Electronic Supplementary Information for

Thermal Responsive Microgels as Recyclable Carriers to Immobilize Active Proteins with Enhanced Nonaqueous Biocatalytic Performance

Qing Wu, Teng Su, and Qigang Wang*

Department of Chemistry, and Advanced Research Institute, Tongji University, Shanghai 200092, P. R. China

1. Materials and reagents

Hemoglobin (Hb, MW = 68 kDa, lyophilized powder) , Bovine serum albumin (BSA, MW = 67 kDa, lyophilized powder) were purchased from Shanghai Baoman Biotechnology Co., Ltd.. *N*-acryloxysuccinimide and *N-Isopropylacrylamide* were purchased from TCI and used without further purification. Mineral oil was purchased from Alfa Aesar. Hydrogen peroxide (H_2O_2, 30 wt %) was obtained from Sinopharm Chemical Reagent Co., Ltd.. All other chemicals and solvents were of the highest purity commercially available and were used as received.

2. Instruments and characterizations

UV-Visible spectra were obtained by UV-2700 (Shimadzu Corporation). Dynamic light scattering (DLS) studies of the microgels was conducted on Zetasizer Nano instrument (Malvern Instruments Ltd., United Kingdom) equipped with a thermoelectric temperature controller. Scanning electron microscope (SEM) images were obtained on Hitachi S-4800 with 1 kV accelerating voltages.

3. Preparation of the microgel by inverse emulsion polymerization

Acryloylation of BSA: BSA was firstly conjugated with N-acryloxysuccinimide (NAS) to introduce acryloyl groups on the molecule. BSA (120 mg) and *N*-acryloxysuccinimide (9 mg) were dissolved in 4 mL of distilled water and the conjugation is achieved by magnetic stirring for 1h at room temperature. The solution was then thoroughly dialyzed against deionized water with a semi-permeable membrane (cut-off molecular weight: 8000-14000, Sinopharm Chemical Reagent Co., Ltd.) for three days to remove the unreacted reagents and by-products. Acryloylated BSA solutions were store at 4°C for further uses.

Preparation of microgel via inverse emulsion polymerization: The W/O inverse emulsion was created by dropwise addition of aqueous solution to the continuous oil phase and then sonication. 200 mg of Span80 (1.25%, w/w) dissolved in 16 mL of mineral oil constituted the continuous oil phase. For the preparation of aqueous phase, 4 mL of acryloylated BSA solutions was mixed with 0.12 g of NIPAM power to give a transparent aqueous solution. The emulsion was homogenized by sonication and then managed under N_2 to remove residual oxygen. Subsequently, the polymerization was initiated by γ-ray (2 kGy). After the reaction, the obtained particles were separated from the oil phase by centrifugation (8000 rpm) for 20 min and washed with

isopropanol and deionized water for several times to eliminate the emulsifiers and unreacted monomers. The purified microgels were then used to immobilize enzymes.

4. Calculation of the swelling ratio (α) [1]

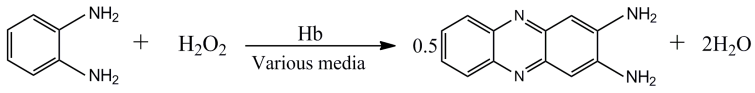
The value of the swelling ratio (α) was calculated by the equation:

$$\alpha = \frac{V_{swollen}}{V_{shrunken}} = \left(\frac{D_{H,\,swollen}}{D_{H,\,shrunken}}\right)^3 \qquad \text{Equation-1}$$

The equation was defined as the volume ratio before and after phase transition. Then, the value of the swelling ratio (α) was obtained as 53. The high value of α indicates the excellent swelling behavior of the microgels variation with the temperature and the microgels are available to embed the enzymes.

