ARTICLE

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Encapsulation of Enzyme via One-Step Template-Free Formation of Stable Organic–Inorganic Capsules: A Simple and Efficient Method for Immobilizing Enzyme With High Activity and Recyclability

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ABSTRACT: Enzyme encapsulation is a simple, gentle, and general method for immobilizing enzyme, but it often suffers from one or more problems regarding enzyme loading efficiency, enzyme leakage, mechanical stability, and recyclability. Here we report a novel, simple, and efficient method for enzyme encapsulation to overcome these problems by forming stable organic–inorganic hybrid capsules. A new, facile, one-step, and template-free synthesis of organic–inorganic capsules in aqueous phase were developed based on PEI-induced simultaneous interfacial self-assembly of Fmoc-FF and polycondensation of silicate. Addition of an aqueous solution of Fmoc-FF and sodium silicate into an aqueous solution of PEI gave a new class of organic–inorganic hybrid capsules (FPSi) with multi-layered structure in high yield. The capsules are mechanically stable due to the incorporation of inorganic silica. Direct encapsulation of enzyme such as epoxide hydrolase SpEH and BSA along with the formation of the organic–inorganic capsules gave high yield of enzyme-containing capsules (~1.2 mm in diameter), >90% enzyme loading efficiency, high specific enzyme loading (158 mg protein g^{-1} carrier), and low enzyme leakage (<3% after 48 h incubation). FPSi-SpEH capsules catalyzed the hydrolysis of cyclohexene oxide to give (1*R*, 2*R*)-cyclohexane-1,2-diol in high yield and concentration, with high specific activity (6.94 U mg^{-1} protein) and the same high enantioselectivity as the free enzyme. The immobilized SpEH demonstrated also excellent operational stability and recyclability: retaining 87% productivity after 20 cycles with a total reaction time of 80 h. The new enzyme encapsulation method is efficient, practical, and also better than other reported encapsulation methods.

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Introduction

Biocatalysis has received great attention for green, selective, and sustainable synthesis of chemicals and pharmaceuticals. In comparison with whole-cell catalysis, enzyme catalysis could give cleaner reaction and easier product recovery. Immobilized enzyme has become increasingly important for practical biocatalytic synthesis, since it could enhance the enzyme stability and reduce the enzyme cost via recycling (Campbell et al., 2014; Chao et al., 2013; Jia et al., 2014; Lv et al., 2014; Sheldon and van Pelt, 2013). Among various enzyme immobilization methods (Bolivar and Nidetzky, 2012; Mariani et al., 2013; Sheldon and van Pelt, 2013; Wang et al., 2009; Zhou and Hartmann, 2013), encapsulation is a simple and gentle one, and it is suitable for immobilizing every type of enzymes. However, the reported enzyme encapsulations often show significant enzyme leakage, low loading efficiency, low mechanical stability, or low recyclability (Kurayama et al., 2012; Taqieddin and Amiji, 2004; Vikartovska et al., 2007; Wang et al., 2013; Zhang et al., 2009; Zhu and McShane, 2005). We recently developed peptide–polymer hybrid capsules for enzyme encapsulation to overcome some of these drawbacks (Huang et al., 2014), but the mechanical stability of the capsules needs further improvement. Therefore, it is necessary to develop more stable capsules for enzyme encapsulation.

Organic–inorganic hybrid capsules could be an ideal carrier for enzyme immobilization with high catalytic performance and excellent recyclability, since it could achieve unusual combinations of stiffness, strength, and toughness of organic and inorganic moieties and thus provide better properties such as higher stability than organic or inorganic moieties alone (Bagaria et al., 2010; Haase et al., 2011; Jiang et al., 2009; Ruiz-Hitzky et al., 2011; Shchukin

et al., 2003). A promising strategy to form organic–inorganic hybrid capsules involves biomimetic mineralization using an organic inducing agent such as a peptide, protein, or polymer (Bradt et al., 1999; Calvert and Rieke, 1996; Sommerdijk and de With, 2008). In previous studies, templates were frequently used as support for inducing agent in the formation of inorganic components. However, this template-assisted approach suffers from several general problems: the use of rigid templates often gives insufficient thickness and strength of the inorganic layer and thus reduces mechanical stability (Zhang et al., 2011). Layer-by-layer strategy is employed to increase the stability of such materials but it requires extensive workup (Haase et al., 2011; Jan et al., 2011; Jiang et al., 2009). The unnecessary rigid template likely increases the mass transfer resistance and the difficulty for further insertion of enzymes; the use of soft templates such as gel matrix often results in the mineralization of only partial templates (Bradt et al., 1999). The physical inclusion of targeted agents (e.g., enzymes) in such soft templates exhibits significant agent leakage or low loading efficiency during the inorganic mineral growth (Kurayama et al., 2012); in addition, using a template for capsule synthesis requires two or more steps, which increases the complexity and the cost of the synthesis. Therefore, it is highly desirable to develop one-step template-free synthesis of organic–inorganic capsules in aqueous phase for simple and practical enzyme immobilization. Thus far, no such kind of synthesis of organic–inorganic capsules has been reported.

Herein, we report a novel one-step template-free process to form stable organic–inorganic capsules containing silicate as inorganic component to enhance the stability via cationic polymer (PEI) induced simultaneous self-assembly of Fmoc-FF and polycondensation of silicate at an aqueous interface (Fig. 1). Furthermore, we report a novel, simple, and efficient enzyme immobilization method by encapsulating enzyme via the formation of such organic–inorganic capsules with epoxide hydrolase SpEH as a model enzyme. The high catalytic performance and recyclability of the immobilized enzyme are demonstrated in the enantioselective hydrolysis of cyclohexene oxide to give (1*R*, 2*R*)-cyclohexane-1,2-diol that is a useful and valuable fine chemical.

