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Electronic Supplementary Information

Integrating interfacial self-assembly and electrostatic complexation at an aqueous interface for capsules synthesis and enzyme immobilization

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1. Experimental Section

1.1 Materials

The lyophilized form of Fmoc-diphenylalanine peptide (Fmoc-Phe-Phe-OH, Fmoc-FF, >98%) was purchased from Bachem (Switzerland). The branched polyethyleneimine (PEI) solution (Mw ~750 kDa, 50 wt% in H_2O), bovine serum albumin (BSA, Mw ~66 kDa, >96%), kanamycin (>99%), cyclohexene oxide (98%), 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). Carboxymethyl cellulose (CMC, sodium salt, 99.6%, viscosity: 42 mPa.s) was from Calbiochem (USA). Isopropyl β-D-1-thiogalactopyranoside (IPTG, >99%)) were purchased from 1st BASE (Singapore). Medium LB and yeast extract were purchased from Biomed Diagnostics (USA).

1.2 Synthesis of Fmoc-FF/PEI capsules

1.0 wt% polyethyleneimine (PEI) solution was prepared by diluting the 50 wt% PEI solution into deionized water. The pH of the PEI solution was then tuned to 7.5 by adding an appropriate amount of concentrated HCl. The lyophilized Fmoc-diphenylalanine (Fmoc-FF) peptide was dissolved in an alkaline aqueous solution (pH 9.0) at a final concentration of 2 mg mL^{-1} by adding 0.5 M NaOH. 1.5 mL Fmoc-FF solution was then injected dropwise into 5.0 mL 1.0 wt% PEI solution (pH 7.5) through a syringe needle with inner diameter of 0.7 mm. After 30 min incubation at room temperature, the excess PEI solution was then poured off, the formed Fmoc-FF/PEI capsules (wet weight: ~550 mg; dry weight: 4.3 mg) were washed twice by potassium phosphate (KP) buffer (10 mM, pH 7.5).

1.3 Synthesis of Fmoc-FF/CMC/PEI hybrid capsules

In a typical experiment, 1.0 wt% polyethyleneimine (PEI) solution was prepared by diluting the 50 wt% PEI solution into deionized water. The pH of the PEI solution was then tuned to 7.5 by adding an appropriate amount of concentrated HCl. The lyophilized Fmoc-diphenylalanine (Fmoc-FF) peptide was dissolved in an alkaline aqueous solution (pH 9.0) at a final concentration of 4 mg mL^{-1} by adding 0.5 M NaOH. 300 μL 3.0 wt% carboxymethyl cellulose (CMC) and 1.2 mL deionized water were added into 1.5 mL freshly prepared Fmoc-FF solution with vigorous shaking. The final concentrations of Fmoc-FF and CMC were 2.0 mg mL^{-1} and 3.0 mg mL^{-1}, respectively. Then the resulting mixed solution (3 mL) was injected dropwise into 5.0 mL 1.0 wt% PEI solution (pH 7.5) through a syringe needle with inner diameter of 0.7 mm. After 30 min incubation, the excess PEI solution was then poured off, the formed Fmoc-FF/CMC/PEI hybrid capsules (wet weight: ~570 mg, dry weight: 21.6 mg) were washed twice by KP buffer (10 mM, pH 7.5).

1.4 Scanning electron microscopy

The Fmoc-FF/CMC/PEI hybrid capsules were freeze-dried for morphology characterization. The samples were sputter-coated with platinum and then observed using a JSM-6700F field emission scanning electron microscope (FESEM, JEOL, Japan).

1.5 Zeta potential measurement

Zeta potential measurements of Fmoc-FF, Fmoc-FF/CMC, and PEI solutions were performed using the Zetasizer Nano-ZS (Malvern Instruments, UK). The pH values of Fmoc-FF (2 mg mL^{-1}) solution and Fmoc-FF/CMC (2 mg mL^{-1} of Fmoc-FF and 3 mg mL^{-1} of CMC) solution were kept at 9.0. For PEI solutions (1.0 wt%), the different pH values, from 2.0 to 13.0, were adjusted with concentrated HCl or NaOH. A pH meter (SevenEasy, Mettler Toledo, Switzerland) was used to measure the pH values of all the solutions.

