Supporting information

Fabrication of Hierarchical Nanoreactor based on COFs for Cascade Enzyme Catalysis

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1. Experimental Section

1.1. Materials and reagents

All chemicals and reagents were purchased from commercial sources and used without further purification. Specially, glucose oxidase was purchased from Shanghai Yuanye biological Co., Ltd (Shanghai, China). Horseradish peroxidase(C10965838) and 1,3,5-trialdehyde-phloroglucinol (C10814868, > 97 %) were supported by Shanghai MACKLIN Biochemical Technology Co., Ltd (Shanghai, China). Benzidine (K1927162, > 95 %), polystyrene microspheres (0.050-0.10 μm, 2.5 wt %) and 3,3',5,5'-Tetramethylbenzidine (TMB, L1804065, ≥ 98 %), and rhodamine B (RGB) (E202044) were purchased from Aladdin Chemistry Co., Ltd (Shanghai, China); p-toluenesulfonic acid monohydrate (> 98 %) was purchased from Shanghai Energy Chemical Co., Ltd (Shanghai, China). Tetrahydrofuran (20200617) was from Shanghai Lingfeng

Chemical Reagent Co., Ltd (Shanghai, China). Sodium dihydrogen phosphate dehydrate (20181114), anhydrous glucose (20161219) and acetonitrile (20190403) were from the shanghai HUSHI Co., Ltd. Fluorescein isothiocyanate (FITC) (1002314553) was purchased from the Sigma Aldrich Co., Ltd. All aqueous solutions were prepared with Millipore water (resistivity of 18.2 MΩ cm^{-1}). Dodecahydrate and disodium hydrogen phosphate was supported by XiLONG SCIENTIFIC Co., Ltd (Guangdong, China). Trypsin-EDTA digestion solution (20190505) was purchased from Jiangsu KeyGen Biotechnology Co., Ltd (Nanjing, China).

1.2. Synthesis of mesoporous TpBD

Mesoporous TpBD was synthesized according to the method derived from literature.[1] Typically, mesoporous TpBD was synthesized by the Schiff base reaction between the organic linkers 1, 3, 5-triformylphloroglucinol (Tp), and benzidine (BD) in the presence of 50 nm PSs. The monodispersed colloidal PSs were used as a hard template, and p-toluenesulfonic acid (PTSA) was used as a catalyst for the COF formation.[2,3] Specially, the colloidal PSs (~ 2.5 wt % in water, 4 ml) were mixed with the BD linker (83.8 mg, 0.45 mmol) and PTSA (475.5 mg, 2.5 mmol) which formed an organic salt. Then the resulting salt and Tp (63 mg, 0.3 mmol) were thoroughly shaken for 10 min, and the dispersion was poured into a Petri dish to evaporate excess water at room temperature overnight. Further drying at 80 °C for 48 h yielded an orange-colored PS@TpBD composite. Then, the sample (PS@TpBD) was thoroughly washed with hot water to remove PTSA. Subsequently, soxhlet extraction of PS@TpBD was conducted using tetrahydrofuran (THF) as solvent to remove the PS template as well as unreacted monomers. The TpBD with a mesoporous structure was collected after drying at 60 °C.

1.3. Preparation of GOx&HRP@TpBD.

Mesoporous TpBD (2 mg) was added into PBS solution and ultrasound for 5 h. Then HRP (100 μL, 0.5 mg min^{-1}) and GOx (100 μL, 0.5 mg min^{-1}) solutions were added, and stirred for 1 h at room temperature. Then, GOx&HRP@TpBD was separated through centrifugation (7000 rpm, 10 min).

1.4. Preparation of FITC-GOx&RGB-HRP@TpBD.

The FITC-GOx was prepared according to the previous literature.[4] Specifically,

FITC (3 mg) and GOx (9 mg) were dissolved in 3 mL of PBS buffer (0.05 M, pH = 5.5), and then, the solution was stirred at room temperature for 24 h. Subsequently, the mixture was dialyzed against distilled water for 3 days using a membrane with a molecular weight cut-off of 8000-14000Da. Then, the FITC-labeled GOx was obtained by lyophilization. RGB-HRP was synthesized by a similar procedure except that GOx was replaced with HRP and FITC was replaced with RGB.

