

Molecular Hydrogel-Immobilized Enzymes Exhibit Superactivity and High Stability in Organic Solvents

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Supporting Information

General: Rheology test was done on Rheometrics ARES 3 system, UV-vis spectra on a KR3000 type spectrophotometer, TEM on JEOL 2010 with 200 kV accelerating voltages, AFM on atomic force microscope mode of MultiMode SPM, and optical images on a digital camera. The reagents and solvents were used as received from commercial sources. The hemoglobin used in our experiment (Sigma H2500, as oxygen carrier in bovine blood) is mainly HbFe^{III} form (methemoglobin) due to the auto-oxidation in preparation (<http://www.sigmaaldrich.com/catalog/search/ProductDetail/SIGMA/H2500>). The other enzymes are peroxidase from horseradish (Shanghai Guoyuan Bio-Tech), Laccase from Trametes Versicolor (Fluka), and alpha-chymotrypsin from bovine pancreas (International Laboratory).

Synthesis of Gel I and Gel II: The typical procedure for confining the enzymes into the molecular hydrogel is as following: Addition of 36 mg Fmoc-L-lysine (**1**), 38 mg Fmoc-L-phenylalanine (**2**), and 20 mg sodium carbonate to 0.9 mL water gave a suspension, whose mass content of **1** and **2** is 7.4%. After being heated to about 333 K, the suspension turned to a clear solution. 40 mg Hb, being dissolved into the 0.1 mL water, was added into the solution at 308~313 K. The hydrogel (Gel I) formed by the self-assembly of the Fmoc amino acid molecules after cooling the warm solution to room temperature, and the whole process took about 10 minutes. The control, Gel II, was formed using the same process but without the addition of Hb. Using the same procedure, the molecular hydrogel was used to encapsulate other enzymes by changing the types and amounts of the enzymes used. The added enzyme contents for horseradish peroxidase (HRP), laccase (Laccase) and alpha-chymotrypsin (Alpha-CT) were 50 U, 3.6 U and 100 U, respectively.

Synthesis of Gel III: The addition of 40 mg Hb powder, 185 mg acrylamide monomer (40% mass content, including 2% N, N'-methylene-bis-acrylamide), 2 mg N, N, N', N'-tetramethylethylenediamine to water afforded a 1.0 mL suspension, in which the polymerization was initiated by 1 mg of ammonium persulfate after degassing oxygen. Gel III, with mass content of the poly(acrylamide) in 7.4%, was formed after about 30 minutes.

Rheological test: A control strain rheometer (Rheometrics ARES) with a cone and plate mixture (the plate diameter is 25 mm and the cone angle is 0.0999 rad) was used to measure the dynamic moduli of the hydrogel with/out Hb as a function of frequency in the range from 0.2 to 100 Hz (Figure S-1). The gap opening at the apex of the cone and plate was set to be 0.0483 mm, and the strain amplitude was fixed at 1% as this amplitude has been found to be well within the linear strain amplitude response regime.

Test of the leach of enzyme in Gel I: The leach of enzyme from hydrogel was measured by incubating 0.5 g of Gel I (containing 20 mg of Hb) in 40 mL of water or 40 mL organic solvents. Then the released amount of Hb from molecular hydrogel was measured by the Bradford methods. The absorbance of the complex of protein and coomassie brilliant G-250 was measured at 595 nm. The selected standard protein was hemoglobin itself (Figure S-1). After 24h incubation, the 50 μ L release solution was added to 3 ml the G-250 solution and measured the absorbance at 595 nm. The absorbance of the leached Hb in water is 0.0025. By comparing with the standard curve plotted by various concentration Hb and absorbed intensity, the calculated protein amount is $(0.0025/4.2)*(3/0.05) = 0.0357$ mg/ml. So the leached amount of Hb in water is $(0.0357/0.5)*100\% = 7.1\%$. The absorbance of the leached Hb in other solvents is less than 0.0001 (the lowest tested limit), thus the leached amount of Hb in these solvents is less than 0.3%.

