Electronic Supplementary Information

Enzyme-based inverse opals: a facile and promising platform for fabrication of biocatalysts

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Experiment Detail

1 Reagents and materials

Horseradish peroxidase (HRP, EC. 1.11.1.7, 150 U/mg) was purchased from Source leaves Biotechnology Co. (Shanghai, China). Lipase (from *Candida rugosa,* Triacylglycerol lipase*,* EC. 3.1.1.3,) and Bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (Rodríguez Couto and Toca Herrera). α-Amylase (from *Bacilus subtilis*, EC. 3.2.1.1, ≥4000 U/g) was purchased from Kayon Biological Technology Co. (Shanghai, China). Nitrile hydratase (from *Rhodococcus Rhodochropus*, EC. 4.2.1.84) was purchased from Hangzhou Biosci Biotech Co. (Hangzhou, China). Glutaraldehyde (50% wt%), ethyl acetate, phenol, H_2O_2 (30% w/v), starch, 3,5-dinitrosalicylic acid and sodium potassium tartrate tetrahydrate were purchased from Jiang Tian Chemical Technology Co. (Tianjin, China). Other chemicals were of analytical grade and were used as received without further purification.

2 Preparation of enzyme-based inverse opal

Monodisperse PS microspheres with diameter of 250 nm were prepared as previous report, and the PS colloidal templates were assembled by centrifugation.[1-3] Specially, the PS microspheres suspensions were loaded into 50 mL plastic falcon tubes, then centrifuged at 1000 rpm for 2 d. The supernatant was removed by decantation, and

the colloidal crystals left to dry in air at room temperature for 3 weeks. The preparation method of enzyme-based inverse opal is as follows: typically, 0.5 g PS colloidal templates was added in the Schlenk flask and using vacuum pump to remove the air in the template gap for 1 h, then 5 mL enzyme solution (HRP 5 mg/mL, BSA 45 mg/mL; crude amylase 40 mg/mL, BSA 30 mg/mL) was injected into the flask to immerse all the templates and kept vacuum for 24 h to fill the gap completely. The enzyme-filled templates was washed 3~5 times to wipe off the enzyme on the surface of template, then 5 mL glutaraldehyde with different concentrations was injected into the flask under vacuum. After cross linking at 4 ºC for 5 h, the unreacted glutaraldehyde was removed by washing. The cross-linked enzyme-filled templates were immersed in ethyl acetate at room temperature to dissolve PS templates. The final enzyme-based inverse opal was stored in phosphate buffer solution (PBS, pH 7.0) at 4 ºC until use.

The preparation method was appropriate for many enzymes, in this study, two enzymes (HRP, amylase) were used to prepare inverse opals and their apparent kinetic parameters (K_m, V_{max}) were studied.

The effect of the parameters such as glutaraldehyde concentration was also studied to optimize the preparation process of enzyme-based inverse opal.

In order to verify the influence of organic solvents on the activity,

HRP-based inverse opal was immersed in ethyl acetate, acetone, and tetrahydrofuran for different times at room temperature. The activity was measured at 25 ^{o}C after washing with PBS (pH 7.0) for 3-5 times. The initial activity was taken as 100%.

3 Preparation of cross-linked enzyme aggregates (CLEAs)

The CLEAs of HRP and amylase were prepared by conventional method. In a 50 mL centrifuge tube with a magnetic stirrer bar, 1 mL of enzyme solution with the concentrations used in the preparation of enzyme-based inverse opal, 9 mL saturated ammonium sulfate were added at 4 ^{o}C. After 30min, an amount of glutaraldehyde (50%, wt%) was added (the final concentration was 1% (wt%)) and stirred for 5 h. The suspension was diluted with 10 mL buffer, centrifugated and washed with buffer for 3 times. Finally, the insoluble CLEAs were re-suspended in phosphate buffer and stored at 4 ^{o}C until use.

