Electronic supplementary information

Just-in-time synthesis of FeCo Prussian blue analogue nanozyme for rapid colorimetric immunoassay of α-synuclein toward home-based Parkinson's disease screening

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Chemical and Reagents. Potassium ferricyanide ($K_3[Fe(CN)_6]$), cobalt acetate tetrahydrate, copper nitrate trihydrate, nickel nitrate hexahydrate, ferric chloride hexahydrate, hydrogen peroxide (30 wt%), 3,3',5,5'-tetramethylbenzidine (TMB), bovine serum albumin (BSA), phosphate-buffered saline tablets (PBS), Tween-20, acetic acid, sodium acetate, ethanol, and other routine analytical-grade reagents were purchased from Aladdin Chemical Reagent Co., Ltd. (Shanghai, China) unless otherwise specified. Ultrapure water (18.2 MΩ cm) obtained from a Millipore purification system was used throughout all experiments. Monoclonal rabbit anti-human alpha-synuclein capture antibody (mAb_1, clone number: MJFR1, 1.054 mg mL^{-1}, cat#: ab138501), monoclonal rabbit anti-human alpha-synuclein detection antibody (mAb_2, clone number: EP1536Y, 2.38 mg mL^{-1}, cat#: ab51253), and the human alpha-synuclein enzyme-linked immunosorbent assay (ELISA) kit containing alpha-syn standards with different concentrations (colorimetric, sensitivity: 504 pg mL-1, linear range: 615-7000 pg mL^{-1}, assay time: 90 min, pre-coated microplate, cat#: ab260052) were purchased from Abcam (Shanghai, China). Alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA), cancer antigen 12-5 (CA12-5), S100 alpha, and S100 beta proteins used in interference experiments were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Human serum samples were obtained from Fujian Medical University Union Hospital and stored at -80 °C prior to analysis.

Instruments and Equipment. The morphology and microstructure of the synthesized nanozymes were characterized using transmission electron microscopy (TEM, JEM-2100F, JEOL, Japan). UV–vis absorption spectra were recorded using a UV–vis spectrophotometer (UV-2600, Shimadzu, Japan). X-ray photoelectron spectroscopy (XPS) measurements were performed on an XPS instrument (ESCALAB 250Xi, Thermo Fisher Scientific, USA). The absorbance signals of immunoassays were measured using a multifunctional microplate reader (Synergy H1, BioTek Instruments, USA).

Assessment of Nanozyme Activity. The POD-like enzyme activity was determined by comparing the oxidative discoloration of TMB over time.[1] Specifically, 100 μL (5 μg mL^{-1}) of the nanozyme substrate was mixed with preconfigured different concentrations of H_2O_2 (100 μL, 3.33 M) and TMB (100 μL, 10 mM). The absorbance variation of the test system was recorded under the specific modulation parameters of UV-vis. The temperature of the entire reaction system was set to 37°C. The specific activity (SA) of POD nanozyme was calculated by the following equation:

$$SA = \frac{(V/KL) \times (A/T)}{m}$$

where V is the total volume of the test system (μL); k is the molar absorption coefficient of the colorimetric substrate, which for TMB typically reaches a maximum at 39K $M^{-1}cm^{-1}$ at 652 nm; L is replaced by the path length of the light propagating in the cuvette (cm); A is the absorbance in the test system after deducting the absorbance; the ratio of A to T is the initial rate of change of the absorbance at 652 nm (min^{-1}); and m is the weight of the nanoparticle enzyme for each assay (mg). In addition, the molecular part of the fractional equation represents the catalytic reaction rate per unit of nanozyme. According to this, combined with the reaction rate curves for different concentrations of substrates, the Mie equation can be obtained. Further, the double inverse curve was used to further determine K_m and V_{max} values according to the following equation:

$$v = (v_{max} \times [S])/(K_m + [S])$$

Where, S represents the substrate concentration, v_{max} represents the maximum reaction rate, and K_m represents Michaelis constant. K_m reacts the affinity between the nanozyme and the substrate.

ELISA Testing. For ELISA analysis, alpha-syn standard solutions or serum samples were added into the pre-coated microplate wells and incubated at 37 °C according to the manufacturer's instructions to allow specific antigen binding. After washing several times with PBST to remove unbound substances, the enzyme-labeled detection antibody solution was introduced into each well and incubated for a designated period. Subsequently, the wells were washed again, followed by the addition of the chromogenic substrate solution. After color development at room temperature, the stop solution was added to terminate the reaction, and the absorbance signal was measured using a microplate reader at the recommended wavelength. The concentration of alpha-syn in the samples was quantified according to the calibration curve obtained from standard solutions.