Material and Methods

Materials

Fmoc-diphenylalanine peptide (Fmoc-Phe-Phe-OH, Fmoc-FF, >98%) was brought from Bachem (Switzerland). Sodium silicate solution (reagent grade, Na_2O: ~10.6 wt%, SiO_2: ~26.5 wt%), branched polyethyleneimine (PEI) solution (Mw ~750 kDa, 50 wt% in H_2O), bovine serum albumin (BSA, Mw ~66 kDa, >96%),

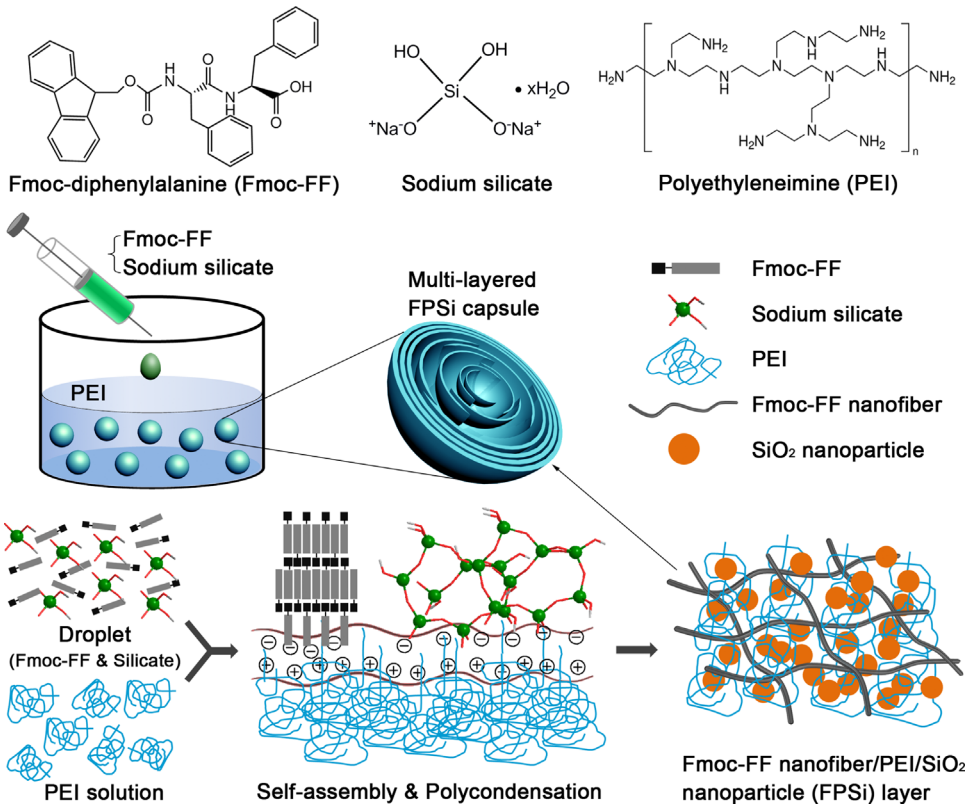

Figure 1. One-step template-free synthesis of multi-layered organic–inorganic capsules via cationic polymer induced simultaneous interfacial self-assembly of Fmoc-FF and polycondensation of silicate.

kanamycin (>99%), cyclohexene oxide (98%), ethyl acetate (>99%), propiophenone (>99%), sodium hydroxide (NaOH, >98%), hydrochloric acid (HCl, 35∼37 wt%), and anhydrous sodium sulfate (Na_2SO_4, >99%) were purchased from Sigma–Aldrich (Singapore).

Synthesis of Fmoc-FF/PEI/SiO_2 (FPSi) Organic–Inorganic Capsules

In a typical experiment, 1.0 wt% polyethyleneimine (PEI) solution was prepared by diluting 50 wt% PEI solution into de-ionized water and adjusting the pH to 7.5 by adding concentrated HCl. Lyophilized Fmoc-diphenylalanine (Fmoc-FF) peptide was dissolved in an aqueous NaOH solution (pH 10.0) to 6 mg mL^{-1}. Sodium silicate solution (SiO_2: 6 mg mL^{-1}) was prepared by dissolving sodium silicate (SiO_2: ∼26.5 wt%, $\rho = 1.39$ mg mL^{-1}) in de-ionized water, followed by adjusting pH to 10.0 with concentrated HCl. 500 μL sodium silicate solution was mixed under vigorous shaking with 500 μL Fmoc-FF solution and 500 μL de-ionized water. The resulting solution was injected dropwise into 5.0 mL 1.0 wt% PEI solution (pH 7.5) through a syringe needle. After 2 h incubation at room temperature, the excess PEI solution was then poured off. After washing twice with KP buffer (10 mM, pH 7.5), Fmoc-FF/PEI/SiO_2 (FPSi) capsules were collected with a dry weight of 10.2 mg.

Encapsulation of BSA via the Formation of FPSi or FP Capsules and the Protein Leakage Test

A total of 500 μL BSA solution (3 mg mL^{-1}) and 500 μL Fmoc-FF solution (6 mg mL^{-1}) were mixed with 500 μL sodium silicate solutions at different concentration (SiO_2: 0∼6 mg mL^{-1}) and different pH (7.0–10.0). The resulting solution was added dropwise into PEI solution (1.0 wt%, pH 7.5) to synthesize BSA-loaded FPSi or FP (containing no Si) capsules. After 2 h incubation at room temperature, 50 μL of PEI solution were taken out for protein measurement using Bradford's method with Coomassie Brilliant Blue reagent (Bio-Rad, Hercules, CA, USA) to calculate the amount of encapsulated enzymes, and the excess PEI solution was then poured off to give BSA-loaded FPSi or FP capsules.

The formed BSA-loaded capsules were incubated in KP buffer (10 mM, pH7.5) at 30 °C and 250 rpm for 48 h. 50 μL aliquots were taken out for protein test by the Bradford's method. The leakage of BSA was defined as the ratio of the leaked amount of BSA to the initial amount of encapsulated BSA.

Cell Growth of Recombinant *Escherichia coli* (SpEH)

The epoxide hydrolase (SpEH) from *Sphingomonas* sp. HXN-200 was cloned and expressed in *Escherichia coli* (*E. coli*) in our previous study (Wu et al., 2013). The recombinant *E. coli* (SpEH) expressing his-tagged SpEH was grown in 2 mL LB (lysogeny broth) medium containing 50 μg mL^{-1} of kanamycin at 37 °C for 8 h and then inoculated into 50 mL TB (terrific broth) medium containing kanamycin (50 μg mL^{-1}). When OD at 600 nm reached 0.6 at around 2 h, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM to induce protein expression. The cells continued to grow at 22 °C and 250 rpm in a rotary shaker for 12 h. The cells were harvested by centrifuge (4000 rpm, 10 min), washed with KP buffer (10 mM, pH 7.5) one time, and then resuspended in KP buffer.

