Electronic Supplementary Information

Crosslinking Catalysis-Active Center of Hemin on the Protein Scaffold toward Peroxidase Mimic with Powerful Catalysis

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Experimental section

Materials and reagents

Carboxyl groups-derivatized hemin (Hem) and bovine serum albumin (BSA) were purchased from Sangon Biotech Co., Ltd. (Shanghai,China). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide (EDC), N-Hydroxy succinimide hydrochloride (NHS), and 3,3',5,5'-Tetramethylbenzidine (TMB) were obtained from DiBai Reagents (Shanghai, China). The TMB-H_2O_2 chromogenic substrate and sodium phosphate were bought from Aladdin Reagent Co., Ltd. (Shanghai, China). Deionized water was supplied from the Ultrapure water system (Pall, USA). Other chemicals are of analytical grade and used directly without further purifications. All glass containers were cleaned by aqua regia and water before usage.

Apparatus

The synthesis of Hem-BSA composites of mimic enzyme was performed in the Collector-type thermostatic instrument (Gongyi Corey Ltd.). The colorimetric measurements of catalytic reaction products were performed by a microplate reader (Infinite M200 PRO, Tecan, Austria) with 96-well plates (JET BIOFIL, Guangzhou, China). Transmission electron microscopy (TEM, FEI Tecnai G20, USA) imaging operated at 100kV was employed to characterize the materials and composites so prepared. Uv-vis absorption spectra were collected using UV-3600 spectrophotometer (Shimadzu, Japan), and Fourier transform infrared (FTIR) spectra were obtained by FTIR spectrophotometer (Thermo Nicolet Nexus

470FT, USA). The hydrodynamic diameters of composites were measured by dynamic light scattering (DLS) with a Zetasizer Nano ZS (Malvern Instruments, UK) setup equipped with a helium-neon laser (λ = 632.8 nm, 4.0 mW).

Synthesis of mimic enzymes of Hem-BSA and Hem@BSA

The Hem-BSA composite was synthesized by coupling BSA with catalytic Hem derivatized with carboxyl groups by the EDC-NHS cross-linking chemistry. Firstly, an aliquot of EDC (100 mM) and NHS (80 mM) were pre-mixed and then added into the Hem solution (0.50 mL, 2.0 mg/mL) to be stirred for 1 h at room temperature. Secondly, an aliquot of BSA solution (0.50 mL, 80 mg/mL) was introduced into the Hem mixture activated by the EDC-NHS to be vigorously stirred at 37 ˚C for 12 h. After the products were further purified by dialysis, the resulting Hem-BSA composite was collected and stored at 4 ˚C for the future usage. The dosage of Hem in all of the composites involved was determined by the UV-vis absorbance measurements. Accordingly, the Hem@BSA was prepared simply by mixing BSA with Hem derivatized with carboxyl groups, except for the EDC-NHS activation of carboxyl groups of Hem. The resulting products were characterized separately using TEM, DLS, FTIR spectra and Uv-vis spectrophotometers.

Colorimetric measurements

Typically, an aliquot of 5.0 μL the prepared Hem-BSA composite with different dilutions was introduced into the TMB-H_2O_2 substrates to catalyze the redox reactions at room temperature for 20 min, of which the blue reaction products were monitored with the UV-visible absorbance values recorded at 652 nm using 96-well plates and a microplate reader. Meanwhile, the colorimetric investigations were carried out accordingly for exploring the enzymatic catalysis activities of Hem-BSA composite, with comparing to Hem and Hem@BSA, each containing the same concentration of Hem. Moreover, the main conditions for the synthesis of Hem-BSA composite were colorimetrically optimized using different Hem dosages (0.050 – 5.0 mg/mL), BSA usages (5.0 – 80 mg/mL), and NaOH amounts (0.050 - 0.20 M). Also, the catalysis stability of Hem-BSA composite was examined, including the catalysis conditions for TMB-H_2O_2 reactions at different pH values (from 2.0 to 14) and temperature (from 0 to 70 ˚C). Subsequently, the colorimetric assays for probing the steady state kinetic were comparably carried out for Hem-BSA composite and native Hem (each containing 2.0 μg/mL Hem), where 2.5 mM H_2O_2 or 0.45 mM TMB was used alternatively at a fixed concentration of one substrate versus varying concentration of the second substrate. The Lineweaver–Burk plots were performed for calculating the Michaelis–Menten constants, where the double-reciprocal plots for kinetic catalysis of Hem-BSA were carried separately by using various TMB concentrations at three fixed H_2O_2 concentrations (0.50, 2.0, and 8.0 mM) and the diverse H_2O_2 concentrations at three fixed TMB concentrations (0.20, 0.40, and 0.65 mM).