Condition Optimization. To further improve the analytical performance of the sensing platform, several key experimental parameters influencing the catalytic chromogenic process were systematically optimized, including the concentration of TMB, the concentration of H_2O_2, and the incubation time for target recognition. The results indicated that the absorbance signal gradually increased with increasing TMB concentration before reaching a plateau at higher substrate levels, suggesting saturation of the catalytic reaction. Similarly, appropriate H_2O_2 concentration was essential for achieving efficient POD-like catalysis, while excessive peroxide concentration resulted

in partial inhibition of the catalytic process due to substrate overoxidation effects. In addition, the incubation time between alpha-syn and the antibody-functionalized microplate significantly influenced the formation efficiency of the sandwich immunocomplex. An optimal incubation duration was selected to ensure sufficient antigen capture while maintaining rapid detection capability. Under the optimized experimental conditions, the developed FeCo PBA-based immunosensing platform exhibited excellent chromogenic efficiency and favorable analytical sensitivity for subsequent alpha-syn detection.

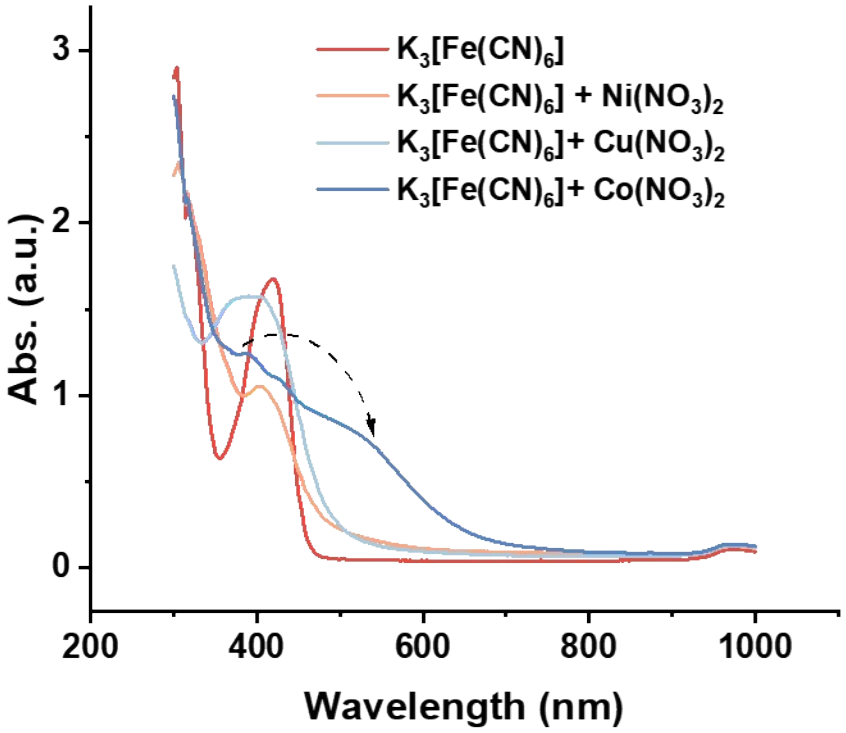

Fig. S1 UV-Vis spectra of the precursor solution and the reaction product.