Purification of Epoxide Hydrolase SpEH

Cells suspension of *E. coli* (SpEH) was passed through a cell disruptor twice, followed by centrifugation (12,300 g, 4 °C) for 30 min. The cell-free extract containing his-tagged SpEH was collected for protein purification. The purification was performed in fast protein liquid chromatography (FPLC) with pre-packed Ni-NTA column. The temperature was kept at 4 °C for the whole process. The cell-free extract (150 mL) was loaded into Ni-NTA column that was prebalanced with 10 mM imidazole KP buffer (10 mM, pH 7.5) to eliminate nonspecific binding. The column was washed with a KP buffer (10 mM, pH 7.5) containing 30 mM imidazole. The target enzyme, his-tagged SpEH, was finally eluted with buffer containing 300 mM imidazole. The elution fraction containing his-tagged SpEH was collected and washed with KP buffer (10 mM, pH 7.5) four times to remove imidazole by using 30 kDa Millipore centrifuge filter. The purified SpEH was then resuspended in a KP buffer (10 mM, pH 7.5) containing 10 wt% glycerol and stored at −80 °C in a refrigerator.

Immobilization of Epoxide Hydrolase SpEH by Encapsulation via the Formation of FPSi Organic–Inorganic Capsules

A total of 750 μL sodium silicate solution (SiO_2: 6 mg mL^{-1}) was mixed under vigorous shaking with 750 μL Fmoc-FF solution (6 mg mL^{-1}; pH = 9.0) and 750 μL purified his-tagged SpEH solution (1.8 mg protein mL^{-1}). The resulting solution (2.25 mL, pH 9.0) was injected dropwise into 5.0 mL 1.0 wt% PEI solution (pH 7.5) through a syringe needle within 2 min. After 2 h incubation at room temperature, 50 μL of PEI solution were taken out for protein measurement using Bradford's method. The excess PEI solution was then poured off. After washing twice with KP buffer (10 mM, pH 7.5), SpEH-loaded FPSi capsules (FPSi-SpEH) were collected, with a dry weight of 20.7 mg.

To assess the enzyme leakage from FPSi capsules, the capsules with different enzyme loading capacity (65 or 158 mg protein g^{-1} dry capsules) were incubated in KP buffer (10 mM, pH7.5) at 30 °C and 250 rpm for 48 h. 50 μL aliquots were taken out for protein testing by the Bradford's method to determine the leaked SpEH.

Enantioselective Hydrolysis of Cyclohexene Oxide With FPSi-SpEH Capsules or Free SpEH

For the reaction with free enzyme, 0.75 mL purified his-tagged SpEH solution (1.8 mg protein mL^{-1}) was diluted with 4.25 mL KP buffer (10 mM, pH 7.5) to a final enzyme concentration of 0.27 mg protein mL^{-1}. For the reaction with immobilized enzyme, FPSi-SpEH capsules (65 mg protein g^{-1} carrier) were dispersed into 4.5 mL KP buffer (10 mM, pH 7.5) to a 6 mL system (equivalent to 6 g by weight; enzyme concentration of 0.21 mg protein mL^{-1}). Cyclohexene oxide was added to the reaction systems to a concentration of 100 mM. The reaction mixture was shaken at 30 °C

and 250 rpm for the appropriate time. 200 μL aliquots were taken out at different time points for GC analysis. To prepare the analytic sample, 200 μL aliquots were extracted with 400 μL ethyl acetate containing 5 mM propiophenone as internal standard; after centrifugation at 15,000 rpm for 10 min, 300 μL organic phases were separated and dried over anhydrous Na_2SO_4; after filtration, the sample was used for GC analysis.

The yields and *ee* values of the cyclohexane diol were determined by using an Agilent GC 7890A (Agilent Technologies, Santa Clara, CA, USA) on a chiral column (β-DEX^{TM} 120, 30 m × 0.25 mm × 0.25 mm, Supelco, Bellefonte, PA, USA). Temperature program: 150 °C for 10 min. Retention times: 2.767 min for cyclohexene oxide, 5.49 min for propiophenone, 7.263 min for (1*S*, 2*S*)-cyclohexane-1,2-diol, and 7.619 min for (1*R*, 2*R*)-cyclohexane-1,2-diol.

The specific activity of free SpEH or FPSi-SpEH was measured for the enantioselective hydrolysis of cyclohexene oxide at 30 °C and 250 rpm for 10 or 30 min, respectively, by following the procedure as described before. Enzymatic activity is given in U per mg of free or immobilized enzymes. 1 U is defined as the hydrolysis of 1 μmol cyclohexene oxide per minute.

Recycling and Reuse of FPSi-SpEH Capsules for the Enantioselective Hydrolysis of Cyclohexene Oxide

Enantioselective hydrolysis of cyclohexene oxide was conducted on a 6 mL scale. FPSi-SpEH capsules (65 mg protein g^{-1} carrier) were added to KP buffer (10 mM, pH 7.5) to give an enzyme concentration of 0.21 mg protein mL^{-1}. Cyclohexene oxide was added to a concentration of 100 mM, and the mixture was shaken at 30 °C and 250 rpm for 240 min. The capsules were collected by filtration after each reaction cycle, washed with KP buffer (10 mM, pH 7.5) for three times, and then dispersed into fresh KP buffer (10 mM, pH 7.5) containing 100 mM cyclohexene oxide for the next reaction cycle. The filtrate after removing the capsules in each reaction cycle was collected and extracted with ethyl acetate to prepare samples for GC analysis to quantify the product yield and *ee*.

Results and Discussion

One-Step Template-Free Synthesis of FPSi –Inorganic Capsules

The novel concept for one-step template-free synthesis of stable organic–inorganic capsules is shown in Figure 1. *N*-fluorenylmethoxycarbonyl diphenylalanine (Fmoc-FF) and sodium silicate are chosen as the organic self-assembling unit and inorganic precursor, respectively. A cationic polymer, polyethyleneimine (PEI), is used to simultaneously trigger the interfacial self-assembly of Fmoc-FF and polycondensation of silicate, leading to the synthesis of Fmoc-FF/PEI/SiO_2 (FPSi) multi-layered organic–inorganic capsules.

For demonstration, an aqueous solution (pH 10.0) of Fmoc-FF (2 mg mL^{-1}) and sodium silicate (corresponding to SiO_2 content, 2 mg mL^{-1}) was added dropwise into an aqueous solution (pH 7.5) of PEI (1.0 wt%) through a syringe needle within 2 min. Upon liquid–liquid contact, white capsules were formed within a few minutes. The capsules were then incubated in PEI solution for 2 h to ensure their complete growth. Figure 2a shows a photograph of the obtained FPSi capsules, which are ∼500 μm in diameter and exhibit good uniformity. By controlling the size of Fmoc-FF/silicate droplets, FPSi capsules with tailored size from 200 μm to 2 mm were synthesized (Fig. S1). All these capsules are mechanically very stable, which is evidenced by the fact that no broken capsule was observed after shaking in an aqueous solution at 30 °C and 250 rpm for 80 h.

