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Supporting information

A Highly Sensitive Ratiometric Fluorescent Immunoassay based on Bioorthogonal Nanozymes

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1.Materials and apparatus

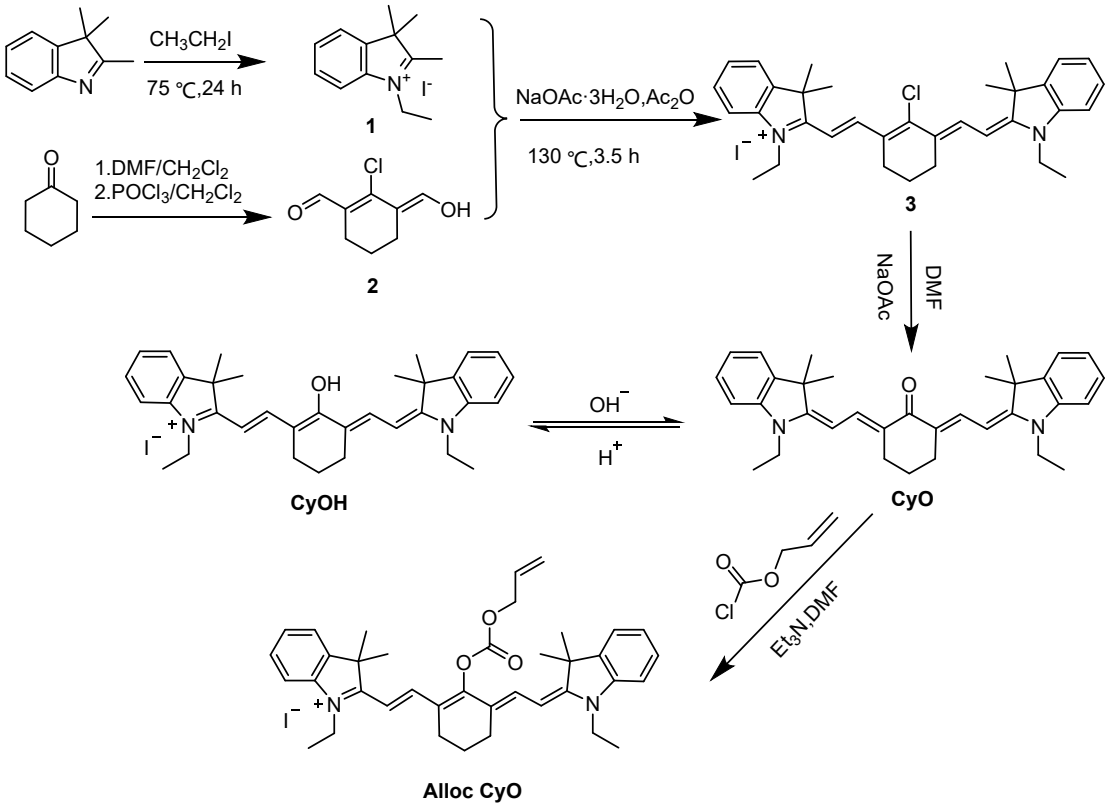
Materials and Reagents. 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC·HCl), N-hydroxysuccinimide (NHS), 11-mercaptoundecanoic acid (MUA), cupric chloride ($CuCl_2$), 2,3,3-Trimethylindolenine, iodoethane, phosphorus oxychloride ($POCl_3$), cyclohexanone and sodium tetrachloropalladate (II) (Na_2PdCl_4) were purchased from Energy Chemical (Shanghai, China). Allyl chloroformate, thioacetamide (C_2H_5NS), sodium borohydride ($NaBH_4$), bovine serum albumin (BSA), Tween-20 and sodium citrate were purchased from Aladdin (Shanghai, China). All other chemicals used in the experiments were of analytical grade and used without further purification. 96-well polystyrene plates were purchased from Corning Incorporated (New York, US). Human carcinoembryonic antigen (CEA), mouse monoclonal antibody to human carcinoembryonic antigen (capture antibody and detection antibody Ab_1) were purchased from Ruixin Biological Technology Co., Ltd (Quanzhou, China). Goat anti-mouse IgG (secondary antibody Ab_2) and human CEA ELISA kit were purchased from Bioss (Beijing, China). Human serum albumin (HSA), lactate oxidase (LOX), alpha-fetoprotein (AFP) and C-reactive protein (CRP) were purchased from Solarbio (Beijing, China).

Apparatus. NMR spectra were measured on a Bruker ARX 400 NMR spectrometer (Bruker, Billerica, MA). UV–vis spectra were measured using a UV-Visible/NIR Spectrophotometer UH5700 (Hitachi High-Tech, Japan). Fluorescence spectra were collected on Fluorescence Spectrophotometer F-7100 (Hitachi High-Tech, Japan). Absorbance and fluorescence intensity was measured by a multifunctional micropore detection board analysis system Infinite 200 PRO (Tecan, Swiss). Dynamic light scattering (DLS) data were collected with Zetasizer Nano ZS ZEN3600 (Malvern Instruments, U.K.). X-ray photoelectron spectroscopy spectra (XPS) were characterized by a photoelectron spectrograph (Thermo Fisher Scientific, UA). Fourier-transform infrared spectroscopy (FT-IR) characterization was conducted on a BRUKE Vertex 70 FT-IR spectrometer. Fluorescence images were acquired by a

IVIS® Lumina III In Vivo Imaging System (PerkinElmer, UA). Deionized (DI) water with resistance over 18 MΩ cm was generated with a Millipore water purification system (Billerica, MA).