In addition, the Hem-BSA composite-based colorimetric assays for H_2O_2 with different concentrations ranging from 0.0015 to 25 mM were performed accordingly, taking Hem as a comparison.

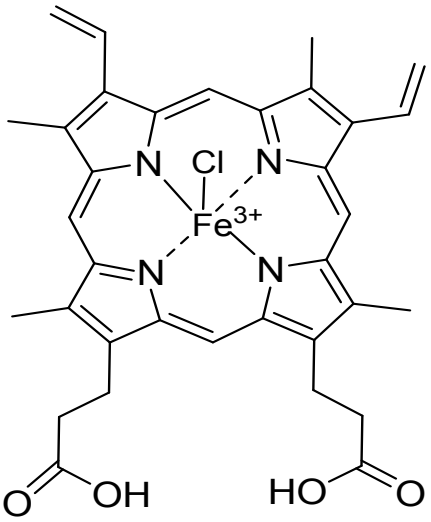

Fig. S1 The structure of native Hem derivatized with carboxyl groups.

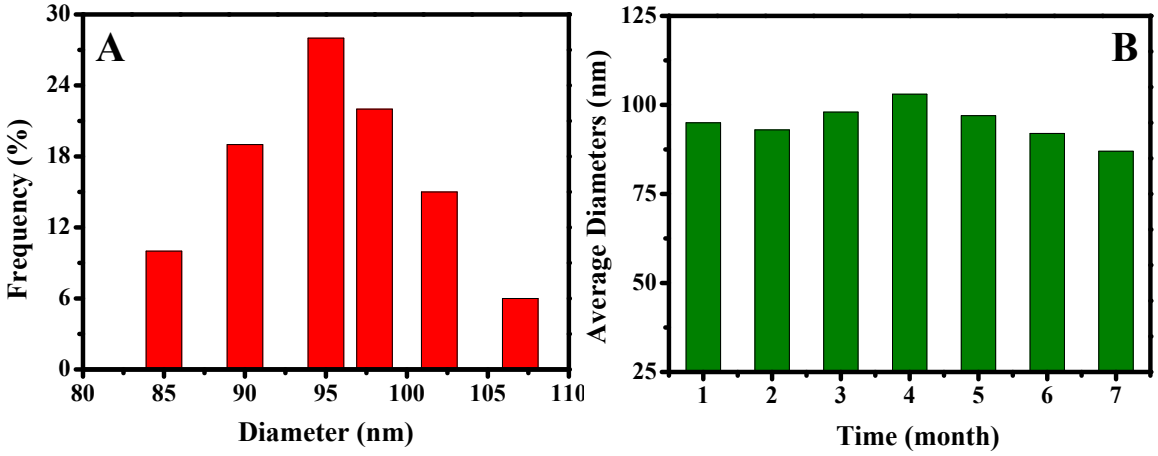

Fig. S2 (**A**) The hydrodynamic diameter distributions of Hem-BSA composites; (**B**) the change of average hydrodynamic diameters of Hem-BSA composites stored in water over different time intervals.