5. Test of catalytic activity [2,3]

We used the oxidation of o-phenylenediamine (OPD) by H_2O_2 as a model reaction to characterize the catalytic activity of the bound and native Hb and HRP. The mixture of the substrates (10 mM) and H_2O_2 (40 mM) in 100 mL of different medias were catalyzed by 0.1 g/L Hb and 0.2 mg/L HRP in different states (unconfined or immobilized). During the reaction course, the reaction mixture was slight stirred at room temperature (298 K). The concentrations of the product in different solvents were corrected according to the molar extinction coefficients in aqueous buffer, toluene, ethylacetate, and dioxane, respectively.



S: o-phenyldiamine P: phenazine-2,3-diamine

$\varepsilon_{450\ nm}$ is 16300 M^{-1} cm^{-1} in 20 mM pH 7.4 phosphate buffer, 10600 M^{-1} cm^{-1} in toluene, 10600 M^{-1} cm^{-1} in acetic ether, and 15000 M^{-1} cm^{-1} in dioxane, respectively.

The increase of the absorbance at 450 nm in the first minute with 0.2 minutes intervals was measured for the reaction of *o*-phenylenediamine with H_2O_2 catalyzed by the two types of Hb. Then the initial reaction rate was obtained by linear fitting the product concentration with time. By changing the substrate concentration from 10 mM to 0.5 mM with a fixed H_2O_2 concentration at 40 mM, we obtained a series of initial reaction rates for constructing the Lineweaver-Burk plot to estimate the kinetic

constant values. The typical characterization of enzymatic reaction is the Michaelis-Menten equation.

$$V_0=V_{max}.[S]/([S]+K_m) \qquad \text{Equation-2}$$

The turnover number, $k_{cat}=\mathbf{V_{max}/[E]}$, can be calculated by the Lineweaver-Burk plot in Fig. S1 and Fig. S2, which is independent with substrate and enzyme concentration.

6. Test of the reusability

To test the reusability of the microgel-immobilized enzymes, we used fresh and recovered Hb ($Hb_{(I)}$, 1.5μM) to catalyze the oxidation of OPD (3 mM) by H_2O_2 (40 mM) in 5 ml toluene solvent. The total amount of phenazine-2, 3-diamine was measured by UV after 15 minutes reaction. The recovered enzyme ($Hb_{(I)}$)was separated from the reaction mixture by centrifuging, then washed with 10 mL fresh toluene 2 times and 10 mL ether solvent 1 time to remove the product. The recycled enzyme ($Hb_{(I)}$) was then mixed with a fresh reaction mixture again to measure the 15 minutes total amount of phenazine-2, 3-diamine.