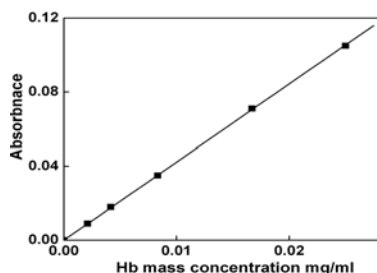


Figure S-1. The standard curve of the hemoglobin mass concentration with the absorbance.

Fluorescence of Gel II: Gel II shows the emission peak at 450 nm (Figure S-2), which confirms the overlap of fluorene groups. This result indicates the self-assembly of **1** and **2** is also governed by π - π interaction and hydrogen bonding, similar to our previous study (Ref. 16).

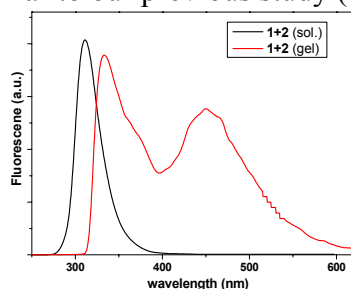


Figure S-2. Emission spectra of Gel II and the solution of **1** and **2**.

Optical images and morphologies of Gels I, II, and III: The TEM samples were prepared by dipping a Cu grid with carbon film into the hydrogels, and the AFM samples by dipping mica wafer into the hydrogels. The vial containing the grid or the wafer was frozen in liquid nitrogen. The frozen samples were then kept in vacuum for at least 12 hours to completely remove water. The pore size (0.2-2 μm) of Gel I was determined by the statistical average value from AFM and TEM images, while the pore size (5-6 nm) of Gel III was obtained from the literature of the same content polyacrylamide electrophoresis hydrogel (Ref. *Electrophoresis of Proteins in Polyacrylamide and Starch Gels*, 1969, North Holland, Amsterdam).

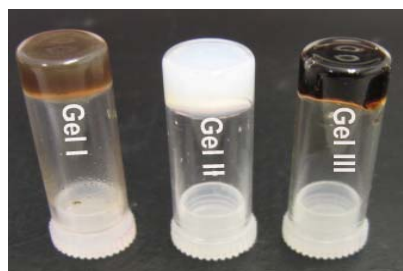


Figure S-3 Optical images of Gels I, II, III.

Test of catalytic activity:

(i): the enzymatic reaction activities are defined as below¹

$$C_{pt} = \Delta A / \epsilon, V_t = -dC_{st} / (dt * C_{enzyme}) = 2dC_{pt} / (dt * C_{enzyme}), \text{Conversion}(\%) = (C_{pt} * 2 / [S]) * 100\%$$

V_t , the reaction rate in reaction time t ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ for Hb and $\text{nmol} \cdot \text{min}^{-1} \cdot \text{U}^{-1}$ for other enzymes); V_0 , the initial reaction rate; ΔA , the increase of absorbance intensity; ϵ , the molar extinction coefficients of the colorful product; t , reaction time in minute; $[S]$, the starting molar concentration of the substrate; C_{st} , the molar concentration of the substrate after t minutes reaction time; C_{pt} , molar amount of the

product after t minutes reaction time; C_{enzyme} , the concentration of enzymes (mass concentration for Hb and enzyme international unit concentration for other enzymes); dC_{st} , differential of substrate; dC_{pt} , differential of product; and dt , differential time.