2.Synthesis of the fluorescent substrate (Alloc CyO)

Allyloxycarbonyl-protected cyanine derivative Alloc CyO was synthesized from CyO as described in the literature.[1] The synthesis route was given in Scheme S1.



Scheme S1. Synthesis route of the fluorescent substrate (Alloc CyO).

Synthesis of 1-ethyl-2,3,3-trimethyl-3H-indol-1-ium iodide (1). 2,3,3-Trimethylindolenine (2.39 g, 15.0 mmol) and iodoethane (2.60 g, 16.7 mmol) were dissolved in CH_3CN (50 mL) and the mixture was refluxed for 24 h, 75 ℃. After the removal of solvent under reduced pressure, the resulting crude product was washed with n-hexane three times to obtain a purple solid product of compound **1** (3.99 g, 84.3%).

Synthesis of (E)-2-chloro-3-(hydroxymethylene)cyclohex-1-ene-1-carbaldehyde (2). Under N_2 atmosphere, DMF (3.00 mL, 40.9 mmol) and CH_2Cl_2 (3.00 mL, 47.3 mmol) were mixed in an ice bath. The CH_2Cl_2 (2.70 mL, 42.6 mmol) solution of phosphorus oxychloride (2.83 mL, 30.7 mmol) was added dropwise into the mixture, and cyclohexanone (0.93 g, 9.4 mmol) was added. The reaction mixture was stirred and refluxed for 5 h, 50 ℃. Then the mixture was poured into ice and stay overnight. The mixture was filtered to collect a light yellow solid product of compound **2** (1.34 g, 82.5%).

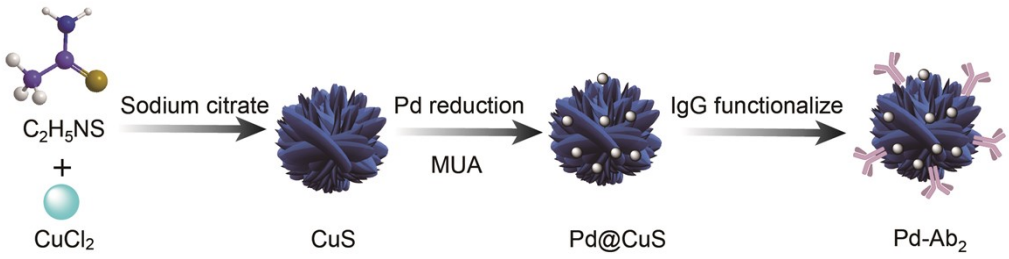
Synthesis of compound 3. Compound **1** (1.89 g, 6.0 mmol), compound **2** (0.51 g, 3.0 mmol), and sodium acetate (0.49 g, 6.0 mmol) were dissolved in acetic anhydride (30 mL). The reaction mixture was heated to 130 °C for 1 h. After the removal of solvent under reduced pressure, the crude product was washed with sodium bicarbonate solution and extracted with dichloromethane. The solvent was removed under reduced pressure and the crude product was purified by silica gel flash chromatography with dichloromethane/methanol at 50:1 to afford compound **3** as a green powder (1.57 g, 81.9%).

Synthesis of CyO. A mixture of **compound 3** (351 mg, 0.55 mmol) and sodium acetate (125 mg, 1.52 mmol) in 15 mL N,N-dimethylformamide was heated at 90 °C for 6 h under N_2 atmosphere. The solvent was removed by rotary evaporation to obtain red oil product, and then the product was purified by silica gel flash chromatography with dichloromethane/ triethylamine at 100: 1 to obtain a red powder (176 mg, 64.8%).

Synthesis of Alloc CyO. To a solution of **CyO** (150 mg, 0.35 mmol) and triethylamine (0.5 mL) in 10 mL of CH_2Cl_2 at 0 °C, a mixture of allyl carbonochloridate (1.2 mL, 8.28 mmol) and CH_2Cl_2 (8 mL) was added dropwise and kept stirring at this temperature for 30 min. Then the mixture was warmed to room temperature and stirred overnight. The mixture was concentrated under vacuum to get a deep green solid. The product was purified by silica gel flash chromatography with

dichloromethane: methanol: triethylamine = 100:1:1 to get a green solid (149 mg, 60.5%).

3.Schematic diagram of construction for Pd-Ab_2



Scheme S2. Schematic diagram of the preparation of palladium biorthogonal nanozymes-secondary antibody conjugates Pd-Ab_2.

4. Experimental procedures

Preparation of CuS NPs. The preparation of CuS NPs was based on the literature[2] published before with slight modification: 8 mg of thioacetamide was added into 25 mL of an aqueous solution dissolved $CuCl_2$ (13 mg) and sodium citrate (20 mg) under stirring at room temperature. Five minutes later, the reaction mixture was heated to 90 °C and stirred for another 15 min. Then, the mixture was transferred to ice-cold water. For purification, unreacted ingredients were dialyzed out through dialysis membranes (10 kDa).