To investigate the capsule's membrane and interior, we fractured them before SEM imaging. As shown in Figure 2b–c and Figure S2–S3b, the capsule membranes contain a few thin layers and have a thickness of 20∼200 μm depending on the capsule size and membrane non-uniformity. However, the interior of the capsules exhibits random layered structure with macro sized pores (Fig. 2d and Fig. S3a). The thin layers have a thickness of ∼400 nm shown in the inset image of Figure 2d. We further explored the surface structure of the layers, as illustrated in Figure 2e–f, and discovered that the layers localized in both the membrane and capsule interior display similar structure. Aggregated nanoparticles (white zone) and nanofibers complexed with unordered component (gray zone) was observed on the layers' surface, suggesting the polycondensation of silicate into SiO_2 nanoparticles and self-assembly of Fmoc-FF into nanofibers.

The specific role played by Fmoc-FF self-assembly in capsule formation was investigated. Either Fmoc-FF or sodium silicate solution was dripped into the PEI solution. Fmoc-FF/PEI (FP) capsules were also formed with the addition of Fmoc-FF alone, but they were mechanically not stable. No capsule formation was observed by adding only silicate (Fig. S4). SEM images of the FP capsules showed a similar structure as FPSi capsules (Fig. S5). These results indicate that the self-assembly of Fmoc-FF into nanofibers is the decisive factor for the formation of FPSi capsules with multi-layered structure.

X-ray photoelectron spectroscopy (XPS) showed C, N, O, and Si element existed in FPSi capsules (Fig. 3a). The Si and other O element were associated with SiO_2, which was confirmed by the high-resolution XPS spectrum of Si2p region (Fig. S6d). A peak of Si2p at 103.5 eV clearly corroborated the Si^{4+} oxidation state existed in SiO_2. Further evidence for the formation of SiO_2 was obtained through Fourier transform infrared (FTIR) analysis, in which three peaks at 1100 cm^{-1}, 800 cm^{-1}, and 450 cm^{-1} were attributed to the absorption of Si—O—Si in silica (Fig. S7). Thermogravimetric analysis (TGA) (Fig. 3b) showed that FPSi capsules lost ∼10% weight when temperature reached 200 °C due to the evaporation of water. Compared with FP capsules, FPSi capsules still remained ∼35% of weight at 450 °C, attributing to its SiO_2 component. The ∼55% weight loss observed from 200 to 450 °C was due to the decomposition of organic components (Fmoc-FF/PEI). The chemical compositions in FPSi are thus deduced as almost the same as the theoretical values of 33% SiO_2, 57% Fmoc-FF/PEI, and 10% H_2O based on starting materials used (Table S1). This confirms the high yield of the capsules based on the initially used amounts of Fmoc-FF and sodium silicate.

To understand the formation mechanism of FPSi capsules, the zeta potentials of the Fmoc-FF, PEI, and silicate solution were measured (Fig. S8). Fmoc-FF solution possessed a negative zeta potential at pH 9.0, while the PEI solution contains positive static

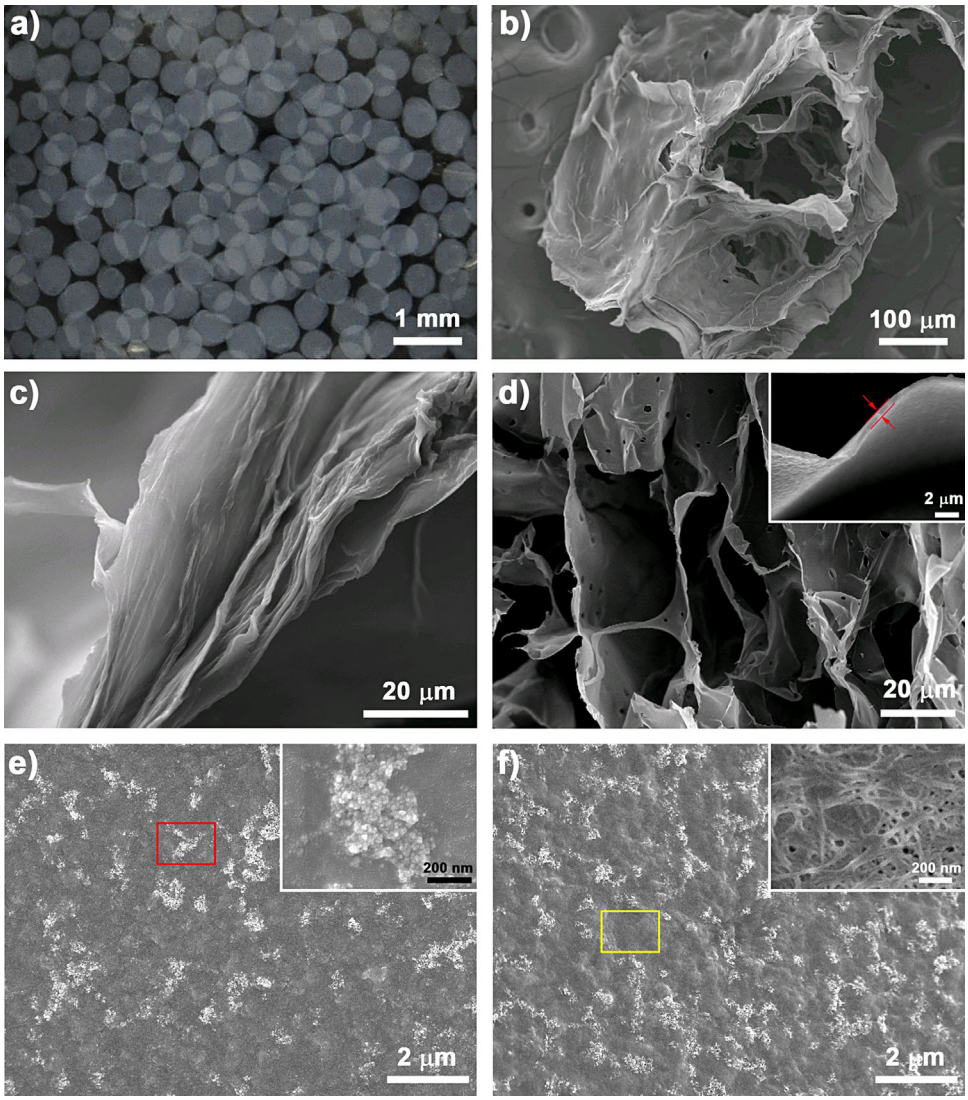

Figure 2. Morphology characterization of the FPSi capsules. (**a**) Photograph of FPSi capsules formed in PEI solution. (**b**) SEM image of FPSi capsule after freeze-drying and cleaving. (**c**) SEM image of the capsule membrane. (**d**) SEM image of the capsule interior. The inset image is a thin layer. (**e**) SEM image of the surface of a layer in the capsule membrane. The inset image is from a typical zone in panel (red-labeled). (**f**) SEM image of the surface of a layer in the capsule interior. The inset image is from a typical zone in panel (yellow-labeled).

