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Short communication

Structured interlocked-microcapsules: A novel scaffold for enzyme immobilization

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ABSTRACT

A facile approach to prepare structured interlocked-microcapsules (SIMC) was developed, which combined the advantages of open mouthed structure, hierarchical porous nanostructure and interlocked architecture. The specific surface area of SIMC was 374.6 m^2/g and the diameter of the pores was 8.707 nm. Nitrile hydratase (NHase) was immobilized on SIMC via covalent bonding to realize the easy separation of the enzyme and improve properties of enzyme such as pH tolerance and heat stability. This work demonstrated that the enzyme-immobilized SIMC presented high catalytic performance and significantly improved stability.

1. Introduction

In recent years, mesoporous microcapsules have been widely concerned owing to their combination of hollow structures and mesoporous nanostructures [1–3]. Some of them like mesoporous silica nanoparticles included SBA, MCM and MSU have been widely used in preparing catalysts [4]. Due to their superior physicochemical properties, mesoporous microcapsules are particularly pursued as enzyme immobilization supports [5,6]. In general, both the microcapsule wall and the capsule lumen can be used for immobilizing enzyme. But up to now, most of the research encapsulated enzyme molecules in the capsule lumen, and the capsule wall only plays the role of preventing the leakage of enzyme molecules. What's more, the dense, thick capsule wall and the stagnant solution in the capsule lumen would cause the high transfer resistance between the capsule lumen and the bulk solution, and then the reaction rate would be influenced [7,8]. If the enzyme molecules were immobilized on the capsule wall, once the substrates come in contact with or diffuse through the capsule wall, the catalytic reaction would occur. Thus the catalytic efficiency will be significantly improved by increasing the contact probability of substrate and enzyme and shortening the mass transfer path. In addition, the special physical effects provided by the pores of capsule wall can endow the immobilized enzyme with improved chemical, biochemical, and mechanical properties. Therefore, it is urgent to design novel wall material according to the advanced concept of nanoarchitectonics, which was proposed for the assembly of structural materials at the nanoscale level [9]. Recently, a concept of open-mouthed microcapsules reactor was proposed for electrochemical and gaseous catalytic applications. Like the function of the cell membrane, the structure makes full use of the inner and outer wall of the microcapsule by forming open-mouth on the capsule wall. Benefiting from the unique structure, the capsule lumen is exposed and mass transfer resistance is also decreased [10]. This concept provides a new opportunity for the construction of robust enzyme catalysts. Parallel to the development of the new catalyst pellets with macropores, structuring of the catalyst is also an efficient way to enhance the effective mass diffusivity [11]. The structured catalysts are considered to be a promising research direction in the field of heterogeneous catalysis [12,13]. In our previous study, three-dimensional ordered macroporous biocatalysts were successfully prepared [14], and superior catalytic performance was obtained.

Hence, it can be conjectured that setting up a structured biocatalyst composed of open-mouthed microcapsules (or the structured interlocked-microcapsules, SIMC) could enhance the catalytic performance of the immobilized enzyme significantly. However, to the best of our knowledge, no investigation of preparing SIMC for enzyme immobilization has been previously reported. Thus, for the first time, we present a facile approach to prepare novel structured interlocked-microcapsules with interconnected ordered structure for enzyme immobilization. Subsequently, the SIMC was functionalized with amine groups and activated with glutaraldehyde (GA) which is one of the most widely used reagents in enzyme immobilization [15]. GA could reacted with amine groups(both in the carrier or enzyme molecules) rapidly at around neutral condition [16], on this account, the NHase could be

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Nomenclatures

SIMC	Structured interlocked-microcapsules
PSCCT	Polystyrene colloidal crystals templates
PBS	Phosphate buffer solution
NHase	Nitrile hydratases
SIMC-NH_2	Amino-functionalized SIMC
APTES	3-Aminopropyltriethoxysilane
GA	Glutaraldehyde

immobilized in SIMC. It is likely to be the most common form of the immobilized enzyme with GA [17,18].