FITC-GOx&RGB-HRP@TpBD was prepared by the following procedure. Specially, TpBD (2 mg) was ultrasonically dissolved in PBS buffer (0.05 M, pH = 5.5) for 5 h, FITC-GOx and RGB-HRP were configured into enzyme solution according to the same procedure as GOx and HRP above. Then, added RGB-HRP and FITC-GOx to the above TpBD solution successively. Prior to observations by confocal laser scanning microscopy (CLSM), FITC-GOx, RGB-HRP and FITC-GOx&RGB-HRP@TpBD were dispersed in PBS buffer (0.05 M, pH = 5.5). the resultant labeled enzymes were used for the CLSM of FITC-GOx&RGB-HRP@TpBD.

1.5. Characterizations

The morphology of the TpBD was examined using scanning electron microscope (SEM) (HITACHI, Regulus 8100) and transmission electron microscope (TEM, HITACHI, HT7700). The Energy dispersive spectrometer (EDS) element mapping data were collected on the energy spectrum analyzer (OXFORD ULTIM MAX170). Fourier transform infrared (FTIR, IR-Affinity-1S) spectra were obtained using an FTIR spectrometer (Shimadzu, Japan) in the 4000-400 cm^{-1} range by the KBr pellet technique. UV−vis absorbance was recorded on a UV-1800 spectrophotometer (Shimadzu, Japan). The gas adsorption isotherms were collected on a surface area analyzer (ASAP 2020) at 77 K. The CLSM were obtained via confocal laser scanning microscopy (Ollibas fluorescence microscope, RTS-2) under ambient conditions. Thermogravimetric analysis (TGA) was performed using a thermogravimetric analyzer (Perkin Elmer TGA/4000) in the range of 30-800 °C under an air flow (heating rate of 10 °C min^{-1}). The zeta potential of mesoporous TpBD was measured by using a Malvern Zetasizer ZS90.

1.6. Determination of the loading capacity of cascade enzymes using BCA method

Preparation of the BCA Working Reagent:

The BCA working reagent was first prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (Cu^{2+} solution). As a typical run, combine 5 mL of Reagent A with 100 μL of Reagent B.

Test-tube Procedure:

I: Pipette 20 μL of each standard and unknown sample replicate into an appropriately labeled test tube.

II: Add 200 μL of the working reagent to each tube and mix well.

III: Cover and incubate tubes at 37 ºC for 25 min.

IV: Cool all tubes to RT for 60 min.

V: Prepare a standard curve. Use the standard curve to determine the protein concentration of unknown samples.

A representative standard curve showing the absorbance of different concentrations of cascade enzymes using BCA assays (**Figure S3**, standard curve was corrected before a new group starting).

1.7. Cascade Enzymes Activity Analysis and Detection of Glucose.

The catalytic activity of related enzymes was evaluated based on the oxidation of glucose and the chromogenic substrate TMB. In the feasibility study stage of the experiment, the catalytic activities of GOx, HRP, TpBD, GOx&HRP and GOx&HRP@TpBD were measured under the same experimental conditions. In condition optimization test, 50 μL of GOx&HRP@TpBD (2 mg mL^{-1}) and GOx&HRP (the enzyme content was the same as that of GOx&HRP@TpBD) were added to a PBS solution containing glucose (100 μL, 2 mM) and TMB (100 μL, 5 mg ml^{-1}). The volume of the mixture was adjusted to 2 ml using 0.05 M PBS buffer with different pH values (pH = 5.0 - 8.0). Then, based on the optimal pH conditions, the mixed solution with appropriate concentrations was added to a cuvette for measure of absorbance. The activity was evaluated by monitoring the increase in absorbance at 652 nm using a UV-Vis spectrophotometer. Specifically, 50 μL of GOx&HRP (2 mg mL^{-1}) and GOx&HRP@TpBD (2 mg mL^{-1}) were added to PBS solution containing glucose (100