charge at pH 7.5. With the interaction with PEI, the electrostatic repulsion of negative Fmoc-FF was eliminated, allowing them to self-assemble into nanofibers. A similar self-assembly mechanism was also reported in previous studies (Capito et al., 2008; Huang et al., 2014). In addition, the sodium silicate solution at pH 7∼10 showed negative zeta potential. The positive PEI chains can adsorb the silicates via electrostatic attraction. The high density of silicate around PEI, together with the hydrogen bonding between Si—O— and $—NH_3^+$, promoted the silicate polycondensation into SiO_2 (Kröger et al., 1999; Roth et al., 2004). A possible mechanism on the formation of FPSi capsules was thus proposed. Upon liquid–liquid contact, the electrostatic complexations between PEI and Fmoc-FF and between PEI and silicate occur at the interface, simultaneously leading to self-assembly of Fmoc-FF into nanofibers and polycondensation of silicate into SiO_2 nanoparticles to form a hybrid FPSi layer (PEI/Fmoc-FF nanofibers/SiO_2 nanoparticles) (Fig. 1). This layer creates a diffusion barrier and results in the physical separation of Fmoc-FF/silicate droplet and PEI solution. Due to the difference in ion concentration between droplet and PEI solution, an excess osmotic pressure of ions in the Fmoc-FF/silicate compartment is generated (Capito et al., 2008). Under this excess osmotic pressure, PEI diffuses through the initial FPSi layer to further induce the self-assembly of Fmoc-FF and polycondensation of silicate inside the capsules. The simultaneous inward penetration of PEI and growth of FPSi layers lead to the multi-layered structure of capsules (Fig. 1).

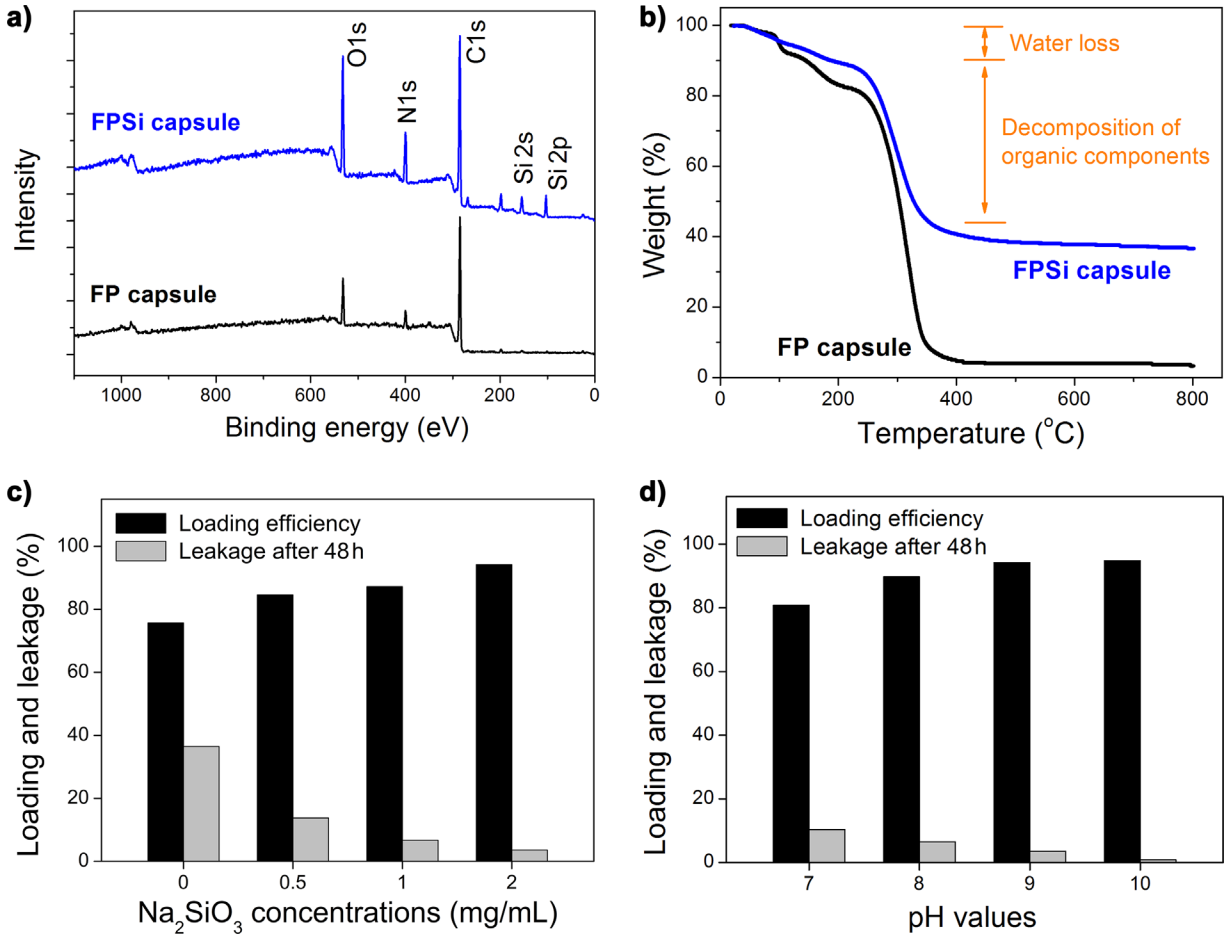

Figure 3. (**a**) XPS survey spectra of FP and FPSi capsules. (**b**) TGA curves of FP and FPSi capsules. The capsule synthesis conditions: 1.5 mL aqueous solution (pH 10) of 2 mg mL^{-1} Fmoc-FF and 2 mg mL^{-1} silica (no silica for FP capsules), 5 mL 1 wt% PEI solution (pH 7.5), 25 °C, 2 h. (**c–d**) BSA loading efficiency and leakage after 48 h for BSA-encapsulating FPSi capsules that were formed at (**c**) different silicate concentration (pH = 9.0); and (**d**) different pH (2 mg mL^{-1} of silica).