4 Activity assay

Enzymatic activities of free HRP, HRP-CLEAs and HRP-based inverse opal were tested according to the method of Worthington with phenol and H_2O_2 as substrates and 4-aminoantipyrine as color indicator.[4] The absorbance values were monitored at 510 nm and converted to the amount of H_2O_2 consumption by standard curve. One unit of activity was defined as the amount of HRP required to hydrolyze 1 μmol of H_2O_2 in 1 minute at 25 ^{o}C and pH 7.0.

The activities of free amylase, amylase-CLEAs and amylase-based inverse opal were determined according to DNS method[5,6] and the reaction time was 3 min for all the experiments. For immobilized amylase, an amount of amylase CLEAs and amylase-based inverse opal were first suspended in 0.5 mL PBS (0.02 M, pH 7.0) and then assayed the activity. One unit (U) of amylase activity was defined as the amount of enzyme which produced reduced-sugar equaled to 1 μmol maltose in 1 min at 25 °C and pH 7.0.

5 The properties of free HRP and HRP-based inverse opal

The pH stability was determined by incubating free HRP and HRP-based inverse opal in PBS between 3.0 and 10.0 (excessive acidity and alkalinity were regulated by HCl and NaOH) for 3 h and the activity was determined in PBS (pH 7.0). The thermal stability was determined by incubating free HRP and HRP-based inverse opal in PBS (pH 7.0) at 50 °C and 60 °C respectively with different time. Residual activity was then determined when temperature decreases to 25 °C. In all these experiments, the results were converted to relative activities where the maximum activity was taken to be 100%.

6 Applications of HRP-based inverse opal

The effect of molar ratio of H_2O_2/phenol on phenol removal with different phenol concentrations was studied. Briefly, different amount of

H_2O_2 (40 mM) was added into a 10 mL centrifuge tube which contains 2 mL of phenol solution and 25 mg HRP-based inverse opal (0.36 U) to start the reaction. After reacting for 90 min at 25 ^oC, the phenol removal efficiency was determined by using potassium ferricyanide and 4-aminoantipyrine.[7,8]

The reusability of HRP-based inverse opal was also studied. The experiments were conducted at 25 ^oC in 200 mL round-bottom flask containing 40 mL phenol solution (4 mM), 40 mL H_2O_2 (8 mM) and an amount of HRP-based inverse opal with the enzymatic activity of 14.4 U. At a predetermined time interval, 1 mL sample was taken out to analyze the phenol removal efficiency. After each bench of reaction, the HRP-based inverse opal was recovered and washed 3 times with PBS (0.05 M, pH 7.0) to remove the residual reactants or products within the immobilized HRP for another cycle. In all these tests, the absorbance values were transformed to the concentrations of phenol by a calibration curve based on pure phenol.

7 Determination of the apparent kinetic parameters

The apparent kinetic parameters (K_m, V_{max}) of the two enzymes were measured. For free HRP, HRP CLEAs and HRP-based inverse opal, the kinetic constants were determined by changing the concentration of H_2O_2 from 0.2 mM to 4 mM. For free amylase, amylase CLEAs and amylase-based inverse opal, the kinetic parameters were measured by

changing the concentrations of starch from 2.5 mg/mL to 10 mg/mL. All the catalytic reactions were taken at 25 ^{o}C and the initial reaction rates were fitted to the Michaelis-Menten equation.