μL, 2 mM) and TMB (100 μL, 5 mg mL^{-1}). The volume of the mixture was adjusted to 2 ml using 0.05 M PBS buffer. Finally, the activity was evaluated by monitoring the increase in absorbance at 652 nm using a UV-Vis spectrophotometer. For kinetic determination, specifically, 50 μL of cascade enzymes solution (free GOx&HRP and GOx&HRP@TpBD) was added in the 2 mL of PBS solution containing the 100 μL of TMB (5 mg mL^{-1}) and 100 μL of glucose (2 mM). Then, the UV-vis absorbance was measured once a 5 min until the cascade catalytic reaction of GOx&HRP@TpBD and GOx&HRP reached equilibrium. Further, the Michaelis constant K_M and maximum reaction rate (V_{max}) of GOx&HRP@TpBD and free GOx&HRP were calculated by measuring the enzymatic kinetic process of the reaction solution containing a series of concentrations of glucose and the TMB (100 μL, 5 mg mL^{-1}). The Lineweaver-Burk figure (Double reciprocal of Michaelis-Menten equation) was used to calculate the apparent kinetic constants. The K_M and V_{max} were calculated based on the following formula (V represents the reaction rate and [S] represents the substrate concentration):

$$\frac{1}{v} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}}$$

The protective effect of mesoporous TpBD was also examined under harsh conditions such as protease, base, and acetonitrile at the same glucose substrate concentration. Specifically, the GOx&HRP@TpBD and GOx&HRP were incubated in the corresponding solution for 10 min, and measured the corresponding enzyme activity was. The thermal stability of GOx&HRP@TpBD and GOx&HRP were determined by placing GOx&HRP@TpBD and GOx&HRP solution in a water bath kettle at 60 °C, followed by detecting their activity per hour using the above methods. The storage stability of free GOx&HRP and GOx&HRP@TpBD was investigated by placing at 4 °C for 8 days and measuring the corresponding enzyme activities every day. The reusability of GOx&HRP@TpBD was determined through centrifugation after the reaction and wash with PBS buffer. The reclaimed GOx&HRP@TpBD was reintroduced into a fresh glucose/TMB solution for the next catalytic reaction. Finally, to evaluate the specificity of GOx&HRP@TpBD for glucose in cascade catalytic reaction, various interferential analytes, chlorinated salts (NaCl and $CaCl_2$), saccharides

(fructose, maltose, and mannose), and a bio-macromolecule (bovine serum albumin (BSA)) was introduced into the catalytic system for determination of corresponding enzyme activity.

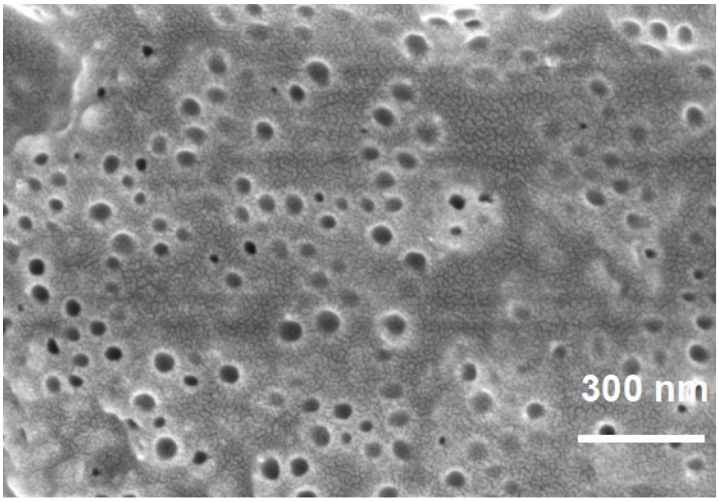

Figure S1. The SEM image of mesoporous TpBD, the uniform cavity structure in mesoporous TpBD confirms the existence of cavity with mesopore.

