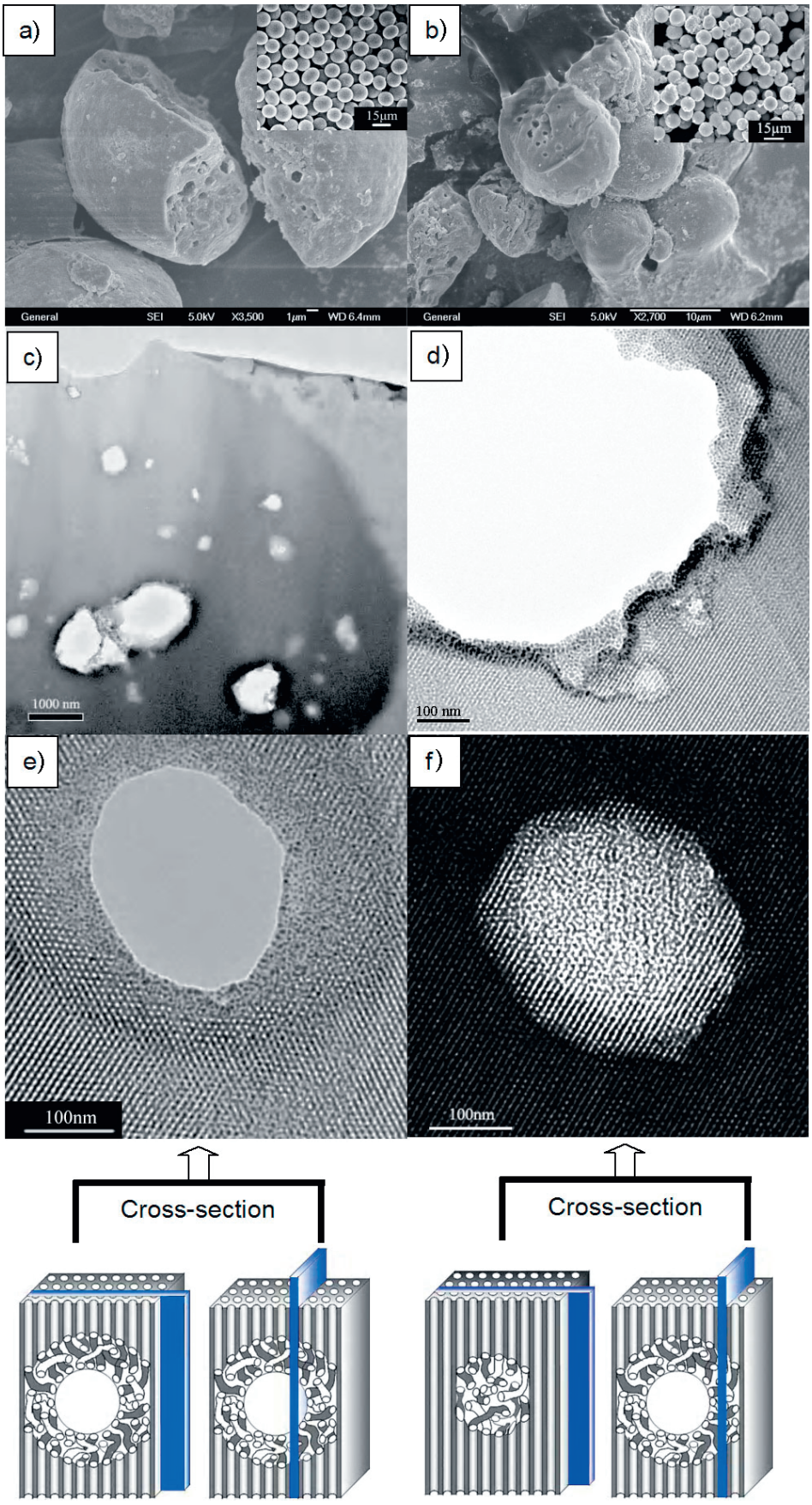

Figure 4. SEM images of a) the encapsulated fumarase after calcination at 550 °C for 3 h (inset: encapsulated fumarase); b) calcined encapsulated trypsin (inset: encapsulated trypsin) samples. c–f) TEM images of cross sections of calcined encapsulated trypsin samples. e,f) The ordered hexagonal mesopores around the macroporous cages are gradually twisted, as illustrated in the corresponding schematic representations of the cross sections of the TEM images.

(P123) in the xerogels by washing several times at 4 °C, the encapsulated enzymes were obtained.

For comparison, the conventional immobilization of enzymes with SBA-15 was carried out by mixing the enzymes with the ordered mesoporous silica. As a typical example, 5.0 mL of enzyme solution (15×10^{-6} M) in pH 6.0 buffer (50×10^{-3} M phosphate, Fisher) was mixed with 0.25 g of SBA-15. After stirring for 2 h at 4 °C, the sample was washed with water and dried in air.

X-ray diffraction (XRD) patterns were obtained with a Siemens D5005 diffractometer using Cu Kα radiation. SEM experiments were performed on a JSM-6700F electron microscope (JEOL, Japan). TEM experiments were performed on a JEM-3010 electron microscope (JEOL, Japan) with an acceleration voltage of 300 kV. The nitrogen adsorption and desorption isotherms at the temperature of liquid nitrogen were measured using a Micromeritics ASAP 2020M system. The samples were degassed for 10 h at 300 °C before the measurements.

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