Preparation of Pd@CuS Bioorthogonal Nanozymes. The CuS NPs solution was added to 1 mL Na_2PdCl_4 solution (9 mg/mL) and stirred gently for 30 min at the room temperature. The reduction of $PdCl_4^{2-}$ ion on CuS nanoparticles was performed by the addition of 0.2 mL of $NaBH_4$ aqueous solution (15 mg/mL) and followed by stirring 10 min. The mixed solution turned dark and was washed three times with DI water, then freeze-dried. Pd@CuS bioorthogonal nanozymes was synthesized by 11-mercaptoundecanoic acid (MUA) modification. First, the as-prepared Pd@CuS nanoparticles (20 mg) were ultrasonically dispersed in aqueous solution (20 mL), and

then MUA (100 mg) was added and stirred at room temperature for 8 h. The product was washed with acetone and DI water several times to remove the residual MUA. Finally, it was dispersed in water to form the Pd@CuS bioorthogonal nanozymes suspension.

Preparation of Pd-Ab$_2$. 1 mL of the Pd@CuS bioorthogonal nanozymes suspension (0.5 mg/mL) was washed three times with MES buffer, followed by centrifuged and the supernatant solution was removed. EDC·HCl (100 μL, 20 mg/mL) and NHS solution (100 μL, 20 mg/mL) were added separately under room temperature to activate the carboxylic groups on the surface of the Pd@CuS nanozymes, and the activation reaction lasted for 20 min. Then, the activated Pd@CuS nanozymes were collected by centrifugation at 15 000 rpm for 10 min under 4 °C and dispersed in 0.5 mL of icy PB buffer (pH 7.4). Next, 10 μL of Ab$_2$ (1 mg/mL) was added to the above solution, and the mixture was gently shaken for 24 h under 4 °C. Subsequently, BSA (100 μL, 10%) was added to block remaining active NHS sites on Pd@CuS nanozymes' surface for 30 min and wash twice with PBST (10 mM PBS with 0.05% Tween-20). The obtained bioconjugates Pd-Ab$_2$ were further washed with PBST three times and freeze-dried or resuspended in 2 mL of PBS buffer (pH 7.4), then stored at 4 °C for subsequent use.

Characterization of nanoparticles. The changes of nanoparticle hydrodynamic diameters (Rh) in different media and different time were investigated by a Malvern ZS90 dynamic light scattering instrument (DLS) to verify the stability of nanoparticles. Specifically, nanoparticles (0.5 mg mL^{-1}) were mixed with different media and incubated at 37 °C in a constant temperature water bath oscillator.

Fabrication of the Bioorthogonal Nanozymes-based Ratiometric Fluorescence Immunoassay for CEA Detection. First, 100 μL of 20 μg/mL mouse monoclonal antibody to human carcinoembryonic antigen (capture antibody) dissolved in 50 mM $NaHCO_3/Na_2CO_3$ buffer (pH 9.6) was added to the 96-well solid black flat bottom polystyrene microplates and incubated at 4 °C overnight. After removing the solution

and washing with PBST five times, 300 μL of 1% BSA solution (dissolved in 0.05 M PBS, pH 7.4) was added to block the active nonspecific binding sites of the wells at 37 °C for 2 h. Then, 100 μL of human CEA antigen standard solutions or serum samples containing different concentrations of CEA dissolved in ELISA diluent was added into the wells and incubated at 37 °C for another 1 h. Then, the wells were rinsed with PBST for five times. Next, 100 μL of detection antibody Ab_1 (2 μg/mL) was added for binding with targets and incubated at 37 °C for 40 min. After washing the microplate with PBST for five times, 100 μL of Pd-Ab_2 at a dilution of 1:100 was added into the wells and incubated at 37 °C for 30 min. Following this step, the wells were rinsed again with 100 μL of PBST for five times. After removing the rinsing solution, 100 μL of Alloc CyO substrate solution (5 μg/mL) in 10% DMSO was added into the wells, and the resultant mixture was incubated for 20 min at 37 °C under mild stirring. Finally, the fluorescence intensity was measured using an Infinite 200 PRO Multimode Reader (TECAN, Switzerland). The intensity of 650 nm (I_{650}) and 805 nm (I_{805}) were extracted for the calculation of the ratio of I_{650} to I_{805} (I_{650} / I_{805}).

Selectivity of CEA detection. To investigate the specificity of our developed ELISA for the detection of CEA antigen CEA, we performed a selective experiment. Usual biomarkers and proteins in human serum, including lactate oxidase (LOX), alpha-fetoprotein (AFP), immunoglobulin G (IgG), C-reactive protein (CRP) and human serum albumin (HSA) were selected as interfering substances. Under the same experimental conditions, different interfering substances, carcinoembryonic antigen (CEA) and the mixture of interfering substances and CEA were added to the pre-coated 96 blacked well plates (n=3). The sample concentration was 10 ng/mL, 100 μL per well. After incubation of Ab_1、Pd-Ab_2 and Alloc CyO sequentially, the fluorescence intensity was measured using an Infinite 200 PRO Multimode Reader (TECAN, Switzerland). The intensity of 650 nm (I_{650}) and 805 nm (I_{805}) were extracted for the calculation of the ratio of I_{650} to I_{805} (I_{650} / I_{805}).