References

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Supplementary Figures

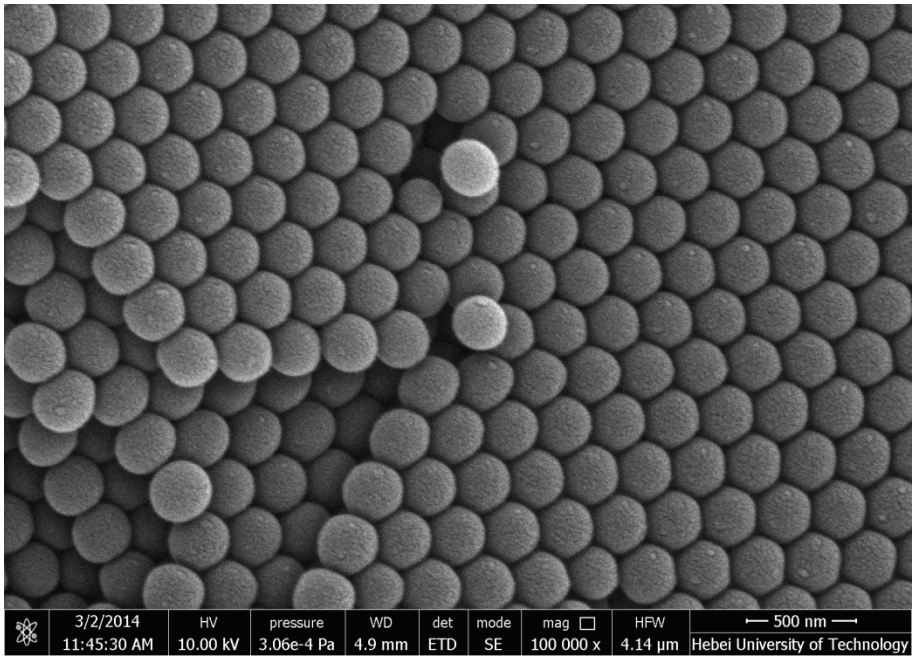

Fig. S1 SEM image of polystyrene colloidal crystal templates

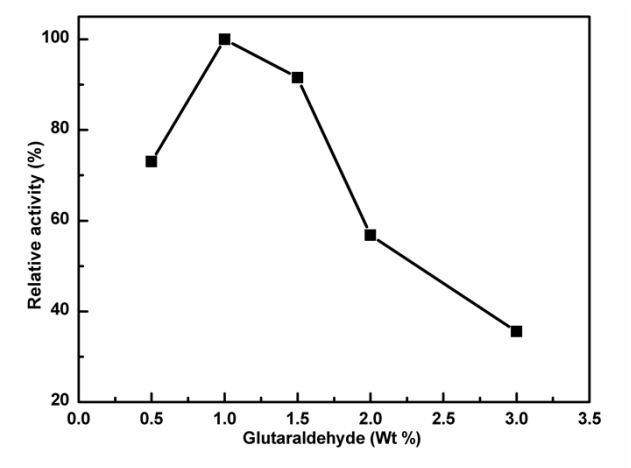

Fig. S2 The effect of glutaraldehyde concentration on the activity of HRP-based inverse opal