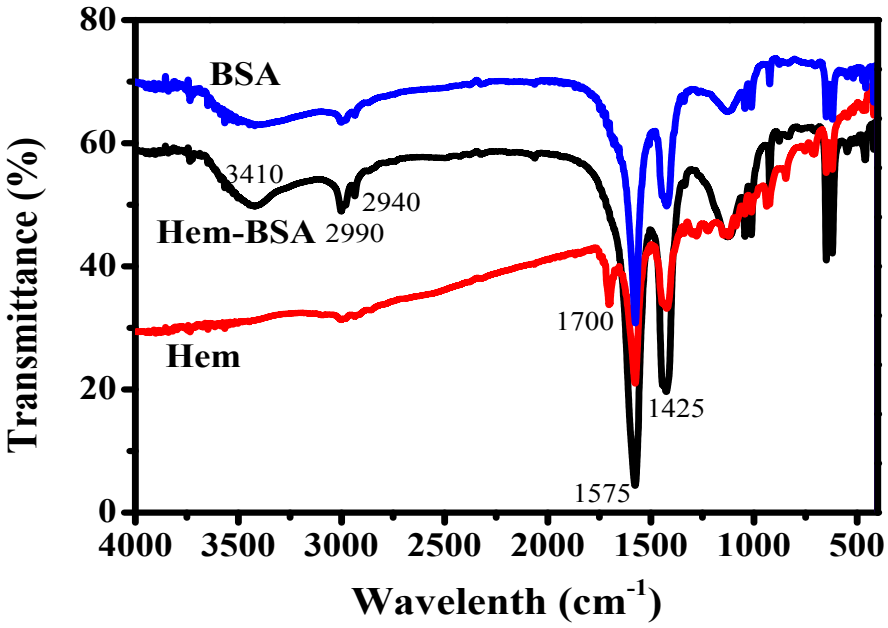

Fig. S3 The comparison of FTIR spectra among BSA, native Hem, and Hem-BSA in PBS (pH 7.0).

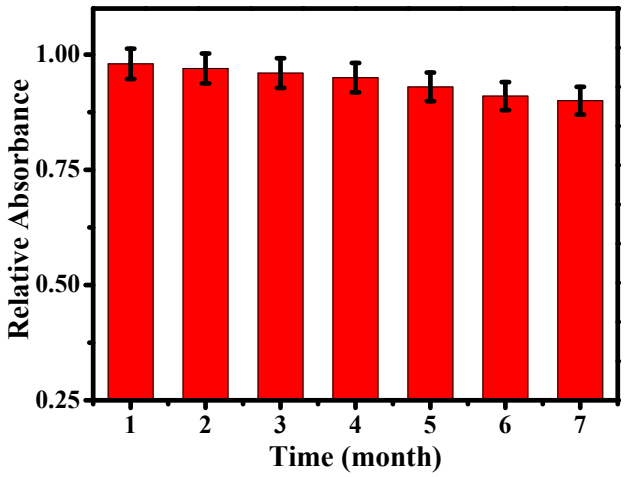

Fig. S4 Investigations on the environmental stability of Hem-BSA composite stored in water over different time intervals, where the catalytic TMB-H_2O_2 reactions were performed to monitor the catalytic activities of the Hem-BSA composite.