Reproducibility of CEA detection. The reproducibility of ELISA generally includes intra-assay reproducibility and inter-assay reproducibility. Intra-assay reproducibility is the repeatability between wells in a single experiment (intra-assay), showing the reproducibility between wells in one test plate. Inter-assay reproducibility is the repeatability between multiple experiments (inter-assay), showing the reproducibility between different ELISA plates. In the intra-assay reproducibility experiment, three replicates were set in the same pre-coated ELISA plate for each sample (five copies of 10 ng/mL CEA standard), and 100 μL of liquid was added to each well. The remaining steps were carried out according to the method previously optimized. Then, the above five copies of CEA samples were detected in three pre-coated ELISA plates to carry out inter-assay reproducibility experiment.

Stability of CEA detection. The stability of ELISA refers to the stability of the pre-coated plate during the effective period. In scientific research, the ELISA plates are usually placed at 37 °C to accelerate disruption, and then its period of validity is inferred (37 °C for one day is equivalent to 2 months of period of validity). In order to study the stability of our developed ELISA product, we conducted stability verification experiments. First, we placed several pre-coated ELSA plates in a 37 °C constant temperature water bath for 1, 3, 5, 7 and 9 days, and then took out the ELISA plates for 10 ng/mL CEA standard samples analysis.

Spiked Tests in Human Serum. To certify the reliability and the applicability of our designed bioorthogonal nanozymes-based ratiometric fluorescence immunoassay in real sample analysis, the detection of CEA in human serum samples spiked with known concentrations of standards was carried out. The human serum was collected from healthy volunteers in the school infirmary and then centrifuged at 3000 rpm for 10 min. The supernatant was recovered and immediately stored at -80 °C. Before detection, the human serum was diluted 10 times with PBS buffer. The initial concentration of CEA was 1.78 ng/mL measured by classic CEA ELISA kit. Then, different concentrations of CEA standard solutions were spiked into diluted human

serum samples, respectively, and the CEA levels were analyzed by our designed immunoassay. Detection limit was calculated by following the equation：

$$Limit\ of\ detection\ (LOD) = 3\sigma/k \quad (1)$$

where σ is the standard deviation of three blank measurement ratios, k is the slope of the linear plot of the fluorescence intensity ratios changes (I_{650} / I_{805}).

5. Supplementary tables and figures

Table S1. Comparison of different methods for the detection of CEA.

Readout	Nanomaterials	Linear range (ng/mL)	LOD (ng/mL)	Reference
Colorimetric	AuNPs	0-120	3.00	[3]
Colorimetric	anti-CEA-ALP	1-200	0.25	[4]
Chemiluminescence	AuNPs	5-20	0.10	[5]
Electrochemical	polymer CAF	0.1-20	0.07	[6]
Fluorescence Polarization	CdTe/CdS QDs	0.5-200	0.21	[7]
Fluorescence	Eu^{3+}	1-1000	0.50	[8]
Fluorescence	CPEs	0.4-100	0.32	[9]
Colorimetric	——	0.3-250	0.20	Abcam commercial product
Fluorescence	Pd-Ab_2	0.1-50	0.05	This work