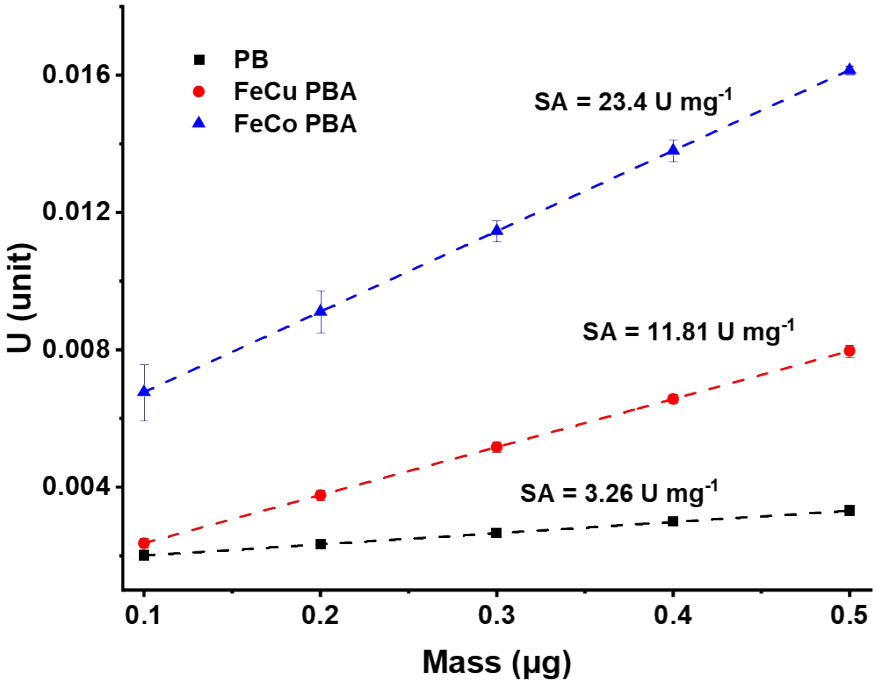

Fig. S2 Reaction kinetics of mass-dependent nanozymes.

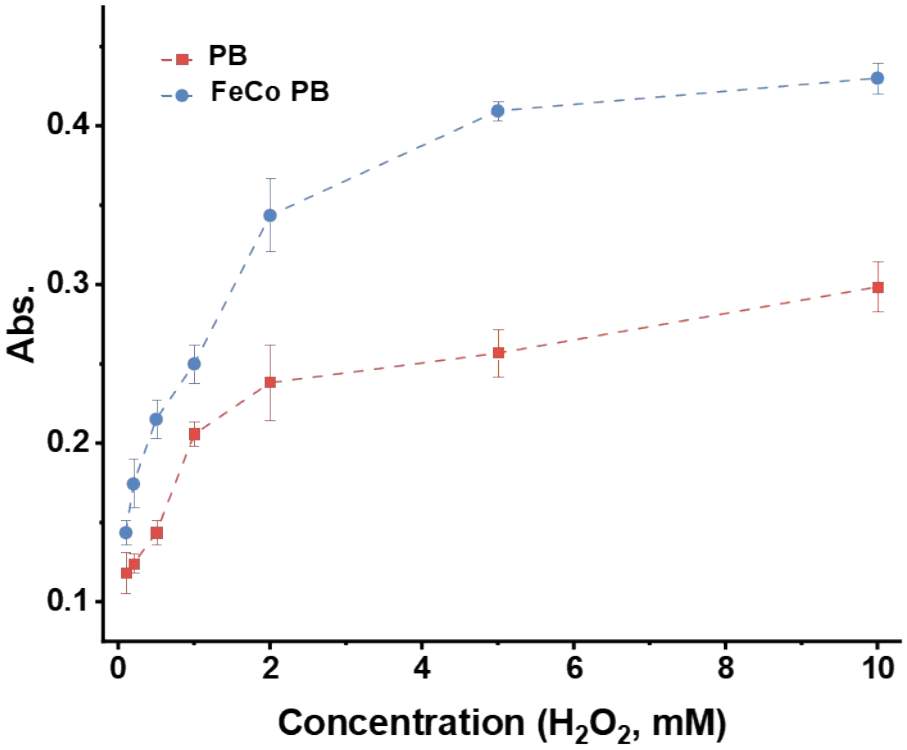

Fig. S3 Reaction rate curves of H_2O_2 substrates catalyzed by PB and FeCo PBA nanozymes at different concentrations.

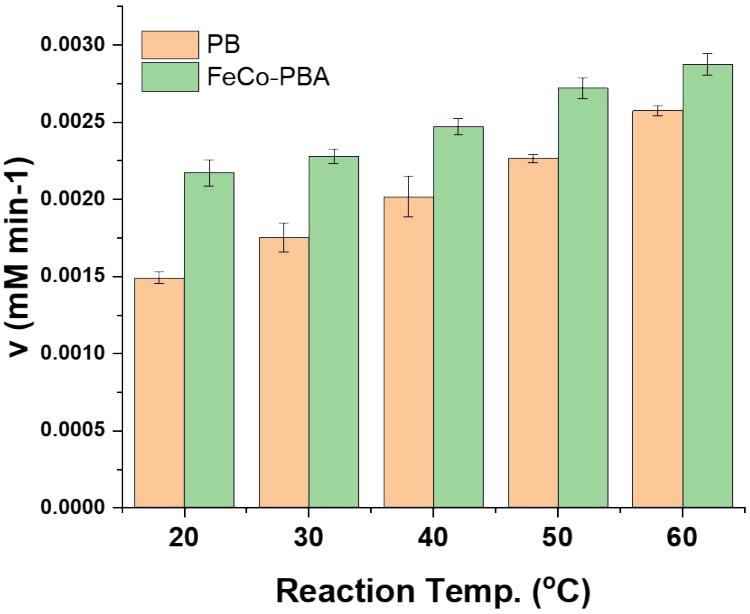

Fig. S4 Catalytic reaction curves of PB and FeCo PBA nanozymes under different incubation temperatures.