1.6 Stability test of peptide-polymer capsules

To assess the stability of capsules, 1.5 mL BSA solution (1 mg mL^{-1}) was added into 1.5 mL Fmoc-FF solution (4 mg mL^{-1}) to synthesize BSA-loaded Fmoc-FF/PEI capsules. 1.5 mL BSA solution (1 mg mL^{-1}) and 300 μL CMC solution (3 wt%) were added into 1.2 mL Fmoc-FF solution (5 mg mL^{-1}) to synthesize BSA-loaded Fmoc-FF/CMC/PEI hybrid capsules. The formed BSA-loaded capsules were placed in KP buffer (10 mM, pH7.5) and then incubated at 30 ^{o}C and 250 rpm for the appropriate time. 50 μL aliquots were taken out at different time points for protein test by the Bradford's method using Coomassie Brilliant Blue reagent. The leakage ratio of protein was defined as the ratio of cumulative leakage amount of BSA to the initial amount of encapsulated BSA.

1.7 Cell growth of recombinant *E.coli* (SpEH)

The epoxide hydrolase (SpEH) from *Sphingomonas sp.* HXN-200 was identified, cloned, and expressed in *Escherichia coli* (*E.coli*) in our previous study.[S1] The recombinant *E.coli* (SpEH) expressing histagged SpEH was grown in 2 mL LB (lysogeny broth) medium containing 50 μg mL^{-1} of kanamycin at 37 °C for 9 h and then inoculated into 50 mL TB (terrific broth) medium containing kanamycin (50 μg mL^{-1}). When OD at 600nm 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) two times, and then resuspended in KP buffer (10 mM, pH 7.5).

1.8 Purification of epoxide hydrolase (SpEH)

Cells suspension was passed through a cell disruptor twice, followed by centrifugation (13500 rpm, 4 ^{o}C) for 20 min. The cell free extract containing histagged 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 ^{o}C for the whole process. The cell free extract (250 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 KP buffer (10 mM, pH 7.5) containing 15 mM and then 30 mM imidazole. The target enzyme, histagged 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) 4 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 ^{o}C in a refrigerator.

1.9 Enzyme immobilization via encapsulation with Fmoc-FF/CMC/PEI hybrid capsules

1.0 wt% polyethyleneimine (PEI) solution was prepared by diluting the 50 wt% PEI solution into deionized water, and the pH of PEI solution was tuned to 7.5 by adding an appropriate amount of concentrated HCl. The lyophilized Fmoc-FF was dissolved in an alkaline aqueous solution (pH 9.0) to a final concentration of 5 mg mL^{-1} by adding 0.5 M NaOH. Then, 1.5 mL purified histagged SpEH solution (protein concentration of 0.797 mg mL^{-1}) and 300 μL 3.0 wt% carboxymethyl cellulose were added into 1.2 mL freshly prepared Fmoc-FF solution with vigorous shaking. The final concentrations of SpEH, CMC, and Fmoc-FF were 0.32 mg mL^{-1}, 3.0 mg mL^{-1}, and 2.0 mg mL^{-1}, respectively. The resulting mixture solution (3 mL) was then injected dropwise into 5.0 mL 1.0 wt% PEI solution (pH 7.5) through a syringe needle with inner diameter of 0.7 mm. After 30-min 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, and the formed enzyme-loaded capsules (wet weight: ~ 575 mg, dry weight: 23.2 mg) were washed twice by KP buffer (10 mM, pH 7.5).

1.10 Enzyme loading capacity and immobilization efficiency

The amount of immobilized histagged SpEH was determined by measuring the final concentrations of protein in PEI solution using Coomassie Brilliant Blue reagent, following the Bradford's method.[S2] The total dry weight (w) of enzyme-loaded capsules was measured after 24 h of oven drying. The enzyme loading capacity (LC) and immobilization efficiency (E_i) was calculated according to equation (1) and (2), respectively.

$$LC = \frac{m}{w - m} = \frac{m_0 - c \times v}{w - (m_0 - c \times v)} \quad (1)$$

$$E_i = \frac{m}{m_0} \times 100\% = \frac{m_0 - c \times v}{m_0} \times 100\% \quad (2)$$

where LC (mg g^{-1}) represents the enzyme loading capacity; m (mg) is the amount of enzyme (SpEH) loaded

into the capsules; *w* is the total dry weight of enzyme-loaded capsules; m_0 (mg) is the initial amount of enzyme before immobilization; *c* (mg mL^{-1}) and *v* (mL) are the enzyme concentration and volume of the PEI solution, respectively.