Encapsulation of BSA via One-Step Template-Free Formation of FPSi Organic–Inorganic Capsules and the Protein Leakage Test

To assess the encapsulation ability and stability of the capsules, bovine serum albumin (BSA, 66 KD) was chosen as an indicator. Encapsulation of BSA with the capsules was performed at various silicate concentrations and pH values. An aqueous solution of BSA (pI = 4.8; 1 mg mL^{-1}), Fmoc-FF (2 mg mL^{-1}), and sodium silicate (SiO_2 content: 0∼2 mg mL^{-1}) at pH of 7.0–10.0 were added dropwise into PEI solution (1.0 wt%, pH 7.5) to give BSA-loaded FP (containing no Si) or BSA-loaded FPSi capsules in high yield (Fig. S9). Measurement of protein concentration in the remaining PEI solution using Bradford's method suggested high BSA-loading efficiency. As shown in Figure 3c, the loading efficiency of BSA increased with the increase of concentration of sodium silicate at pH of 9: from 75% in the absence of sodium silicate to 93.5% at 2 mg/mL of sodium silicate. Also the pH value influenced the loading efficiency of BSA. As shown in Figure 3d, the loading efficiency was 80% at pH of 7 and increased to a very high level at pH of 10. These results suggested clearly that higher silicate concentration and pH value enhanced the encapsulation of BSA in the capsules. According to the formation mechanism of capsules, as discussed before, the negative zeta potential of Fmoc-FF/silicate solution is a key parameter for the protein encapsulation.

To study the leakage of BSA from the capsules, the obtained BSA-loaded capsules were incubated in KP buffer (10 mM, pH7.5) at 30 °C and 250 rpm for 48 h. Determination of protein concentration of the supernatant allowed for the quantification of the protein leakage. Figure 3c showed that the protein leakage decreased with the increase of the concentration of sodium silicate at pH of 9. 36% protein leakage occurred after 48 h shaking of BSA-loaded FP capsules containing no silica. In this case, some broken capsules were also observed, indicating the low stability of this type of capsules. With the increase of silica content, the FPSi capsules showed a decreased protein leakage and no breakage. At 2 mg/mL of sodium silicate, only 2.3% BSA was leaked out after 48 h. These results suggested that the addition of silica significantly enhanced the capsule stability. The pH value used at the encapsulation experiment influenced

also the protein leakage. As shown in Figure 3d, the protein leakage decreased when pH value was increased. The capsules prepared at pH of 9–10 showed very low level of BSA leakage and very high stability. The low protein leakage also indicated the pore size of <7 nm for the capsule membrane, since the stokes radius of BSA is 3.48 nm.

For the encapsulation of enzyme using initial pH of 9–10, the exposure time of enzyme to the high pH is very short (ca. 1 min for solution preparation and 2 min for injection). After the injection, the pH value in the mixed solution decreased to pH of 8.0 (mixture of 5.0 mL solution of pH 7.5 and 2.25 mL solution of pH 9.0). Therefore, the encapsulation at pH of 9–10 may still be applicable to the enzymes that are less stable at such pH values. Moreover, high enzyme loading efficiency (>80%) and low enzyme leakage (<10%) were also achieved at pH of 7–8. This allows for the application of the immobilization method to many enzymes by encapsulation at pH of 7–8.

Immobilization of SpEH by Encapsulation via One-Step Template-Free Formation of FPSi Organic–Inorganic Capsules as Active and Stable Biocatalyst for the Enantioselective Hydrolysis of Cyclohexene Oxide

To demonstrate this novel concept of using the stable organic–inorganic capsules for enzyme immobilization, we chose epoxide hydrolase SpEH as the model enzyme (Chang et al., 2003; Jia et al., 2008; Liu et al., 2006; Wu et al., 2013). SpEH catalyzes the regio- and enantio-selective hydrolysis of several racemic epoxides to give the enantiopure epoxides in high *ee* and good yield (Jia et al., 2008; Liu et al., 2006; Wu et al., 2013), and it also catalyzes the enantioselective hydrolysis of some meso-epoxides to produce the corresponding diols in high yield and good *ee* (Chang et al., 2003; Wu et al., 2013). Development of active and recyclable immobilized SpEH could significantly reduce the enzyme cost and enhance the operational stability, thus improving the process economics for the preparation of the useful and valuable chiral epoxides and diols.

The optimized conditions for preparing stable capsules containing BSA were used for the immobilization of SpEH. Initial solution at pH of 9 containing Fmoc-FF, sodium silicate, and SpEH was chosen, since BSA-encapsulating FPSi capsules prepared at this initial pH gave lower enzyme leakage and higher enzyme loading efficiency. His-tagged SpEH was produced and purified from the cells of *E. coli* (SpEH). An aqueous solution of sodium silicate (SiO_2: 2 mg mL^{-1}), Fmoc-FF (2 mg mL^{-1}), and purified his-tagged SpEH (0.6 mg protein mL^{-1}; pI of 5.7) at pH of 9.0 was injected dropwise into a 1.0 wt% PEI solution (pH 7.5) through a syringe needle within 2 min. After 2 h incubation, the formed capsules were isolated and washed with KP buffer (10 mM, pH 7.5) to give SpEH-loaded FPSi capsules (FPSi-SpEH) with ~1.2 mm in diameter (Fig. 4a). Based on the determined amount of protein in the PEI solution and the washing KP buffer, the enzyme loading efficiency was established as >90% and the specific enzyme loading was 65 mg protein g^{-1} carrier. By increasing the initial ratio of SpEH to Fmoc-FF and sodium silicate, FPSi-SpEH with a specific enzyme loading of 87, 158, 346 mg protein g^{-1} carrier and an enzyme loading efficiency of >90% were easily obtained.