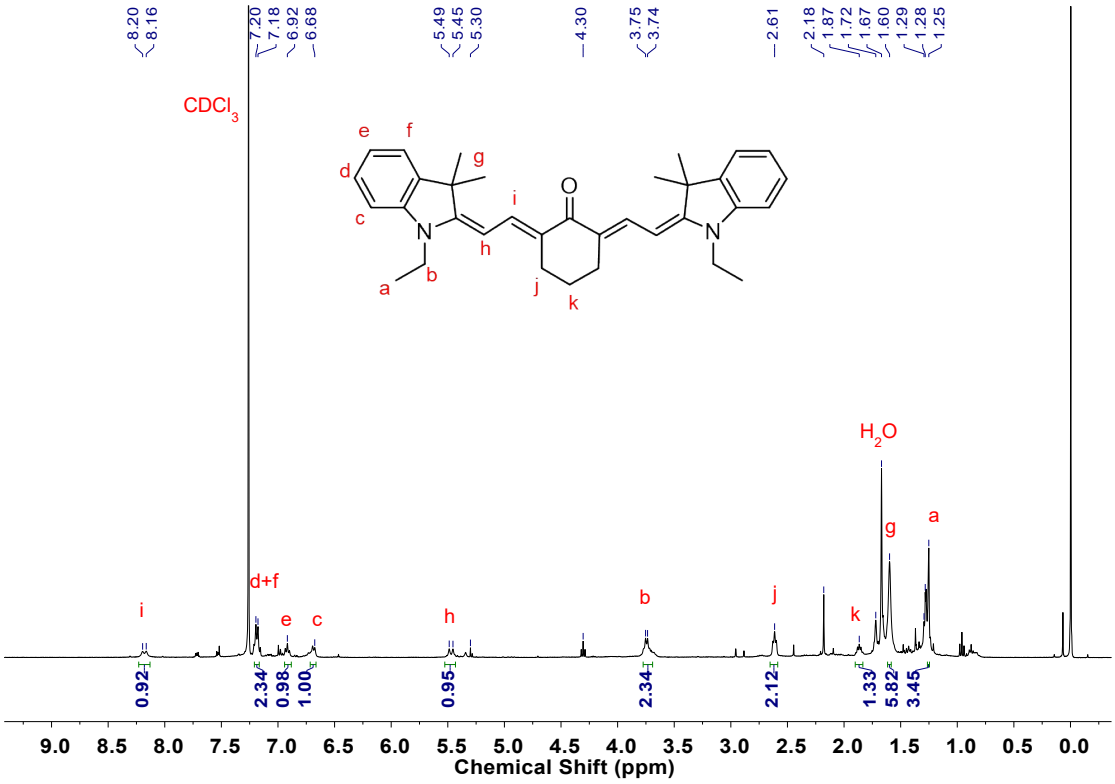


Figure S1. ^{1}H NMR spectrum of CyO in $CDCl_3$.

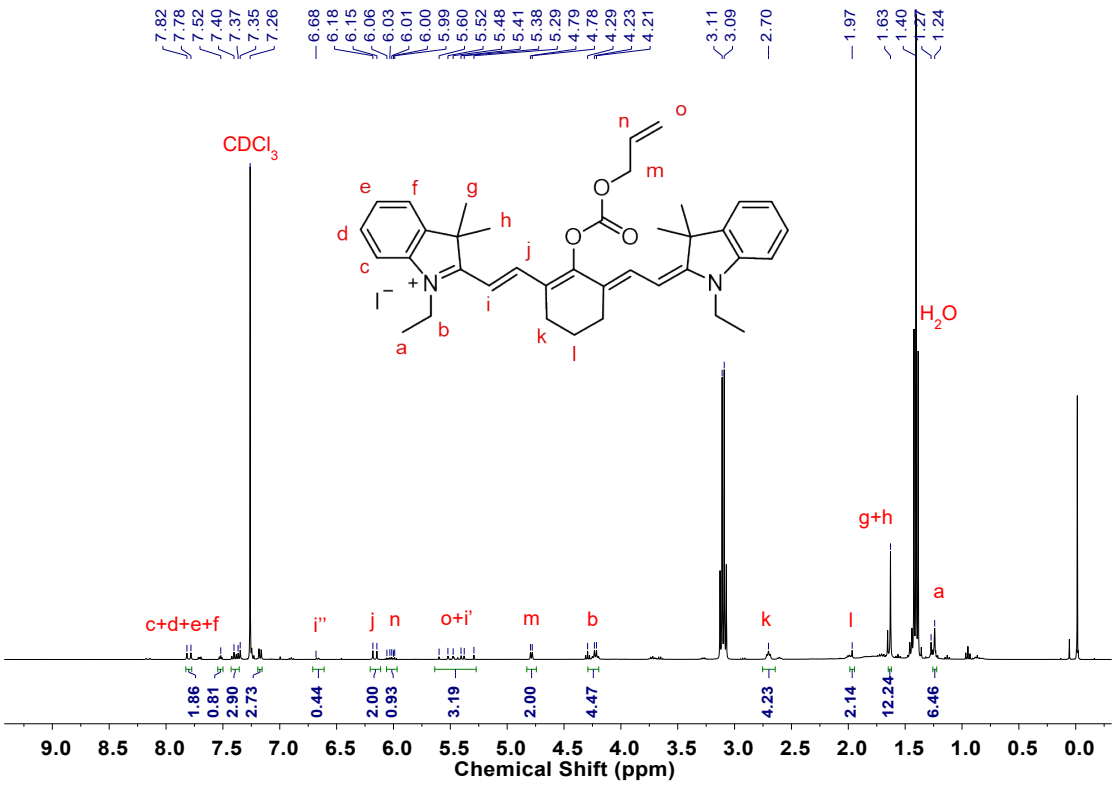


Figure S2. ^{1}H NMR spectrum of Alloc CyO in $CDCl_3$.

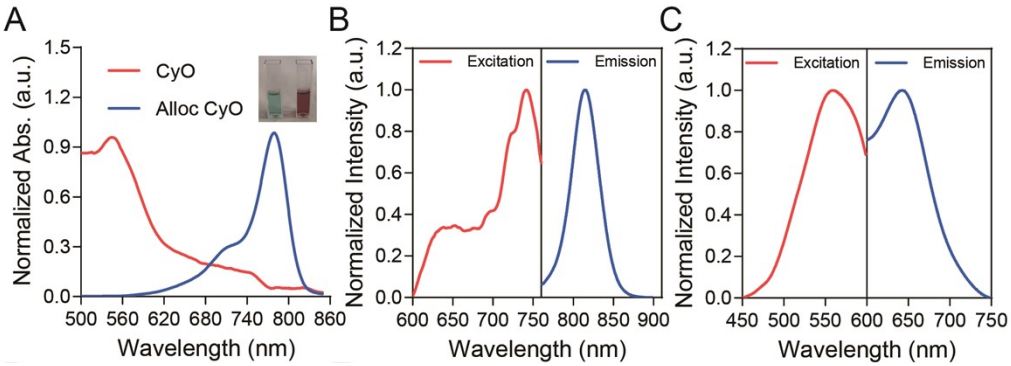


Figure S3. (A) UV-Vis absorption spectra of CyO and Alloc CyO in 10% DMSO. Inset: photographs of Alloc CyO (left) and CyO (right). (B-C) Fluorescence excitation and emission spectra of (B) Alloc CyO and (C) CyO in 10% DMSO.