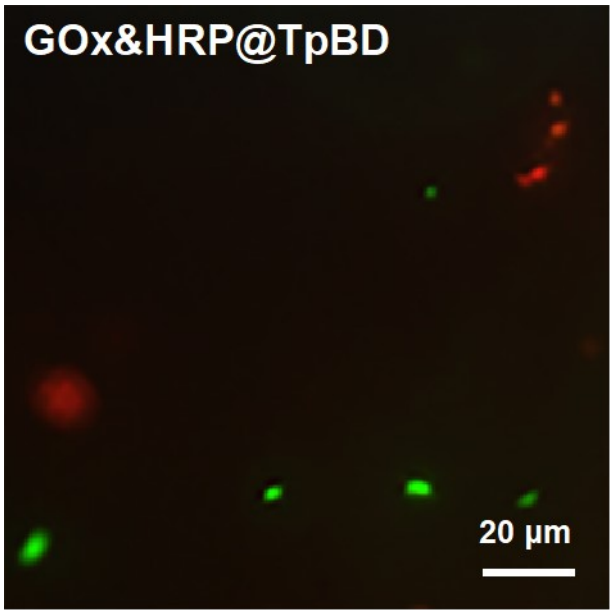

Figure S2. The CLSM images of FITC-GOx&RGB-HRP@TpBD, the CLSM was carried out to visualize the spatial distribution of cascades enzymes within the mesoporous TpBD.

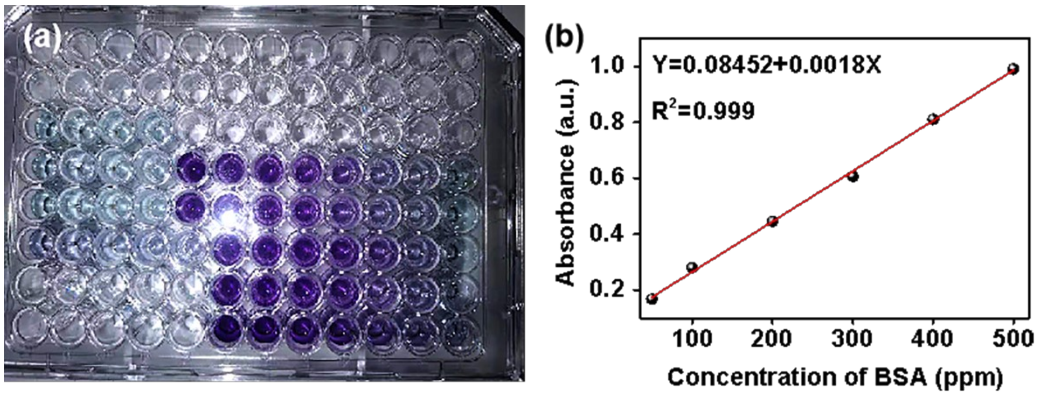

Figure S3. (a). The colorimetric changes in the TMB solution containing GOx&HRP@TpBD in the process of BCA assay. **(b).** Corresponding standard calibration curve of BSA based on BCA assay.