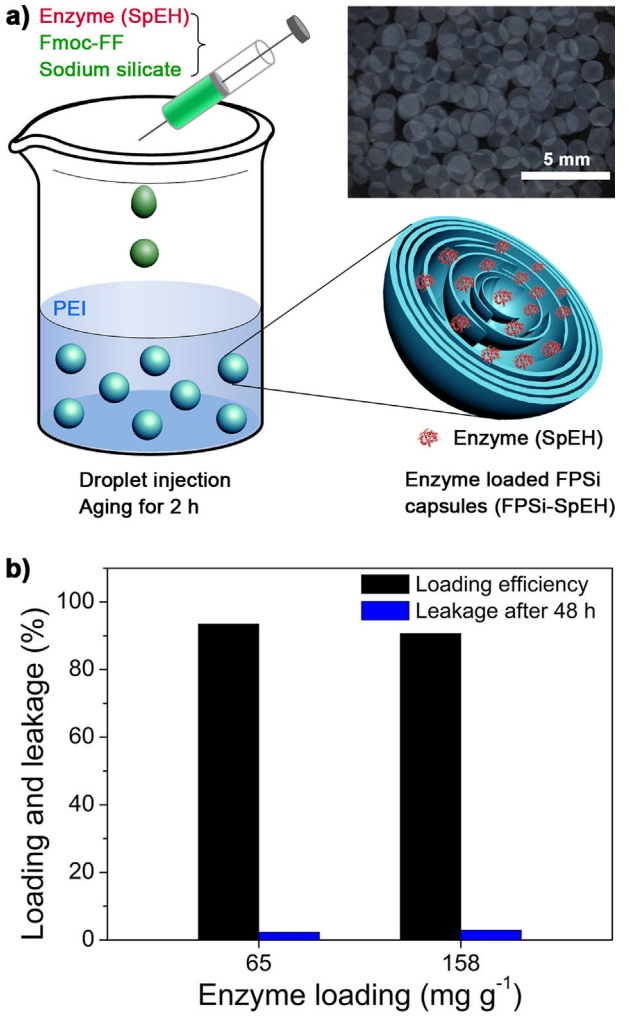

Figure 4. (**a**) Immobilization of epoxide hydrolase SpEH via encapsulation by one-step template-free formation of FPSi capsules as FPSi-SpEH capsules (1.2 mm; 65 mg protein g^{-1} dry capsules). (**b**) SpEH loading efficiency and SpEH leakage from FPSi capsules after shaking at 250 rpm and 30 °C for 48 h.

The leakage of SpEH from the capsules after 48 h incubation at 30 °C and 250 rpm was shown in Figure 4b. FPSi-SpEH capsules with either 65 or 158 mg protein g^{-1} carrier gave less than 3% leakage of SpEH. The results demonstrated once again the high stability of the capsules.

The enantioselective hydrolysis of cyclohexene oxide to (1*R*, 2*R*)-cyclohexane-1,2-diol, that is a useful and valuable synthetic intermediate for chiral pharmaceutical manufacturing, was selected as the target reaction for examining the performance of the immobilized SpEH (Fig. 5a). FPSi-SpEH capsules (65 mg protein g^{-1} carrier; 0.21 mg protein mL^{-1}) was used to perform the enantioselective hydrolysis of 100 mM cyclohexene oxide at 30 °C and 250 rpm for 4 h and compared with the free enzyme SpEH (0.27 mg protein mL^{-1}). The biotransformation was carried out at pH of 7.5, an optimal value for SpEH-catalyzed epoxide hydrolysis. The yield and *ee* of the product were determined by GC

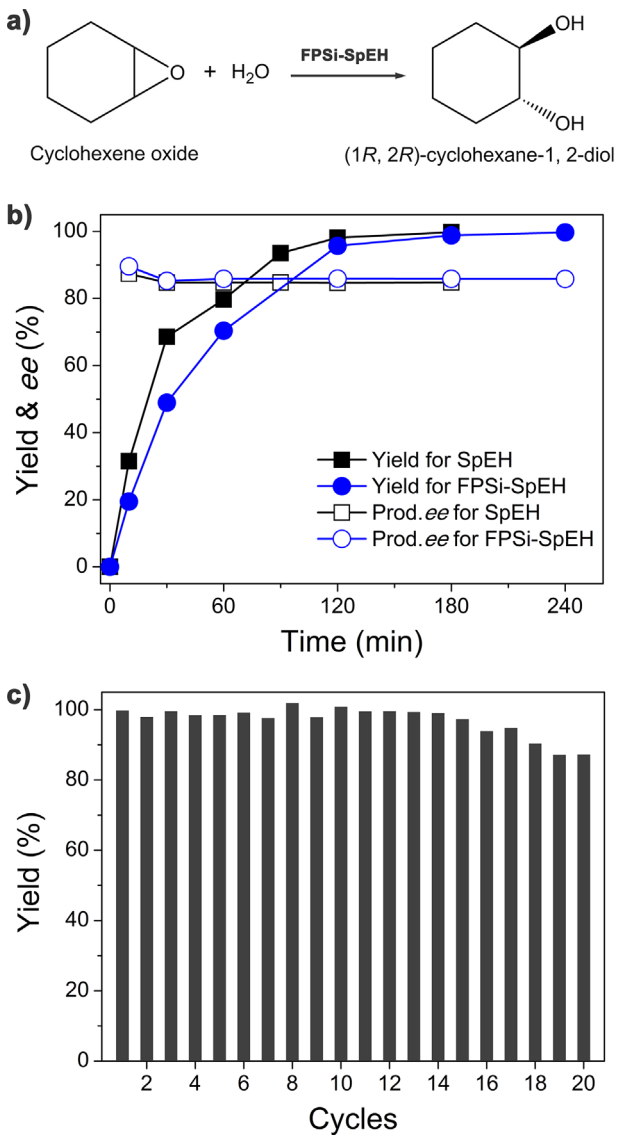

Figure 5. (**a**) Enantioselective hydrolysis of cyclohexene oxide with epoxide hydrolase SpEH-encapsulating FPSi capsules (FPSi-SpEH). (**b**) Time courses of the biotransformation of cyclohexene oxide to (1*R*, 2*R*)-cyclohexane-1,2-diol using free SpEH (0.27 mg protein mL^{-1}) and FPSi-SpEH (65 mg protein g^{-1} capsules; 0.21 mg protein mL^{-1}), respectively. (**c**) Recycling and reuse of FPSi-SpEH (65 mg protein g^{-1} capsules; 0.21 mg protein mL^{-1}) for the hydrolysis of cyclohexene oxide (4 h/cycle). The default reaction conditions for (**b**) and (**c**): 100 mM substrate; 30 °C; 250 rpm.

analysis using a chiral column (β-DEX™ 120). Figure 5b shows the time courses of the biotransformation of cyclohexene oxide using free SpEH and FPSi-SpEH, respectively. In both cases, the product (1*R*, 2*R*)-cyclohexane-1,2-diol was obtained in 88% *ee*. This confirmed no change of the enantioselectivity of SpEH after immobilization. The specific activity of FPSi-SpEH (65 mg protein g^{-1} carrier) for the first 10 min was 6.94 U mg^{-1} protein, being 60% of the initial free enzyme activity. The decrease of the initial activity may be caused by mass transfer limitation. Additionally, as shown in Figure S10, further increase of the enzyme loading led to a decrease in the specific activity of FPSi-SpEH, which is possibly attributed to the limited mass transfer and/or too much enzymes within capsules. Nevertheless, at an enzyme loading of 65 mg protein g^{-1} carrier, the degree of retaining the free enzyme activity is relatively high, and the absolute value of the specific enzyme activity is still very high. Moreover, FPSi-SpEH gave 95% yield at 2 h, reaching nearly the same yield achieved with free SpEH at 2 h (Fig. 5b).