7. Figures

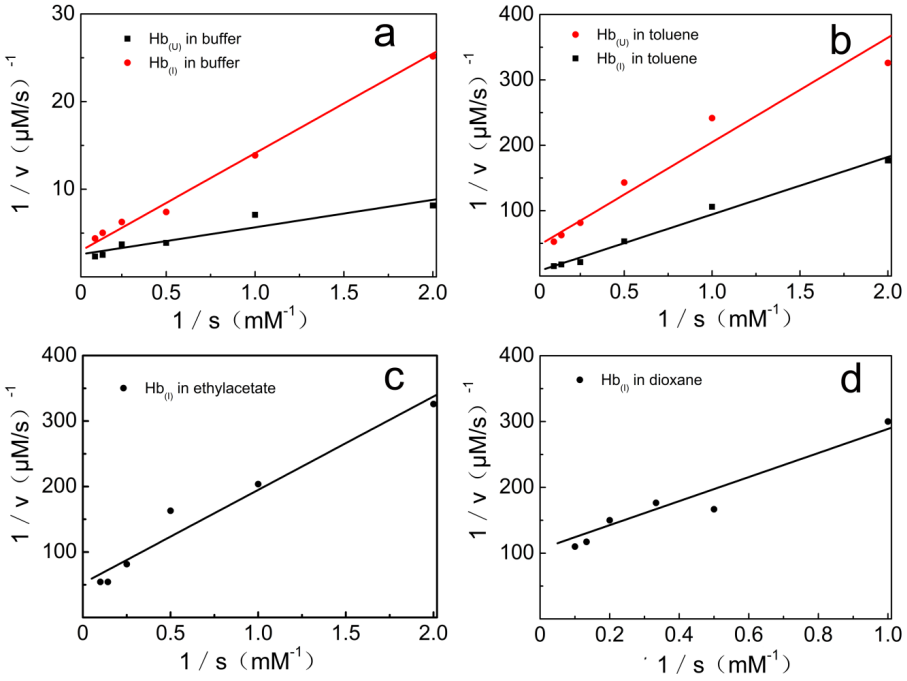


Fig. S1 The Lineweaver-Burk plot of $Hb_{(U)}$ and $Hb_{(I)}$ in water buffer (a), toluene (b),and $Hb_{(I)}$ ethylacetate (c),dioxane(d).

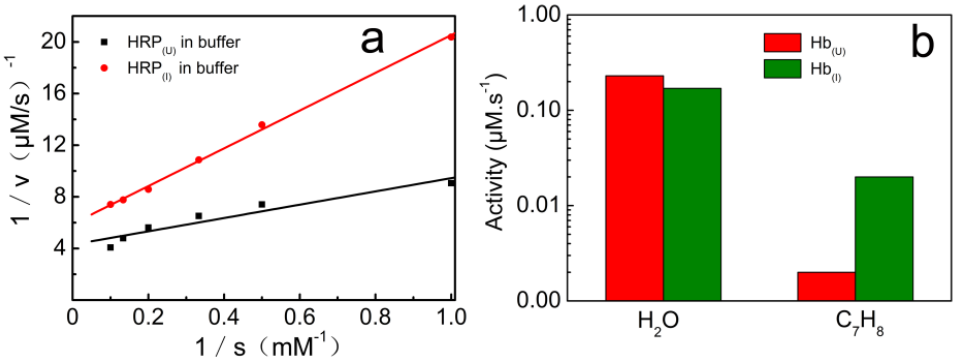


Fig. S2 The Lineweaver-Burk plot of $HRP_{(U)}$ and $HRP_{(I)}$ in water buffer (a), and comparison of activities of $HRP_{(U)}$ and $HRP_{(I)}$ in water buffer and toluene (b).

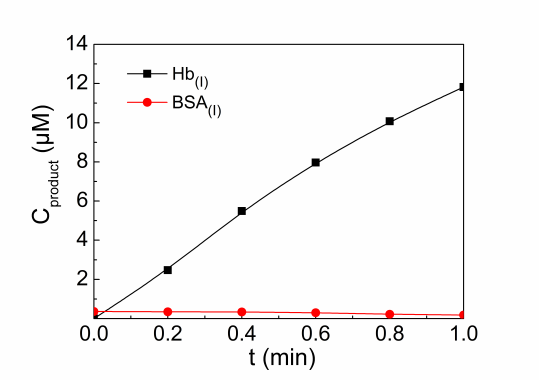


Fig. S3 The initial reaction courses of o-phenylenediamine (OPD) (10.0 mM) and H_2O_2 (40.0 mM) catalyzed by the BSA mirogel and the BSA mirogel with Hb in water.

Table S 1. Comparison of the catalytic activity of the active proteins in water and toluene.

	K_{cat} in water (s^{-1})	K_{cat} in toluene (s^{-1})
$Hb_{(I)}$	0.17	0.12
$Hb_{(U)}$	0.23	0.005
$HRP_{(I)}$	34.00	4.00
$HRP_{(U)}$	46.00	0.40

8. References

[1] K. Kratz, A. Lapp, W. Eimer, T. Hellweg, *Colloid Surf. A-Physicochem. Eng. Asp.* **2002**, 197, 55.

[2] A. Liese, K. Seelbach, C. Wandrey, *Industrial Biotransformations: A Collection of Processes* Wiley-VCH Darmstadt, **2000**.

[3] A. C. Maehly, B. Chance, *Methods Biochem Anal.* **1954**, 1, 357.