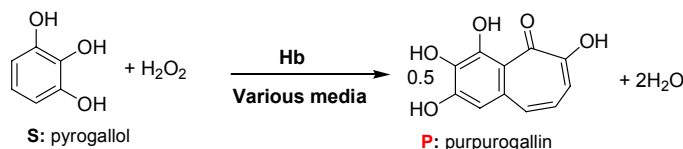
In the starting period, the enzyme catalytic reaction displays zero order kinetics. Thus the first average rate can be used to represent V_0 . The enzyme reactions fit the steady-state Michaelis-Menten equation.

$$V_0 = V_{\text{max}} \cdot [S] / ([S] + K_m)$$

V_{max} , the maximum reaction rate, which is independent with the substrate concentration; K_m , the enzyme Michaelis-Menten constant, in which concentration the reaction rate can achieve the half of the V_{max} . The dynamic constant (V_{max} and K_m) can be calculated by the Lineweaver-Burk plot ($1/V_0$ as y axis and $1/[S]$ as x axis). It is well known that the V_{max} value of enzyme is independent with substrate concentration, which represents the inherent activity of the enzyme.

(ii): the enzymatic reaction condition are described as below

The reaction of the water-soluble pyrogallol with H_2O_2 was selected to characterize the activity of Hb. The concentrations of the product in different solvents were corrected according to the molar extinction coefficients in aqueous buffer, toluene, acetonitrile, and ethylacetate.



$\epsilon_{420\text{nm}}$ is $2640 \text{ M}^{-1} \cdot \text{cm}^{-1}$ in 50 mM pH 7.0 phosphate buffer, $4400 \text{ M}^{-1} \cdot \text{cm}^{-1}$ in toluene, $4320 \text{ M}^{-1} \cdot \text{cm}^{-1}$ in acetonitrile, and $4260 \text{ M}^{-1} \cdot \text{cm}^{-1}$ in ethylacetate, respectively.

The mixture of the substrates (10 mM) and H_2O_2 (30 mM) in 100 mL of different solvents were catalyzed by 0.1 g/L Hb in different matrix (unconfined, Gel I, Gel III).² The absorbance of product was measured during 15 minutes. During the enzymatic reaction course, the reaction mixture was slight stirred at room temperature (298 K). **The randomly shaped hydrogels would form 0.03 to 0.3 mm sized particles during reaction. The hydrogel particles were dispersed in the low density solvents bottom. The hydrogel formed by self-assembly of Fmoc amino acids is stability in different solvent, especially for hydrophobic toluene. In aqueous solutions, the hydrogel is stability until heating to its sol-gel transition temperature about 333K.** The blank control is Gel II (Figure S-4), which didn't show any catalytic ability in the system.

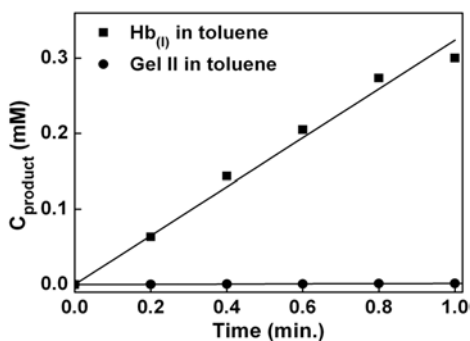


Figure S-4 The oxidation of pyrogallol catalyzed by Hb₍₀₎ and Gel II, respectively.

The increase of the absorbance at 420 nm in the first minute with 0.2 minutes intervals was measured for the reaction of pyrogallol with H_2O_2 catalyzed by the three Hbs. The colorful product purpurogallin concentration was calculated by their molar extinction coefficient at 420 nm. Then the initial reaction rate

was obtained by linear fitting the product concentration with time. By changing the substrate concentration from 10mM, 7.5 mM, 5mM, 3mM, 2mM, and 1mM, a series of initial reaction rate is calculated. At the same time, the concentration of H₂O₂ is fixed at 30 mM. At last, the maximum reaction rate (V_{\max}) is calculated by the Lineweaver-Burk plot of initial reaction rate with substrate concentration (Figure S-5).