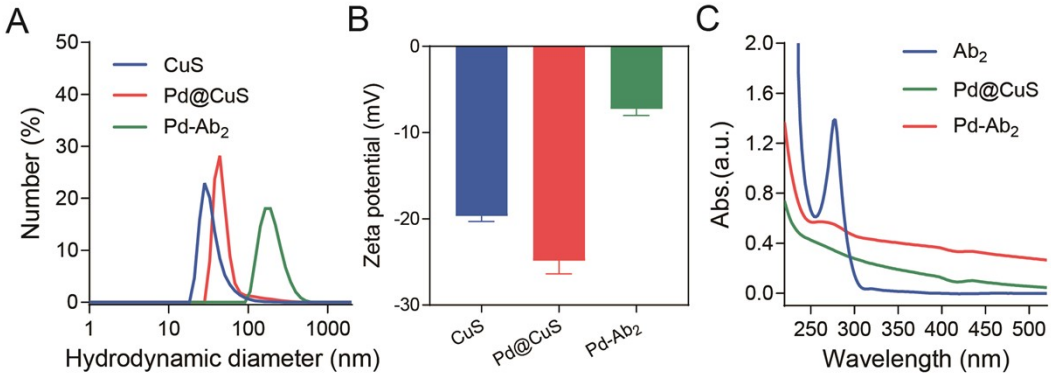


Figure S4. (A) The size distribution histogram of CuS NPs, Pd@CuS NPs, and Pd-Ab_2 bioconjugates. (B) The zeta potentials of CuS NPs, Pd@CuS NPs, and Pd-Ab_2 bioconjugates. (C) The UV–Vis absorption spectrum of Ab_2, Pd@CuS NPs, and Pd-Ab_2 bioconjugates.

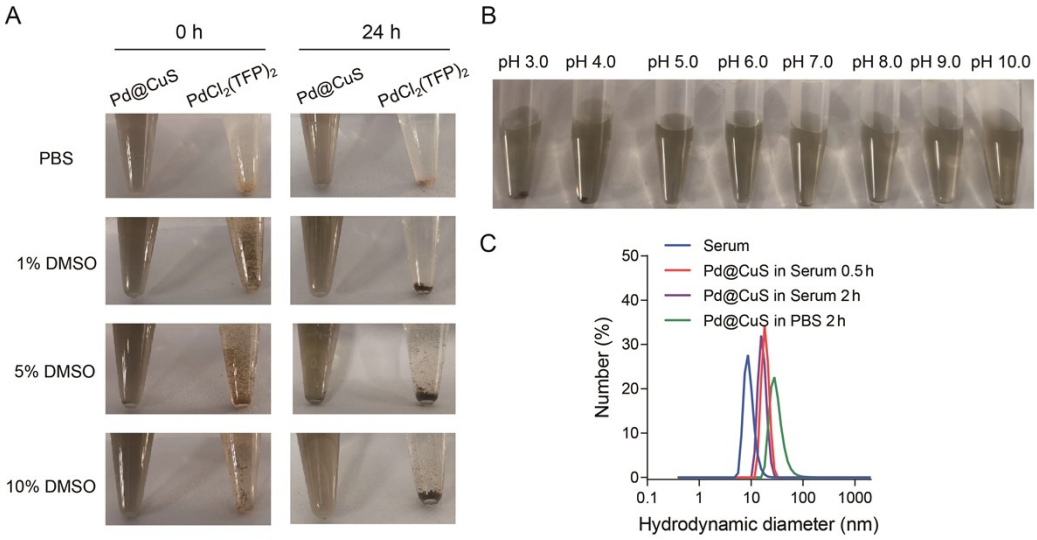


Figure S5. (A) Photographs of palladium nanozymes in different solvents for 0 or 24 h. (B) Photographs of palladium nanozymes in PBS with different pH values. (C) Changes of size distribution of palladium nanozymes.