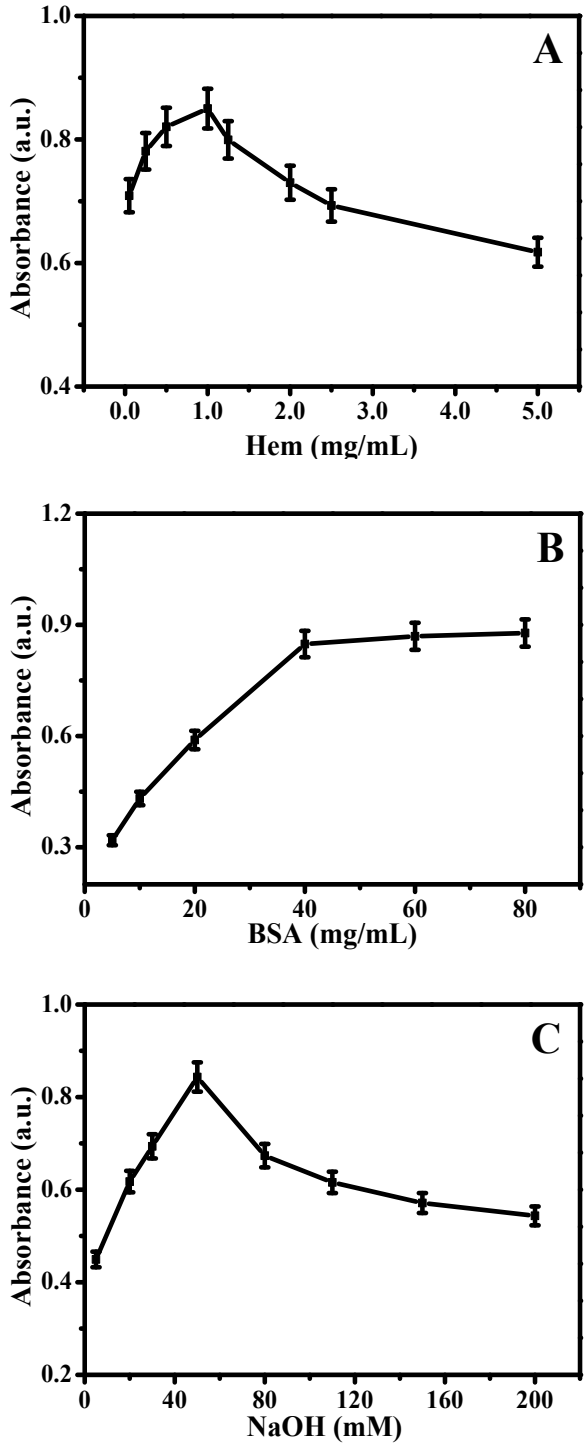

Fig. S5 Effects of the main synthesis conditions on the catalytic activities of Hem-BSA composite prepared using different amounts of (**A**) native Hem, (**B**) BSA, and (**C**) NaOH, where the catalytic TMB-H_2O_2 reactions were performed to monitor the catalytic activities of the resulting Hem-BSA composite.

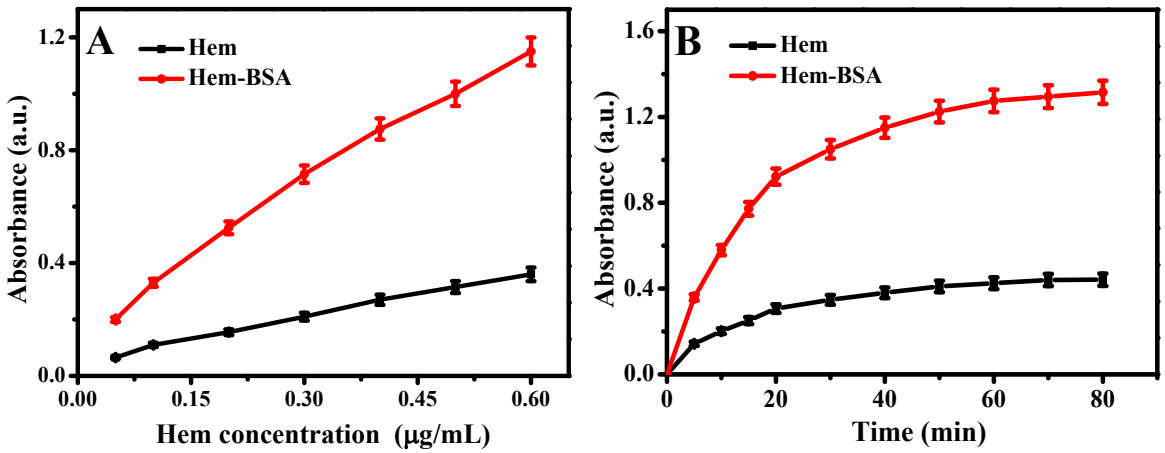

Fig. S6 (**A**) Colorimetric comparison of catalysis activities between Hem-BSA composite and native Hem containing Hem with different concentrations; (**B**) the time-depending TMB-H_2O_2 redox reactions catalyzed by Hem-BSA composite in comparison to Hem.