2. Experimental

2.1. Preparation of SIMC

The preparation process of the structured interlocked-microcapsules (SIMC) was shown in Scheme 1 (SEM images of all the steps were provided in Supporting Information, Fig. S1). Firstly, polystyrene colloidal crystals templates (PSCCT) were soaked in concentrated sulfuric acid for 2 h at room temperature. The reaction of sulfonation was taken place at 40 °C under stirring for 12 h. After the reaction was completed, the PSCCT was washed completely with ultra-pure water.

Then the sulfonated PSCCT (1.5 g) was immersed in an excess amount of TEOS for 5 h to allow the saturated absorption of TEOS. Subsequently, a mixture of ethanol/water (1:1 v/v) was added to form silica-based capsule wall through sol-gel process. After being aged and dried at 60 °C for 12 h, the composites were calcined to remove the template, and finally the SIMC was obtained.

2.2. Preparation of immobilized NHase and stability measurement

Briefly, SIMC was dispersed in ethanol, and then APTES was added slowly. The reaction was performed under reflux overnight. Thereafter, the solid was washed three times and then dried at 60 °C under vacuum for 10 h. The obtained solid was named SIMC-NH_2. Subsequently, SIMC-NH_2 was activated with GA (100 mg/mL) for 1.5 h and immersed in NHase solution for 4 h.

To assess the pH stability of free and immobilized NHase, their activities were measured after incubating in pH 4.0 and pH 10.0 sodium phosphate buffer solution (PBS) for different times at 30 °C. To determine thermal stability, both the free and immobilized NHase were immersed in 40 and 50 °C PBS and the activity was assayed at regular time intervals.

Storage stability of free and immobilized NHase was also tested by immersing them in pH 7.0 PBS at 4 °C. At regular time intervals, the samples were taken out to measure the residual activities.

2.3. Determination of kinetic parameters

Taking equal activity (1.441 U) of free and immobilized NHase reacted with different concentration of substrate range from 2 to 15 mM in pH 7.0 at 30 °C and the reaction system was maintained 2 mL. After 2 min, the reaction rate was determined by HPLC. The Km and Vmax values were calculated by the plotting method of Lineweaver-Burk, and Kcat was obtained from the formula: Kcat = Vmax/[E].

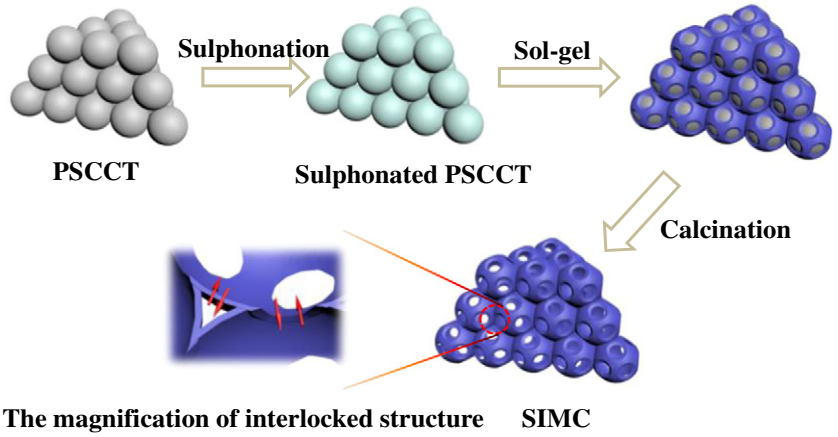
3. Results and discussion

3.1. Characterization of the SIMC

Fig. 1a shows that the SIMC is composed of vast regularly shaped microcapsules which have unique particle size of 400 nm. The macropores (open mouths) can be clearly observed on the capsule wall which stemmed from the point of contact between template microspheres. Owing to the SIMC is fabricated by replicating PSCCT which has a hexagonal close-packed structure [19], twelve macropores could be obtained on each microcapsule wall in theory. The microcapsule can highly coalescent through the edge of macropores (marked with red arrow) and further present a grape-like network which is internal interconnected. TEM image demonstrated the hollow structure and interlocked architecture of the SIMC as expected (Fig. 1b).