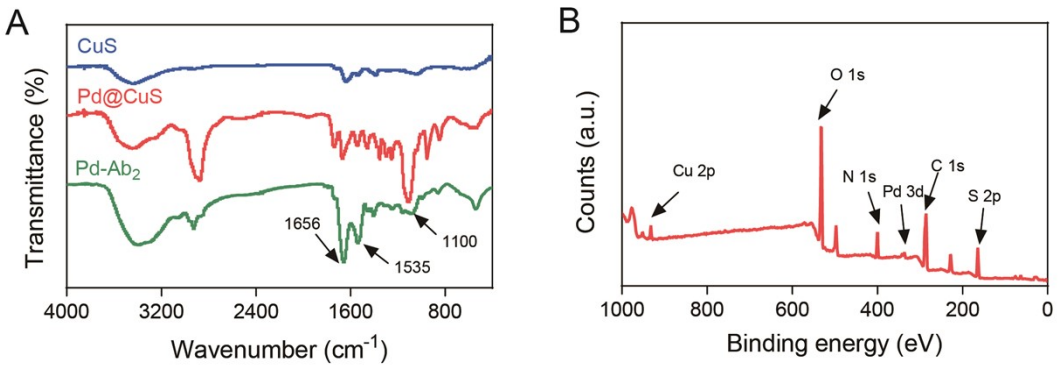


Figure S6. (A) FT-IR spectra of CuS NPs, Pd@CuS NPs, and Pd-Ab_2 bioconjugates. (B) X-ray photoelectron spectroscopy of Pd-Ab_2 bioconjugates.

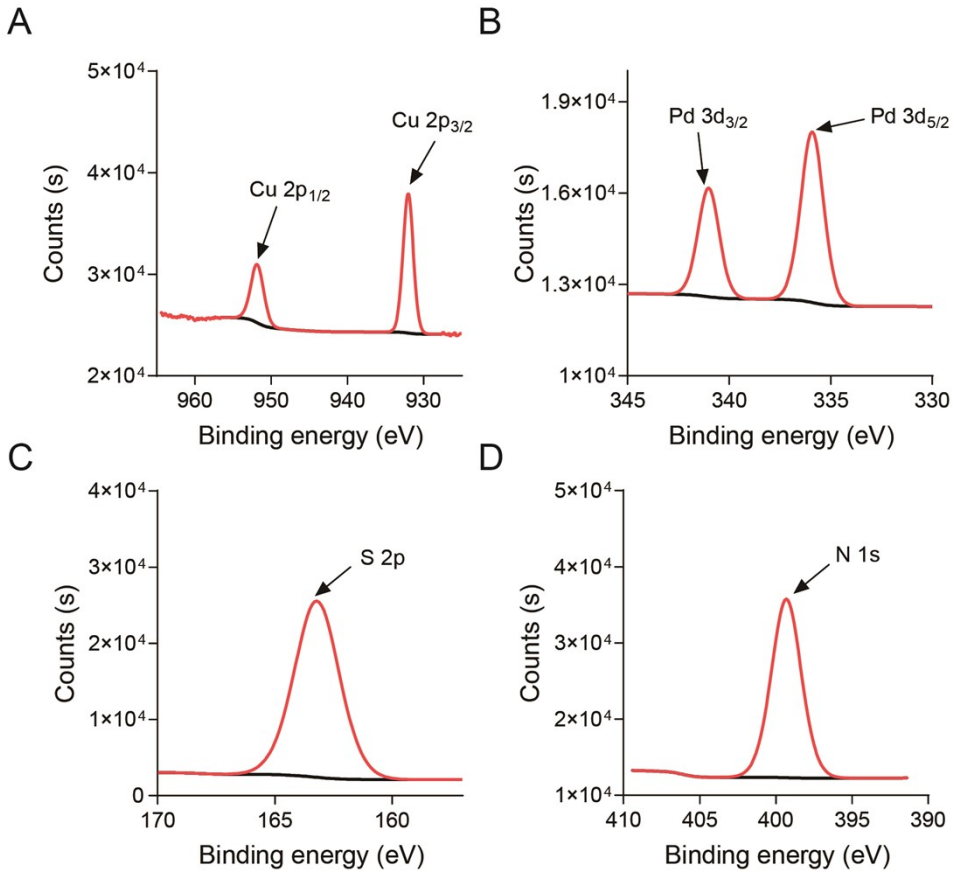


Figure S7. High-resolution XPS spectra of Pd-Ab_2 bioconjugates, (A) Cu 2p, (B) Pd 3d, (C) S 2p and (D) N 1s.