Recycling and Reuse of FPSi-SpEH Capsules for the Enantioselective Hydrolysis of Cyclohexene Oxide to Prepare (1*R*, 2*R*)-cyclohexane-1,2-diol

Recycling of FPSi-SpEH capsules (0.21 mg protein mL^{-1}) for enantioselective hydrolysis of 100 mM cyclohexene oxide was conducted at 30 °C and 250 rpm for 240 min for each cycle. The capsules were collected by filtration, washed with KP buffer (10 mM, pH 7.5), and then reused for new cycle of biotransformation of cyclohexene oxide. FPSi-SpEH demonstrated excellent stability and recyclability. As shown in Figure 5c, the capsules retained 87% productivity after 20 cycles with a total reaction time of 80 h.

The stability of FPSi-SpEH and free SpEH was compared by storing the catalysts at 4 °C for 8 days followed by measuring their specific activities for the hydrolysis of cyclohexene oxide for 30 min. The data were shown in Figure S11. Free SpEH retained 83% of its activity after storage at 4 °C for 8 days, while FPSi-SpEH retained 94% of its activity after the same storage. These results indicate the enhanced stability of the encapsulated enzyme in comparison with free enzyme during storage.

Studies on encapsulation of enzymes via the formation of different organic–inorganic capsules are summarized in Table I. Although different enzymes were used, they all have good stability at 25 °C for the reaction and storage. This allows for comparison of different encapsulation methods regarding enzyme loading efficiency, enzyme leakage, retained free enzyme activity, and recyclability. FPSi-SpEH capsules were the best regarding the overall performance in these aspects. FPSi-SpEH capsules showed also much better performance than the enzymes encapsulated with other type of capsules such as Alginate (Zhang et al., 2009) and Alginate-chitosan (Taqieddin and Amiji, 2004). In comparison with FCPCs-SpEH (Huang et al., 2014), the best example of organic capsules developed recently by us, FPSi-SpEH gave much higher mechanical stability and recyclability (retaining 87% productivity after 20 cycles vs. 86% productivity after 10 cycles). In addition, FPSi-SpEH retained higher initial free enzyme activity than FCPCs-SpEH (60 vs. 50%). For hollow capsules, thick membrane is generally required to achieve high stability, which could lead to an increased mass transfer resistant through the membrane and thus reduced catalytic performance. Template-filled capsules exhibit high stability while having limited mass transfer inside the capsules, especially for the hydrophobic substrate (e.g., cyclohexene oxide, in this study) or product. In our multi-layered capsules, the membrane contain few thin layers and the capsule interior exhibits random layered structure with macro sized pores, giving rise to high stability as well as good catalytic performance. These results suggested that the immobilization of enzyme by encapsulation via the formation of the

Table I. Comparison of encapsulation of enzymes using different organic–inorganic capsules.

Capsules	Enzymes	Enzyme loading efficiency	Enzyme leakage/time	Activity referred to free enzyme/time	Retained activity/No. of cycles/time	References
Alginate-silica	Formate dehydrogenase	<60%	–[a]	<40%/-	99%/10/-	Kurayama et al., 2012
Alg-pLys-silica	β-Galactosidase	–	–	55.6%/-	–	Coradin et al., 2001
APSi	Laccase	–	–	13.9%/10 min	45%/10/10 min	Wang et al., 2013
Alginate-CaP	Transglucosidase	92.6%	35%/7 h	<50%/60 min	82%/10/10 min	Zhang et al., 2009
PSi-PDA	α- or β-Amylase, glucosidase	28–54%	10%/20 h	53.8%/10 min	85%/8/10 min	Zhang et al., 2011
PTi-PDA	α- or β-Amylase, glucosidase	47–69%	0/20 h	68.8%/10 min	98%/8/10 min	Zhang et al., 2011
APSi	β-Glucuronidase	69%	–	125%[b]/-	30%/13/-	Zhang et al., 2008
FPSi	Epoxide hydrolase	93.5%	2.3%/48 h	60%/10 min	87%/20/4 h	This study

[a]"-" represents that the corresponding data are not determined or not mentioned in the reference.
[b]CMC was added to improve the enzyme activity.

stable organic–inorganic capsules is a simple and useful method to prepare active and recyclable biocatalyst for practical in vitro biotransformation. The method could be extended by using other self-assembling molecules, organic, and inorganic materials for one-step template-free formation of other inorganic–organic hybrid capsules.

Conclusions

A facile, one-step, and template-free synthesis of organic–inorganic capsules in aqueous phase was developed via PEI-induced simultaneous interfacial self-assembly of Fmoc-FF and polycondensation of silicate. Addition of an aqueous solution of Fmoc-FF and sodium silicate into an aqueous solution of PEI gave the multilayered Fmoc-FF/PEI/SiO_2 (FPSi) capsules in high yield. By controlling the size of Fmoc-FF/silicate droplets, FPSi capsules with tailored size from 200 μm to 2 mm were obtained. The capsules are mechanically very stable due to the incorporation of silica as the inorganic component.

A simple and practical method for efficient enzyme immobilization was also developed by direct encapsulation of enzyme via the one-step template-free formation of the organic–inorganic capsules. Encapsulation of BSA and epoxide hydrolase SpEH was demonstrated, respectively, giving enzyme-containing capsules (∼1.2 mm in diameter) in high yield with >90% enzyme loading efficiency and high specific enzyme loading (158 mg protein g^{-1} carrier). The capsules were highly stable and showed only small amount of protein leakage (<3%) after 48 h incubation. FPSi-SpEH capsules catalyzed the hydrolysis of cyclohexene oxide to (1*R*, 2*R*)-cyclohexane-1,2-diol with high specific activity (6.94 U mg^{-1} protein), high yield, high product concentration, and the same high enantioselectivity as the free enzyme. The immobilized SpEH demonstrated also excellent operational stability and recyclability: retaining 87% productivity after 20 cycles with a total reaction time of 80 h. In comparison with other known immobilized enzymes using organic–inorganic capsules and other type of capsules, FPSi-SpEH capsules showed the best overall performance regarding enzyme immobilization efficiency, specific enzyme loading, enzyme leakage, catalytic activity, mechanical stability, and recyclability.

Although the optimum pH for the enzyme encapsulation is 9–10, high enzyme loading efficiency and low enzyme leakage were also achieved at pH of 7–8. Thus, the immobilization method developed here might be generally applicable to other enzymes.

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