3.2. The pH and thermal stability of free and immobilized NHase

For enzyme immobilization, the amino-functionalized SIMC(SIMC-NH_2) was prepared by post grafting of 3-aminopropyltriethoxysilane (APTES) on SIMC. The SIMC-NH_2 was activated via GA and then the GA-activated SIMC-NH_2 was used to immobilize NHase through covalent bonding approach (the detailed immobilization process can be found in ESI)·The pH stability of free and immobilized NHase were shown in Fig. 2a, the immobilized NHase was inactivated at a slower rate than the free NHase. After 3 h of incubation in pH 4.0, the immobilized NHase maintained 93.85% of its initial activity. In contrast, free NHase only retained 11.74% of its initial activity. Similar behavior was also observed at pH 10.0. This can be attributed to the intense multipoint covalent attachment between enzyme molecule and SIMC via a short spacer arm, which will lead to significantly improved



Scheme 1. Schematic synthesis process of the structured interlocked-microcapsules.

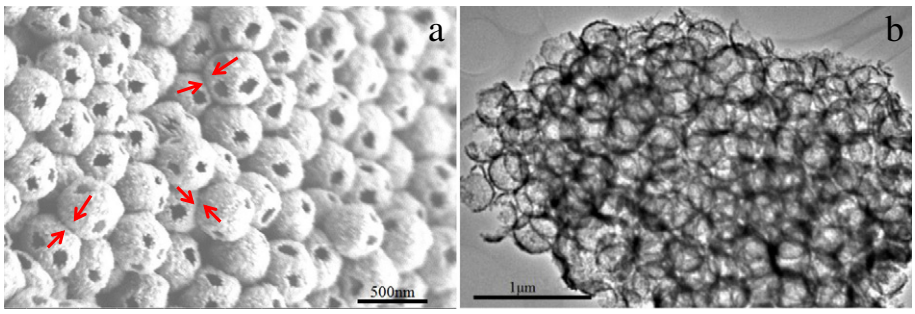


Fig. 1. SEM image (a) and TEM image (b) of SIMC.

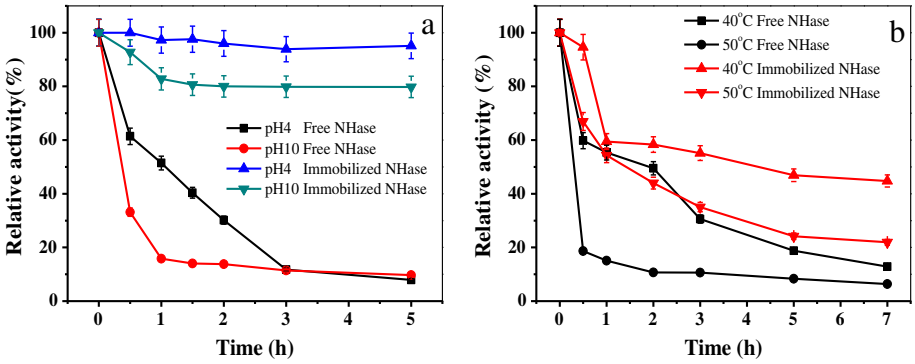


Fig. 2. (a) pH stability, (b) thermal stability of free NHase and immobilized NHase.

stability compared with the free NHase [20]. For practical applications, thermal stability of enzymes is one of the most important criteria. As shown in Fig. 2b, with the incubation time increased, the immobilized NHase retained higher activity than the free NHase. At 50 °C, free NHase retained only 18.69% of its initial activity after 0.5 h heat treatment, while the immobilized NHase retained 66.93% of the initial activity. After 7 h of incubation, the residual relative enzyme activity of immobilized NHase was more than three times higher than that of free NHase (21.92% and 6.35% resp.). Better behavior could be observed at 40 °C. The enhanced thermal stability could be caused by the covalent immobilization with the addition of short spacers on the SIMC surface and multipoint covalent bonding with SIMC will make a rigidification of the NHase's conformations [20–22]. This gives the immobilized NHase ability to maintain a constant conformation at higher temperature and retain higher activity.