1.11 Enzyme leakage from capsules

To assess the enzyme (histagged SpEH) leakage from Fmoc-FF/CMC/PEI hybrid capsules, the capsules with different enzyme loading capacity were added into KP buffer (10 mM, pH7.5) and then incubated at 30 ^oC and 250 rpm for the appropriate time. 50 μL aliquots were taken out at different time points for protein testing by the Bradford's method using Coomassie Brilliant Blue reagent. The leakage ratio of enzyme was defined as the ratio of cumulative leakage amount of histagged SpEH to initial amount of encapsulated SpEH.

1.12 Enantioselective hydrolysis of cyclohexene oxide with free or immobilized enzyme

For the reaction with free enzyme, 1.5 mL purified histagged SpEH solution (0.797 mg mL^{-1}) was diluted by adding 3.5 mL KP buffer (10 mM, pH 7.5) in a 15 mL flask. For the reaction with immobilized enzyme, the capsules containing the same amount of free histagged SpEH (1.5 mL enzyme solution) were dispersed by adding 3.5 mL KP buffer (10 mM, pH 7.5) to a 5 mL system (equivalent to 5 g by weight). Then 50.5 μL cyclohexene oxide was added to the reaction systems to a final concentration of 100 mM. The reaction mixture was shaken (250 rpm) in an incubator (New Brunswick Scientific) at 30 ^oC for the appropriate time. 200 μL aliquots were taken out at different time points for GC analysis.

200 μL aliquots were extracted by adding 400 μL ethyl acetate containing 5 mM propiophenone as internal standard, and then centrifuged at 15000 rpm for 10 min. 300 μL organic phases were separated and dried over anhydrous Na_2SO_4 before GC analysis.

The yields and *ee* values of the cyclohexane diol were determined by an Agilent GC 7890A on a chiral column (Supelco, β-DEXTM120, 30 m × 0.25 mm ×0.25 mm). Temperature program: 150°C for 10 min. Retention times: cyclohexene oxide (2.767 min), propiophenone (5.49 min), (1*S*, 2*S*)- cyclohexane-1, 2-diol (7.263 min), and (1*R*, 2*R*)-cyclohexane-1, 2-diol (7.619 min).

1.13 Storage stability of the immobilized enzyme

Free and immobilized enzymes (histagged SpEH) were stored in KP buffer (10 mM, pH 7.5) at 4 ^oC for 30 days. The storage stability was compared in terms of relative activity defined as the ratio of the activity of free or immobilized enzyme after storage to their initial activity.

Activity test: Free and immobilized SpEH were added to KP buffer (10 mM, pH 7.5) with 99.6 μg mL^{-1} of enzyme concentration (total volume is 2 mL). Then cyclohexene oxide was added to the reaction system with a

final concentration of 100 mM. The reaction mixture was shaken (250 rpm) in an incubator at 30 ^{o}C for the appropriate time. 200 μL aliquots were taken out at 10, 20, and 30 min for GC analysis (as described above).

Enzymatic activity is given in U per mg of free or immobilized enzyme. 1 U corresponds to the hydrolysis of 1 μmol of cyclohexene oxide per minute.

1.14 Recycling of immobilized enzyme in enantioselective hydrolysis of cyclohexene oxide

Recycling of encapsulated SpEH was conducted on a 5 mL-scale for enantioselective hydrolysis of cyclohexene oxide in the same procedure described in *Section 1.12*. The enzyme-loaded capsules were added to KP buffer (10 mM, pH 7.5), giving a final enzyme concentration of 0.239 mg mL^{-1}. Then 50.5 μL cyclohexene oxide was added to a final concentration of 100 mM and shaken in an incubator (250 rpm) at 30 ^{o}C for 240 min. The capsules were collected by filtration after each reaction batch, washed with KP buffer (10 mM, pH 7.5) three times to remove residual product or substrate, and then dispersed into 5 mL fresh KP buffer (10 mM, pH 7.5) for the next reaction cycle. All the filtrate was collected and extracted by ethyl acetate for GC analysis. The yields and *ee* values in each successive reaction cycle were measured.

1.15 Measurement of kinetic parameters for free and immobilized enzyme catalyzed hydrolysis of cyclohexene oxide

To determine the kinetic data, a set of hydrolysis reactions was performed with free and immobilized enzyme (47.8 μg mL^{-1}) at various concentrations (10 mM~160 mM) of cyclohexene oxide. The initial reaction velocity (V) at different substrate concentration ([S]) was plotted according to the Lineweaver- Burk double reciprocal model (equation 3).