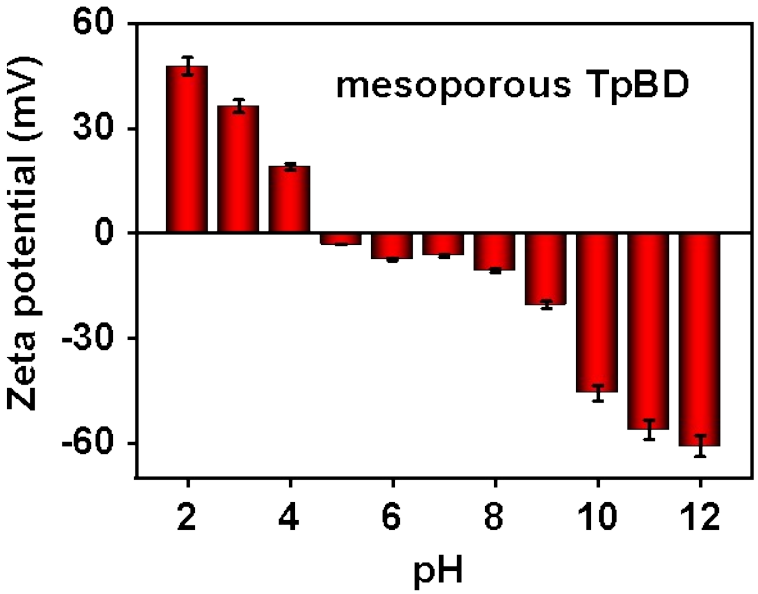

Figure S4. The zeta potential of mesoporous TpBD.

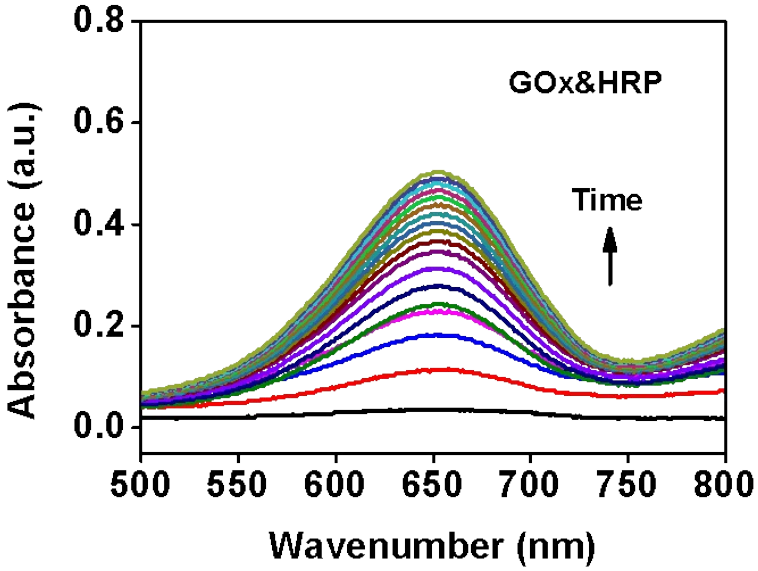

Figure S5. The time-dependent absorbance curves of GOx&HRP.

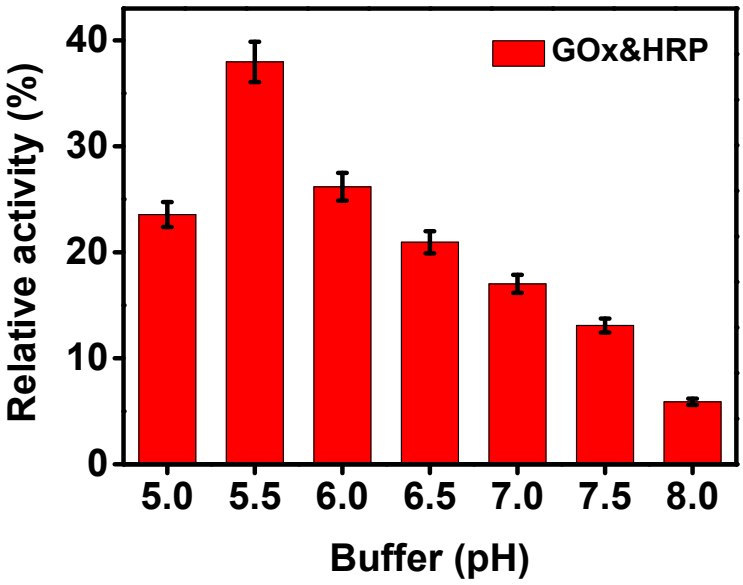

Figure S6. The catalytic activities of GOx&HRP in different pH buffer solutions at the same substrate concentration.