3.3. Storage stability of free NHase and immobilized NHase

As shown in Fig. 3, it is obviously that storage stability of NHase had been greatly improved through immobilization. In the initial 9 days, the immobilized NHase maintained about 100% of the initial activity while the activity of free NHase was reduced to 83.99%. After 24 days storage, the residual activity of the immobilized NHase was 98.7%, which was much better than the free NHase (only maintained 56.16% of the initial activity). This may also be ascribed to the multipoint covalent attachment of NHase with the SIMC, which kept the enzymes in stable conformation [22].

3.4. Kinetic parameters of free NHase and immobilized NHase

After immobilization, the Km value of NHase increased compared to its free counterpart, indicating lower affinity of substrate and immobilized NHase (See Table 1). It may be caused by the unfavorable conformational transition in the structure of NHase during immobilization, which led to hindered accessibility of the substrate to the active site of the immobilized NHase [23,24]. Although a slight decrease in affinity, the Vmax of immobilized NHase provided a dramatic increase. The conformation changes of NHase were the mainly reason for the Vmax alteration. Usually, NHase was inhibited by free cyanide existed in acrylonitrile, which decreased the observed activity [25,26]. However, the inhibition would be reduced by inducing a certain distortion in the active site through multipoint covalent binding (confirmed by the optimum temperature of immobilized NHase). In addition, rigidification of the NHase conformation by multipoint covalent immobilization could prevent or at least diminish some allosteric inhibitions [27]. All of this will lead to a high reaction rate.

On the other hand, benefiting from the unique structure of the SIMC, the catalytic reaction can be controlled in a smaller space (the capsule lumen and the capsule wall) and the reactant has a higher concentration

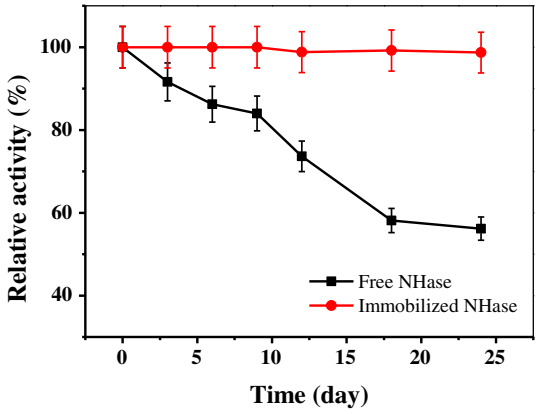


Fig. 3. Storage stability of free NHase and immobilized NHase.

Table 1
The kinetic parameters of free NHase and immobilized NHase.

	Km(mM)	Vmax(mM/min)	Kcat(S^{-1})	Kcat/Km ($L \cdot mmol \cdot S^{-1}$)
Free NHase	3.278 ± 0.1639	0.612 ± 0.0306	$4.898(\pm 0.2449) \times 10^3$	$1.494(\pm 0.0747) \times 10^3$
Immobilized NHase	18.42 ± 0.921	1.620 ± 0.081	$3.991(\pm 0.1996) \times 10^3$	$2.167(\pm 0.1084) \times 10^2$

in this particular space, which would lead to the high reaction rate [3]. The interpenetrating parallel channels originating from the macropores are also responsible for the reduction of internal diffusion resistance (as shown in Fig. S16) and then high reaction rate would be obtained. The Kcat value of free NHase was only 1.2 times higher than immobilized NHase, which indicated a slight decrease or loss in the catalytic capability. All the results demonstrated that immobilized NHase preserved the conversion ability of NHase to the largest degree. Consistent with previous reports, the ratio of Kcat/Km, which was generally used to compare the efficiencies of different enzymes with one substrate [28] also showed lower catalytic efficiency for NHase after immobilization. Presumably the decrease resulted from the external diffusion resistance.

4. Conclusions

In summary, a facile and efficient approach to prepare structured interlocked-microcapsules was developed and the obtained SIMC was used to immobilize enzyme. The NHase immobilized on the SIMC exhibited significantly enhanced stability compared to its free counterpart. Moreover, the improved catalytic activity and mass transfer performance was also obtained owing to the unique structure of SIMC. In the future studies, several factors such as compatibility of the SIMC with different enzymes and mesopore size on the capsule wall require further research work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.catcom.2016.09.010.

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