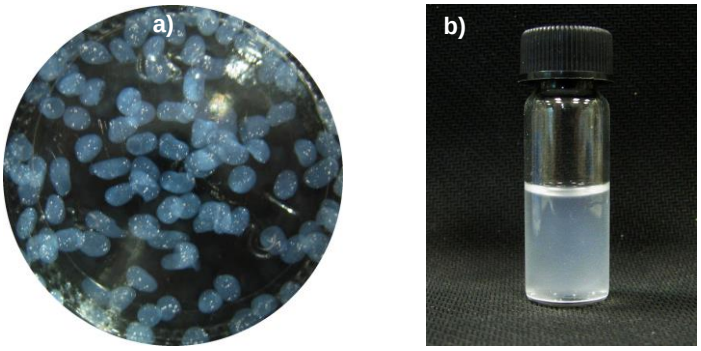
$$\frac{1}{V} = \frac{K_m}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}} \quad (3)$$

$$k_{cat} = \frac{V_{max}}{[E]} \quad (4)$$

where V is the apparent initial catalytic rate, V_{max} is the maximum apparent initial reaction rate, [S] is the substrate concentration, K_m is the apparent Michaelis-Menten constant, k_{cat} measures the number of substrate molecules turned over per enzyme molecule per second, and [E] is the enzyme concentration.

The kinetic parameters, including K_m (mM), V_{max} (mM min^{-1}), k_{cat} (s^{-1}, turnover number), can be calculated from equation (3) and (4).

2. Supplementary Figures



Fmoc-FF/PEI capusles CMC/PEI

Fig. S1 Photograph of Fmoc-FF/PEI capsules (a) and CMC/PEI precipitate (b) formed in 1.0 wt% PEI solutions at pH 7.5, respectively.

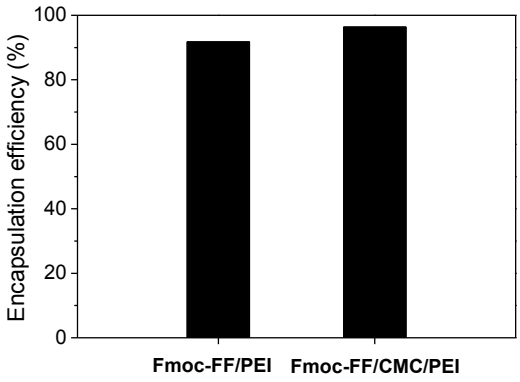


Fig.S2 The encapsulation efficiency of BSA within Fmoc-FF/PEI capsules and Fmoc-FF/CMC/PEI hybrid capsules. The protein content was determined by UV-Vis spectrometry.

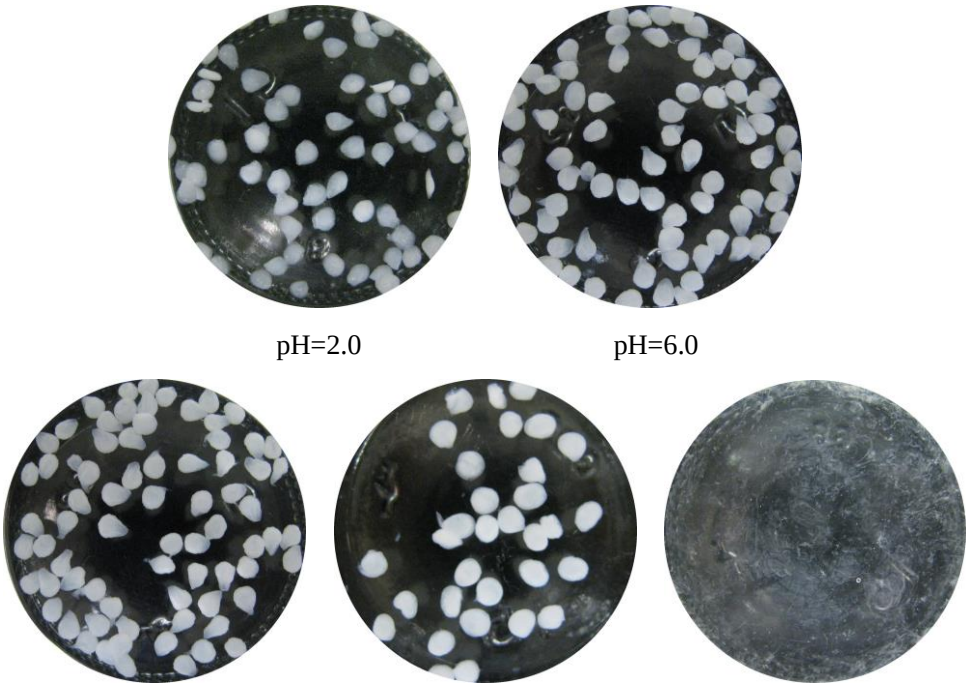


Fig. S3 Photograph of Fmoc-FF/CMC/PEI capsules formed in 1.0wt% PEI solutions with different pH values ranging from 2.0 to 12.0.