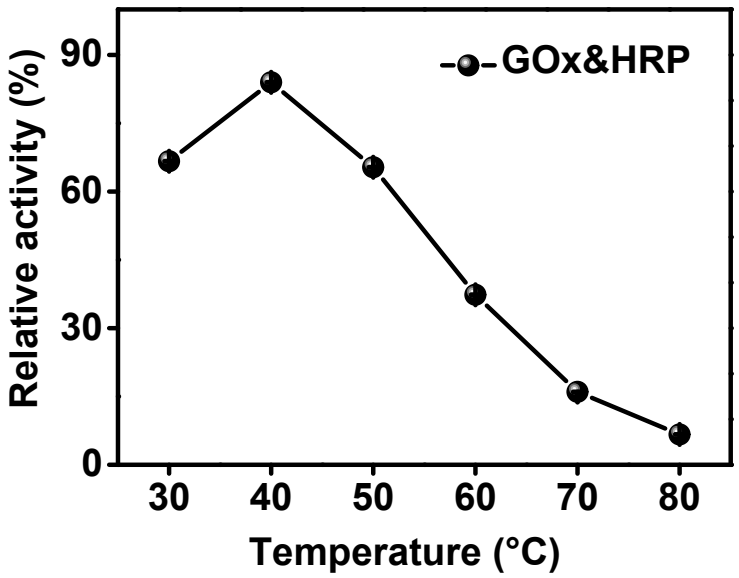

Figure S7. The catalytic activities of GOx&HRP at the same substrate concentration under different temperatures.

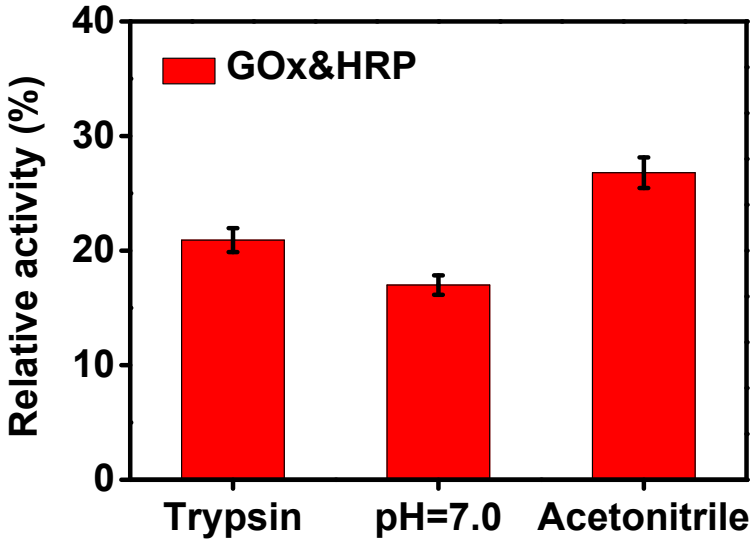

Figure S8. The cascade catalytic reaction of GOx&HRP under harsh conditions (Trypsin, pH = 7.0, and Acetonitrile).

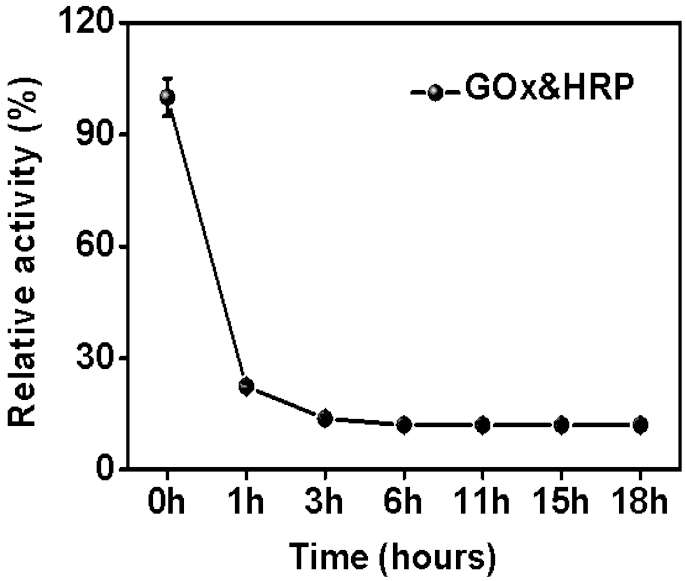

Figure S9. The thermal stability test of GOx&HRP at 60 °C.