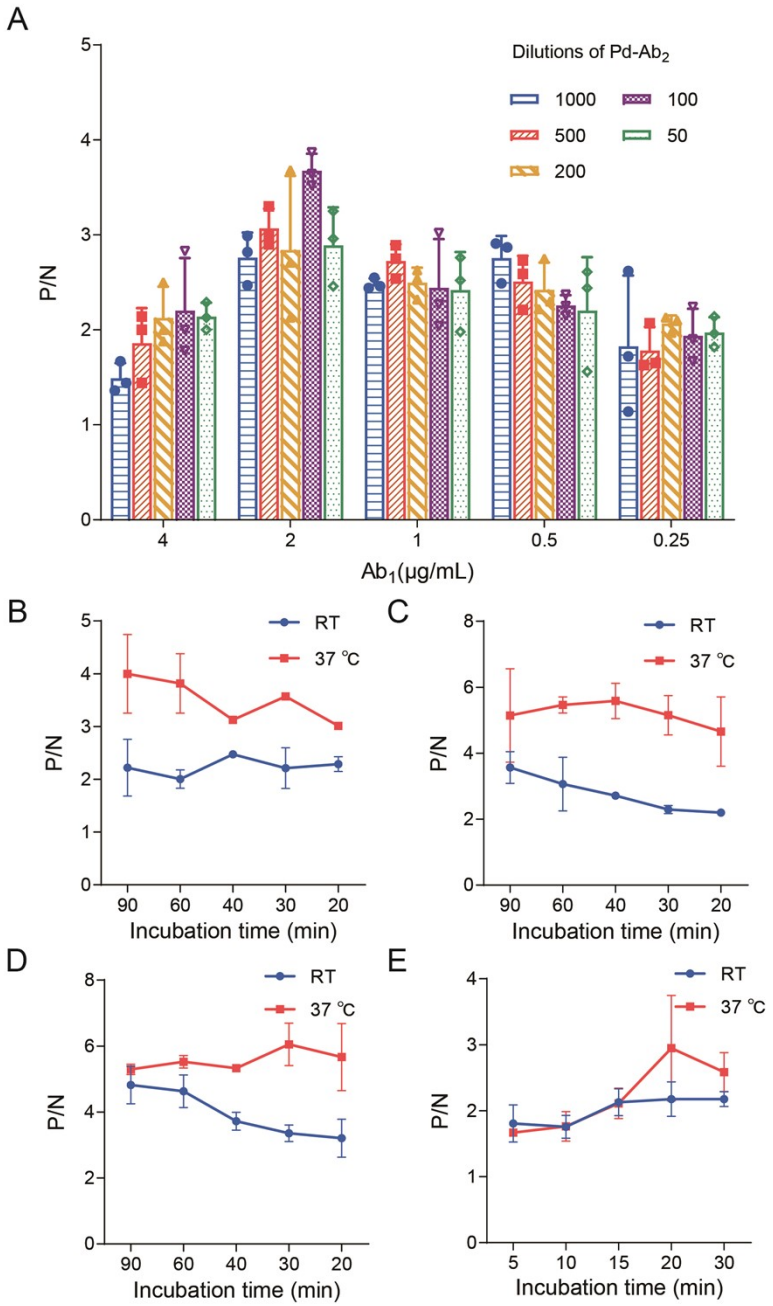


Figure S8. (A) The optimization of Ab_1 and Pd-Ab_2 concentration in immunoassay. (B-E) The optimization of incubation conditions of (B) samples, (C) Ab_1, (D) Pd-Ab_2 and (E) Alloc CyO.

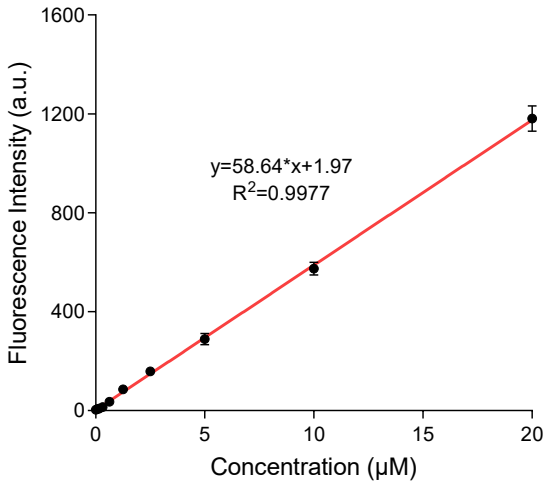


Figure S9. Fluorescent calibration curve of CyO in 10% DMSO (λ_{ex} = 540 nm, λ_{em} = 650 nm).

6.References

1. X. Wang, Z. Guo, S. Zhu, H. Tian and W. Zhu, *Chem. Commun.*, 2014, **50**, 13525.

2. M. Zhou, R. Zhang, M. Huang, W. Lu, S. Song, M. P. Melancon, M. Tian, D. Liang and C. Li, *J. Am. Chem. Soc.*, 2010, **132**, 15351-15358.

3. C. Luo, W. Wen, F. Lin, X. Zhang, H. Gu and S. Wang, *RSC Adv.*, 2015, **5**, 10994-10999.

4. W. Wei, C. Zhang, J. Qian and S. Liu, *Anal. Bioanal. Chem.*, 2011, **401**, 3269-3274.

5. X. Yang, Y. Guo and A. Wang, *Anal. Chim. Acta*, 2010, **666**, 91-96.

6. X. Zhang, Y. Shen, Y. Zhang, G. Shen, H. Xiang and X. Long, *Talanta*, 2017, **164**, 483-489.

7. J. Tian, L. Zhou, Y. Zhao, Y. Wang, Y. Peng, X. Hong and S. Zhao, *J. Fluoresc.*, 2012, **22**, 1571-1579.

8. J.-Y. Hou, T.-C. Liu, G.-F. Lin, Z.-X. Li, L.-P. Zou, M. Li and Y.-S. Wu, *Anal. Chim. Acta*, 2012, **734**, 93-98.

9. B. Bao, P. Su, J. Zhu, J. Chen, Y. Xu, B. Gu, Y. Liu and L. Wang, *Talanta*, 2018, **190**, 204-209.