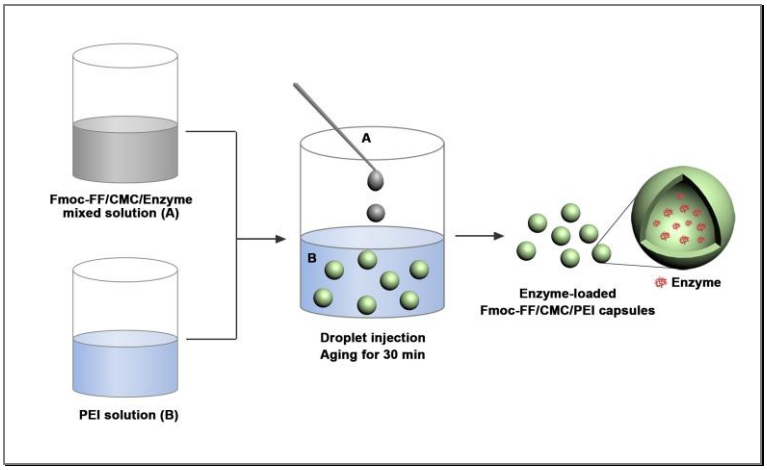


Fig.S4 Schematic illustration for the enzyme immobilization within Fmoc-FF/CMC/PEI hybrid capsules. The default conditions: Fmoc-FF: 2 mg mL^{-1}, CMC: 3 mg mL^{-1}, PEI: 1.0wt%, pH of solution A: 9.0, pH of solution B: 7.5.

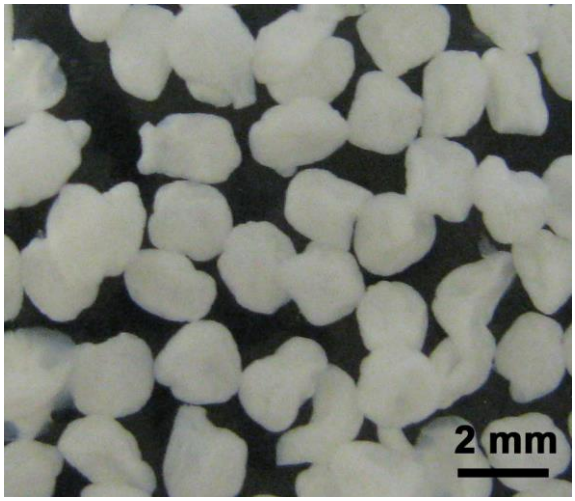


Fig. S5 Photograph of enzyme-loaded Fmoc-FF/CMC/PEI hybrid capsules, the enzyme loading capacity is 54.3 mg g^{-1} dry capsules.

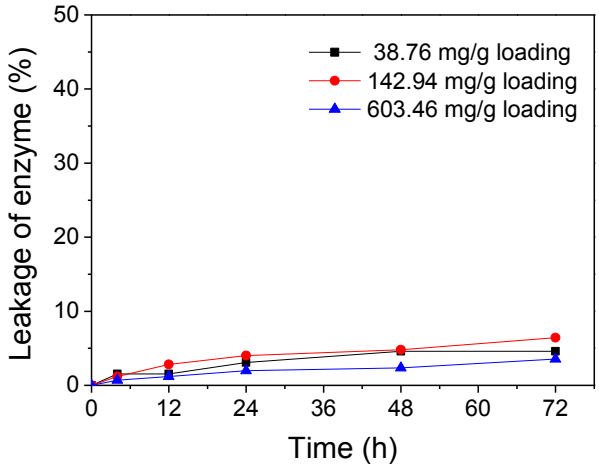


Fig.S6 The leakage of SpEH from Fmoc-FF/CMC/PEI hybrid capsules, the accumulative proteins in KP buffer were determined by UV-Vis spectrometry.