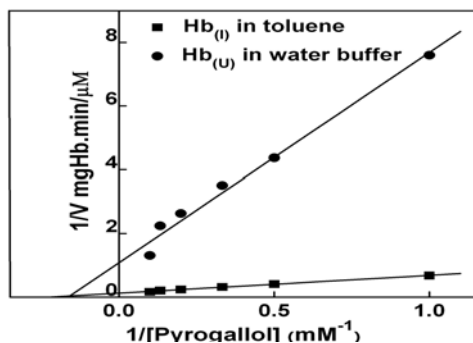
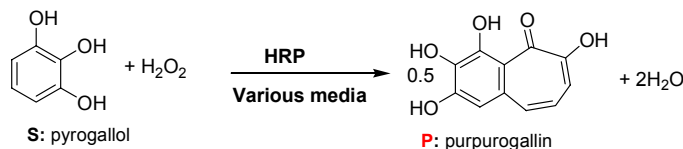
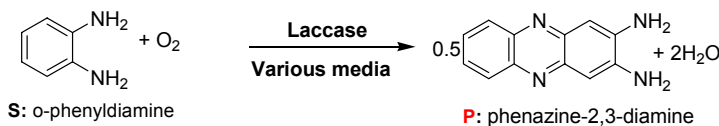


Figure S-5 The Lineweaver-Burk plot of Hb_(U) in water and Hb_(II) in toluene.

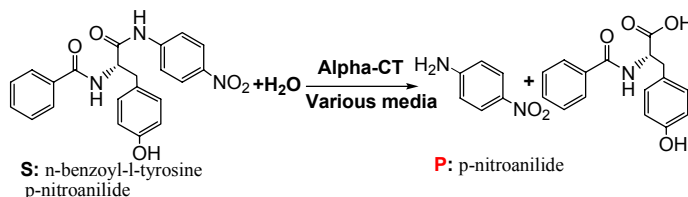
The results show that the V_{\max} values of Hb_(U) in water and Hb_(II) in toluene are 0.92 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ and 7.98 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$, respectively. The V_{\max} of Hb_(II) and Hb_(U) in other solvents are determined by the same methods. For other enzymes, the catalytic reactions and the relative molar extinction coefficients of the colorful products are listed as follow.



$\epsilon_{420\text{nm}}$ is 2640 $\text{M}^{-1}\cdot\text{cm}^{-1}$ in 50 mM pH 7.0 phosphate buffer, 4400 $\text{M}^{-1}\cdot\text{cm}^{-1}$ in toluene.



$\epsilon_{450\text{nm}}$ is 16300 $\text{M}^{-1}\cdot\text{cm}^{-1}$ in 50 mM pH 6.0 phosphate buffer, and 10600 $\text{M}^{-1}\cdot\text{cm}^{-1}$ in toluene.



$\epsilon_{380\text{nm}}$ is 14000 $\text{M}^{-1}\cdot\text{cm}^{-1}$ in 50 mM pH 8.0 Tris-HCl buffer, and 11000 $\text{M}^{-1}\cdot\text{cm}^{-1}$ in toluene.

For HRP, the mixtures of pyrogallol (from 10 to 1mM) and 30 mM of H₂O₂ in 100 mL of different solvents was catalyzed by HRP (50 U/L) in different forms (free or immobilized in the hydrogel).² The absorbance of product at 420 nm is monitored. The average rate in the first minute is defined as the initial rate. For laccase, the mixture of o-phenyldiamine (from 5 to 1mM) in 100 mL of different solvents was catalyzed by laccase (3.6 U/L) in different forms (free or immobilized in the hydrogel). The absorbance of product at 450 nm is monitored. The average rate in the first five minutes is defined as the initial rate. For alpha-CT, the mixture N-benzoyl-l-tyrosine-p-nitroanilide (from 1 to 0.1 mM) of in 100 mL of different solvents was catalyzed by alpha-CT (100 U/L) in different forms (free or immobilized in the hydrogel). The absorbance of product at 380 nm is monitored. The average rate in the first five minutes is

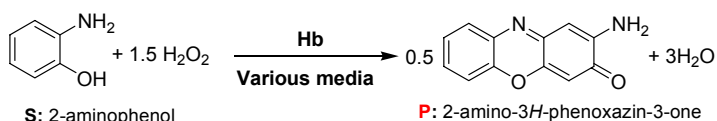
defined as the initial rate. The activity of HRP, laccase, and alpha-CT were determined by the Lineweaver-Burk plot as shown in the literature [Refer to *Collect. Czech. Chem. Comm.* **1992**, *57*, 625-640.].