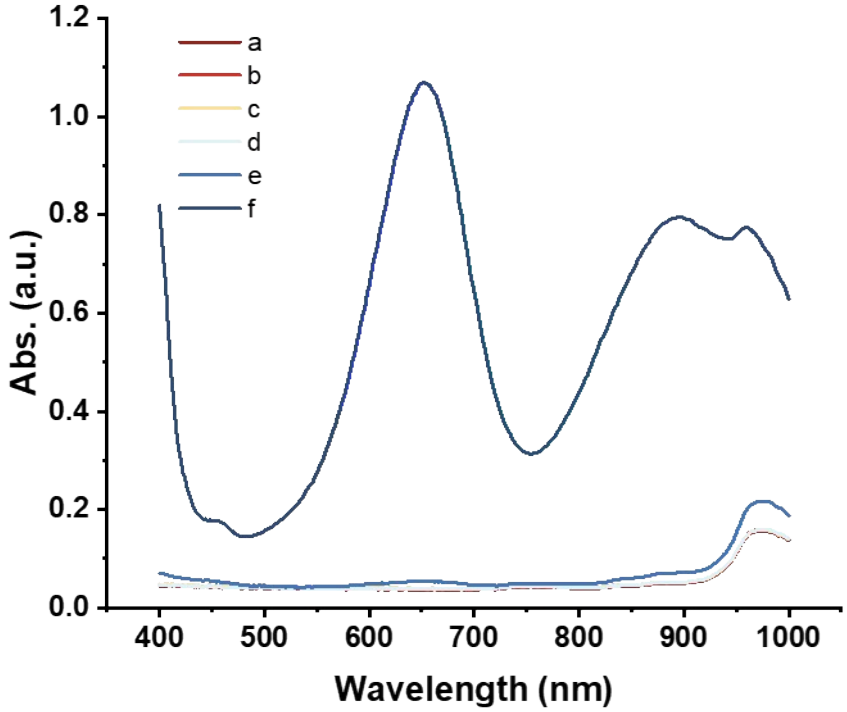

Fig. S5 Feasibility analysis: mAb_1 (line a), mAb_1 with target α-syn (1.0 ng mL^{-1}, Line b), or mAb_1 + target + mAb_2 (Line c), mAb_1 + target + mAb_2-FeCo PBA (Line d), mAb_1 + target + mAb_2-FeCo PBA system (Line e), and mAb_1 + target + mAb_2-FeCo PBA + TMB + H_2O_2 (Line f).

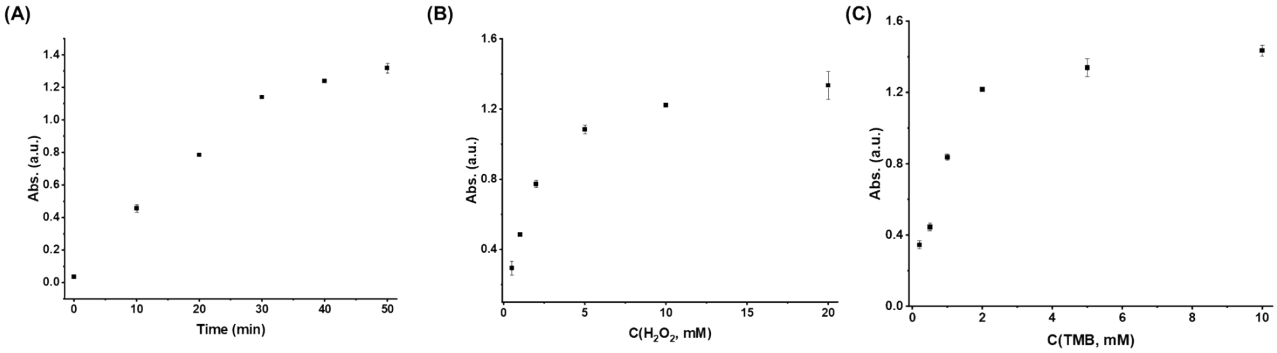

Fig. S6 Results of the optimization of key parameters in the immune-incubation process: (A) Reaction time, Concentration of (B) H_2O_2 and (C) TMB.