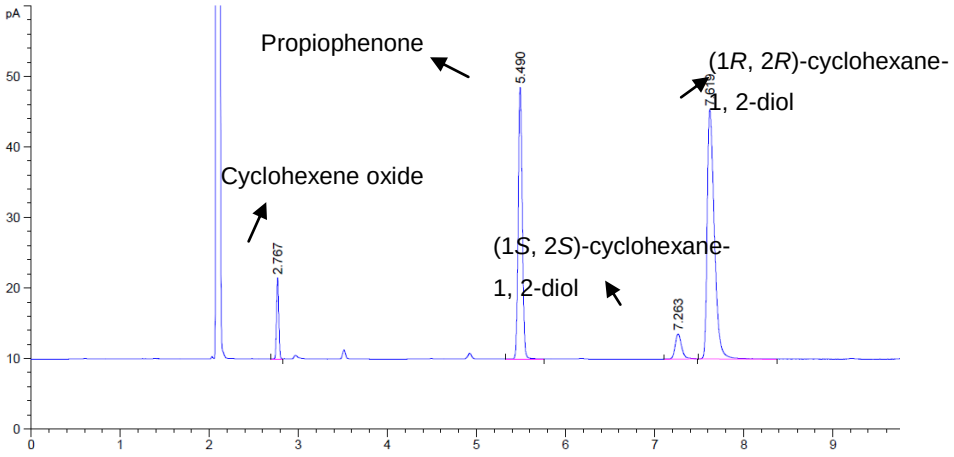


Fig. S7 GC chromatogram of the substrate (cyclohexene oxide) and its products, the operating conditions: Agilent GC 7890A, Chiral column: Supelco, β-DEXTM120, 30 m × 0.25 mm ×0.25 mm; Temperature: 150 °C.

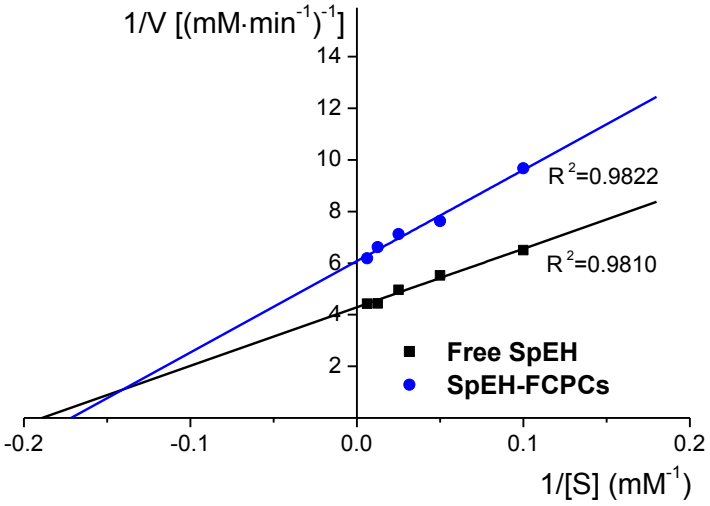


Fig. S8 Lineweaver–Burk double-reciprocal plot for the catalytic activity of free SpEH and SpEH-FCPCs. The reciprocal of initial catalytic velocity (1/*V*) is plotted against the reciprocal of cyclohexene oxide concentration (1/[*S*]).

3. Supplementary Tables

Table S1 Kinetic parameters of free and immobilized enzyme

State of enzyme	K_m (mM)	V_{max} (mM min^{-1})	k_{cat} (s^{-1})
Free SpEH	5.3	0.23	3.7
SpEH-FCPCs	5.8	0.16	2.6

Note: the EF value (0.7) is less than 1, suggesting the capsules provide a barrier to transport of substrate.

Table S2 The parameters of the immobilized enzyme within capsules

Capsules	Immobilization efficiency	Maximum loading capacity	Enzyme leakage	Activity referred to free enzyme	Recycling efficiency/ cycles/time per cycle
Alginate[S3]	70.5%	-	80% (5 h)	<50% (60 min)	20% / 10 / 10 min
Alginate-chitosan[S4]	-	-	-	21.8% (60 min)	44% / 24 h
SA-CS/PMCG[S5]	43%	-	-	12.5% (4 min)	92% / 45 / 8 min
Alg–Chi–CaP[S3]	92.6%	-	35% (7h)	<50% (60 min)	82% / 10 / 10 min
Alginate-silica[S6]	-	-	-	55.6 % (-)	-
Alginate-silica[S7]	<60%	-	-	<40% (-)	99% / 10 / -
PSS/DAR[S8]	-	-	-	52.8% (3 min)	-
This study	>95%	604	<5% (72 h)	50.4% (10 min)	86% / 10 / 240 min

Note: "-" represents the corresponding data is undetermined or unmentioned in the references.

4. References

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