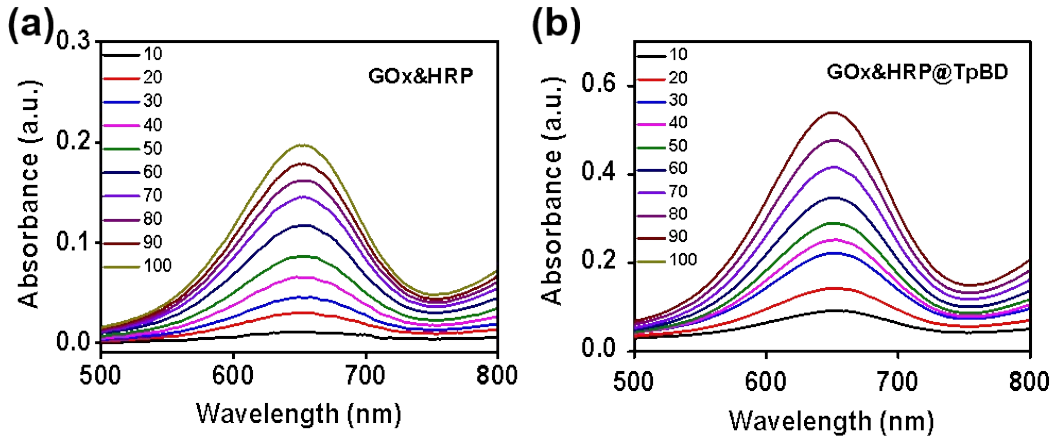

Figure S10. The spectrum of linearity test of GOx&HRP **(a)** and GOx&HRP@TpBD **(b)** at a range of glucose concentrations (10-100 μM).

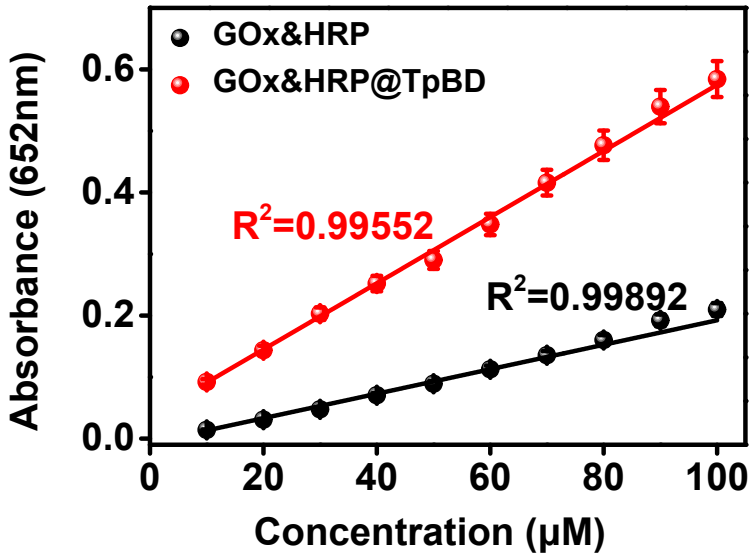

Figure S11. The linear-fittings of linearity test spectrum of GOx&HRP and GOx&HRP@TpBD.