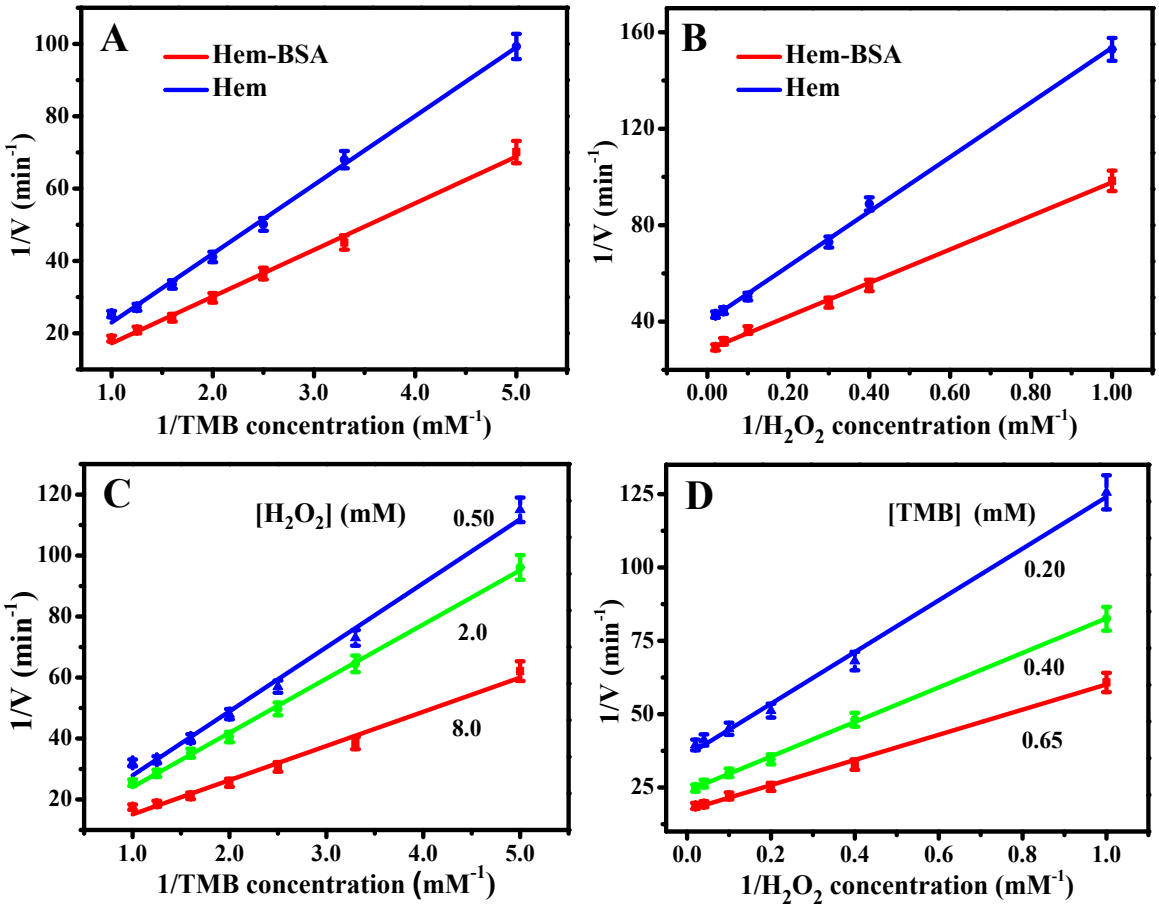

Fig. S7 Double-reciprocal plots for comparison of catalysis activities among (a) Hem-BSA composite and (b) Hem by using (**A**) various TMB concentrations at fixed 2.5 mM H_2O_2 and (**B**) various H_2O_2 concentrations at fixed 0.45 mM TMB; the double-reciprocal plots for kinetic catalysis of Hem-BSA composites using (**C**) various TMB concentrations at three fixed H_2O_2 concentrations, and (**D**) various H_2O_2 concentrations at three fixed TMB concentrations. The y-axis values were calculated from the colorimetric absorbance values recorded.

Table S1. Comparison of dynamic catalysis parameters among Hem-BSA composite, native Hem, and documented HRP with the reaction substrates of TMB and H_2O_2.

Mimetic Enzymes	Substrates	K_m(mM)
Hem	TMB	4.81
Hem-BSA	TMB	2.97
HRP	TMB	0.434[1]
Hem	H_2O_2	2.85
Hem-BSA	H_2O_2	2.46
HRP	H_2O_2	3.70[1]

References

1. L. Z. Gao, J. Zhuang, L. Nie, J. B. Zhang, Y. Zhang, N. Gu, T. H. Wang, J. Feng, D. L. Yang and S. Perrett, *Nat. Nanotechnol.*, 2007, **2**, 577-583.