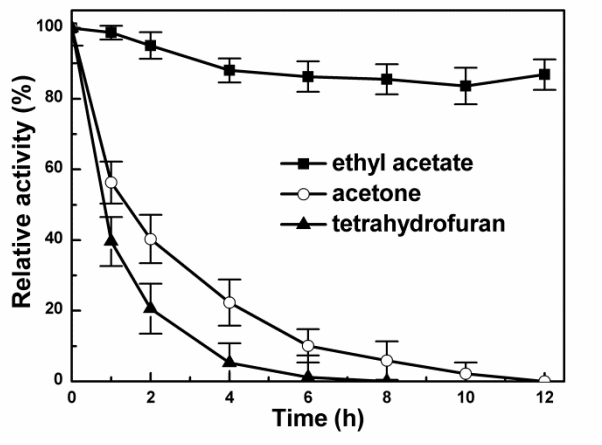

Fig. S3 The effect of organic solvents on the enzymatic activity of HRP

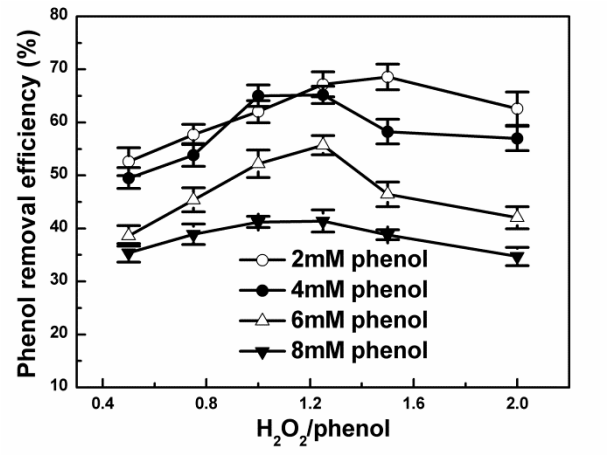

Fig. S4 Effect of H_2O_2 concentration on phenol removal

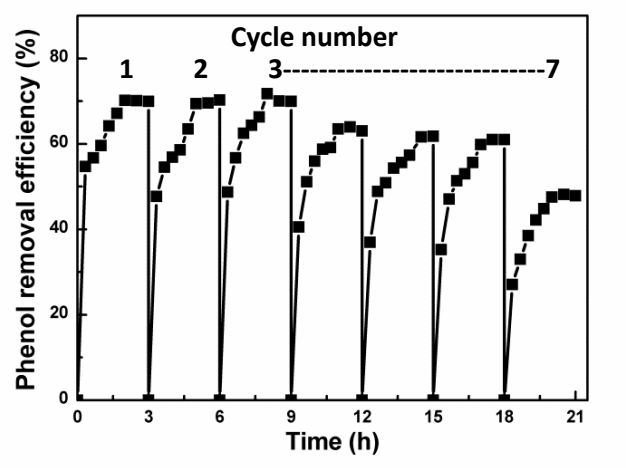

Fig. S5 Reusability of HRP-based inverse opal on phenol removal (phenol concentration 4 mM)

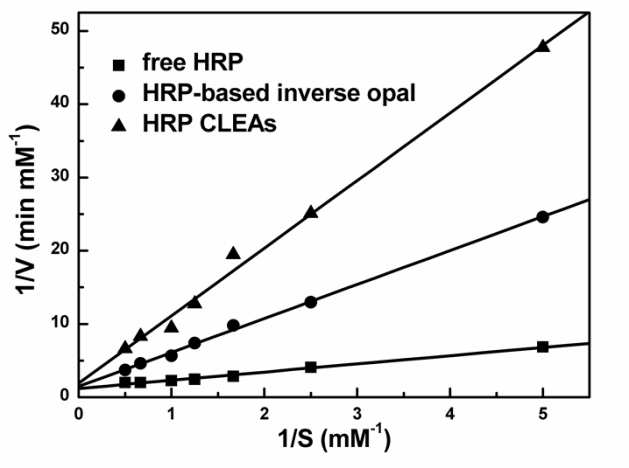

Fig. S6 Lineweaver–Burk plot for calculation of apparent kinetic parameters of the hydrolysis of H_2O_2 catalyzed by free HRP, HRP-based inverse opal and HRP CLEAs

Fig. S7 The optical image of amylase-inverse opal in ethyl acetate

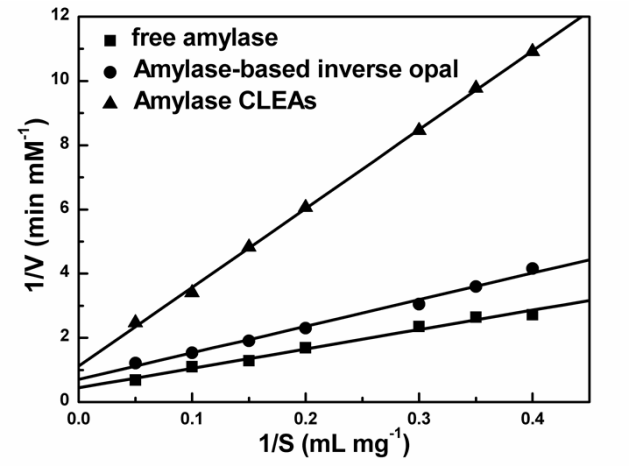

Fig. S8 Lineweaver–Burk plot for calculation of apparent kinetic parameters of the hydrolysis of starch catalyzed by free amylase, amylase-based inverse opal and amylase CLEAs