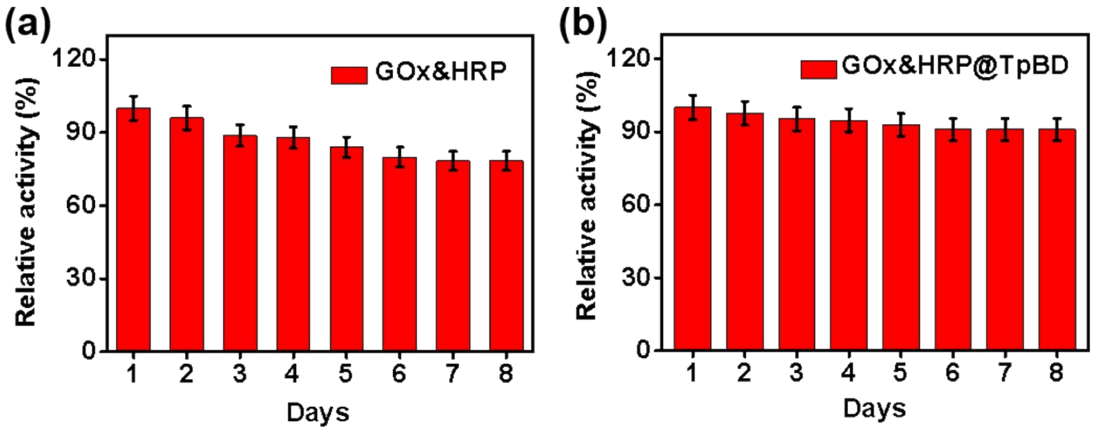

Figure S12. The storage stability test of GOx&HRP **(a)** and GOx&HRP@TpBD **(b)** at 4 °C.

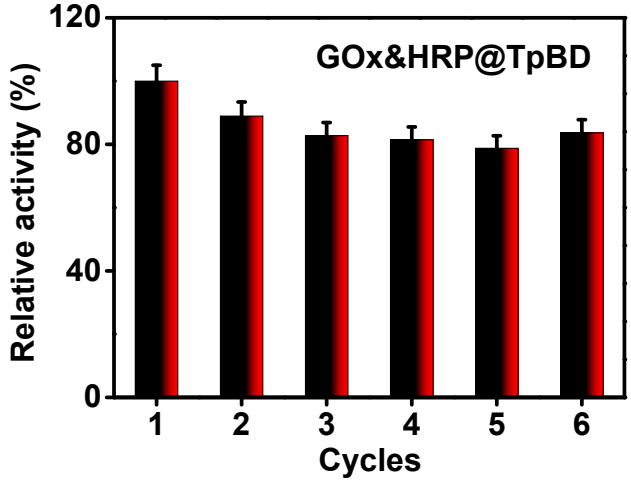

Figure S13 The reusability test of GOx&HRP@TpBD.

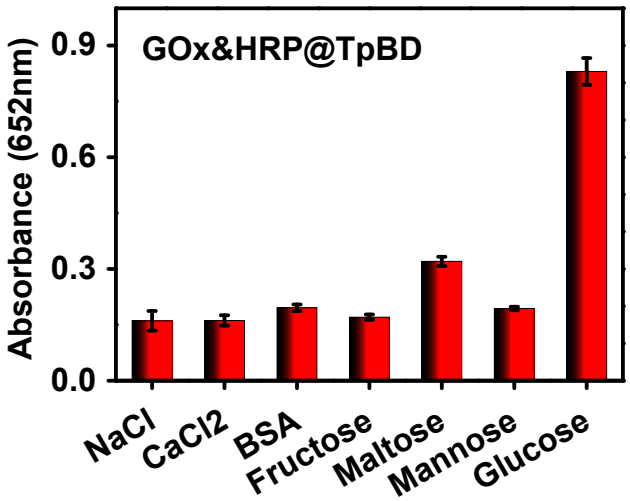

Figure S14. Absorbance value of the solutions after the reaction with 1 mM NaCl, 1 mM $CaCl_2$, 300 μM fructose, 300 μM maltose, 300 μM mannose, 1μg L^{-1} BSA, and 300 μM glucose.

Reference

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