Test of the stability constants and reusability:

(i): Stability constants

The stability constants are defined and calculated according to literature.¹ For the oxidation of pyrogallol catalyzed by the Hbs, the 15 minutes reaction course curves were measured based previous procedure. The C_{pt} derivative in 10 minutes was selected to calculate $t_{1/2}$. The results are shown **Table S-1**.

For the oxidation 2-aminophenol catalyzed by the Hbs (shown as below), the 60 minutes reaction course curves were measured by monitoring the increasing of absorbance at 430 nm. The practical product amount is calculated by the molar extinction coefficient of 2-amino-3H-phenoxazin-3-one in different solvents.



$\epsilon_{430\text{nm}}$ is $26000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ in 50 mM pH 7.0 phosphate buffer, and $13600 \text{ M}^{-1} \cdot \text{cm}^{-1}$ in toluene

Hb_(I) and Hb_(III) in toluene are active even after 60 min of reaction. So their C_{pt} derivative in 50 minutes was selected to calculate $t_{1/2}$. Hb_(U) showed low catalytic ability after 15 minutes of reaction in toluene. So the C_{pt} derivative in 10 minutes was selected to calculate $t_{1/2}$. The results are shown in **Table S-1**.

Table S-1. The half time of various forms Hb with various substrates

	$t_{1/2}$ min of pyrogallol	$t_{1/2}$ min of <i>o</i> -aminophenol
Hb _(I) in toluene	7.0	27.8
Hb _(III) in toluene	4.3	14.1
Hb _(U) in water	1.9	3.4

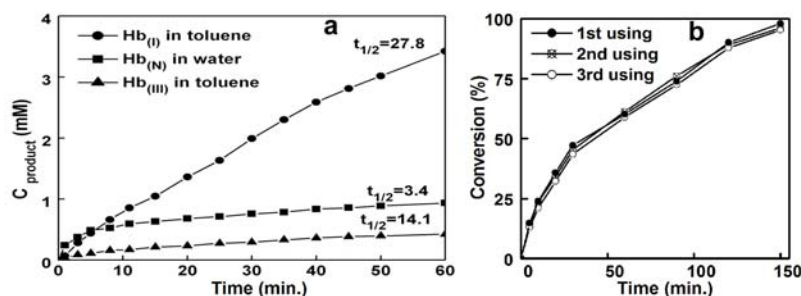


Figure S-6 (a) Hb (0.1 g/L) catalyzed oxidation of 2-aminophenol (10 mM) by H_2O_2 (30 mM) under different conditions. The half life ($t_{1/2}$, minute) represents stability of the enzyme. (b) The oxidation of 2-aminophenol catalyzed by the fresh and reused Hb_(I) in toluene.

(ii): the reusability is test as below¹

To test its reusability, we used fresh and recovered Hb_(I) (0.1 g/L) to catalyze the oxidation of OAP (2 mM) by H_2O_2 (6 mM) in 100 ml toluene solvent. The first run achieved 98% conversion of OAP after 150 minutes reaction. The recovered Hb_(I) was separated from the reaction mixture by centrifuging, then washed with 50 mL fresh toluene 2 times and 50 mL ether solvent 1 time to remove APX, and the mixed with a fresh reaction mixture.

- (1) Liese, A.; Seelbach, K.; Wandrey, C. *Industrial Biotransformations: A Collection of Processes* Wiley-VCH Darmstadt, 2000.
- (2) Maehly, A. C.; Chance, B. *Methods Biochem Anal.* **1954**, *1*, 357-424.