Supporting Information for

A Specific Fluorescent Probe for Detecting Endogenous Butyrylcholinesterase: Multi-Model Validation from Cells to Zebrafish and Type 2 Diabetes Cases Mice

Zhipeng Chen, ‡ [a], Ling Huang, ‡ [ab] Qingqing Ma,[a] and Weiying Lin*[a]

a. Institute of Optical Materials and Chemical Biology, Guangxi Key Laboratory of Electrochemical Energy Materials, School of Chemistry and Chemical Engineering, Guangxi University, Nanning, 530004, China. E-mail: weiyinglin2013@163.com

b. School of Chemistry and Chemical Engineering, Guangxi Minzu University, Guangxi Key Laboratory of Polysaccharide Materials and Modification, Engineering Research Center of Low-carbon and High-quality Utilization of Forest Biomass, University of Guangxi, Nanning, 530006, China.
‡ These authors contributed equally to this work.

Table of Contents

A. Experimental Procedures

Materials and Instruments

All reagents were purchased from commercial suppliers and were used without further purification. Ultrapure water (18.2 MΩ-cm) was prepared using a Milli-Q water purification system. TLC analysis was performed on silica gel plates and column chromatography on silica gel (mesh 200-300); both products were purchased from Qingdao Ocean Chemical Company. ^{1}H and ^{13}C NMR spectra were measured on a Varian Unity 500 spectrometer. High resolution mass spectrometry (HRMS) analysis was measured on a Bruker SolariX X 70. Use of a PHS-3E pH meter for pH measurements. UV-Vis absorption spectra were obtained on a Shimadzu UV-2700 and fluorescence spectra were measured on a Hitachi F4700. Fluorescence imaging of cells and zebrafish were collected with Forward and Inverted Integrated Fluorescence Microscope (Echo Laboratories-Revolve). The imaging of mouse tissues was performed on a small animal imaging system (Leica IVIS Lumina III).

Synthesis Procedure

Compound **2** was synthesized according to the reported procedure.[1]

Preparation of compound **YN**. Compound **YN** was synthesized according to the reported procedure.[2] Compound **2** (300 mg, 1.72 mmol) and 6-hydroxy-2-naphthaldehyde (300 mg, 1.74 mmol) were mixed in a flask containing anhydrous EtOH. The mixture was stirred at 80°C under nitrogen atmosphere for 24 h. When the reaction was completed, the solvent was removed under reduced pressure and the crude product was purified by column chromatography (EA: MeOH) with the yield of 80.1%. ^{1}H NMR (600 MHz, Methanol-*d*$_4$) δ 8.66 – 8.60 (m, 2H), 8.25 (dd, *J* = 8.8, 1.8 Hz, 1H), 8.04 (d, *J* = 8.7 Hz, 1H), 7.96 – 7.89 (m, 3H), 7.80 – 7.71 (m, 3H), 7.36 – 7.29 (m, 2H), 4.28 (s, 2H), 4.18 (s, 1H), 2.67 (p, *J* = 1.9 Hz, 3H), 1.97 (s, 3H). HRMS (ESI) m/z calcd for $C_{23}H_{22}NO^+$ ($[M]^+$): 328.1696; found 328.1698.

Preparation of the probe **YNP**. Probe **YNP** was synthesized according to the reported procedure.[3] Compound **YN** (328 mg, 1.0 mmol) was dissolved in anhydrous DCM before the triethymine (101 mg, 1.0 mmol) was added into flask. The solution was cooled to 0°C in an ice bath, then cyclopropane chloride (156 mg, 1.5 mmol) was added dropwise into flask. The mixture was stirred at room temperature under nitrogen atmosphere for 5 h. The solvent was removed under reduced pressure and the crude product was purified by column chromatography (PE : MeOH) with the yield of 40.3%. ^{1}H NMR (600 MHz, Chloroform-d) δ 8.80 (s, 1H), 8.37 (d, J = 16.2 Hz, 1H), 8.20 (d, J = 9.9 Hz, 1H), 8.14 (d, J = 8.8 Hz, 1H), 7.90 (d, J = 16.1 Hz, 1H), 7.83 (d, J = 8.6 Hz, 1H), 7.63 – 7.60 (m, 1H), 7.59 – 7.53 (m, 4H), 7.30 – 7.28 (m, 1H), 4.45 (s, 2H), 3.48 (s, 2H), 1.94 – 1.91 (m, 1H), 1.88 (s, 5H), 1.24 (dt, J = 8.0, 4.3 Hz, 2H), 1.11 (dq, J = 7.7, 4.2 Hz, 2H). ^{13}C NMR (151 MHz, Chloroform-d) δ 182.28, 173.39, 154.35, 151.18, 143.00, 141.40, 136.45, 134.36, 131.62, 131.16, 130.98, 129.99, 129.67, 128.92, 126.08, 122.59, 122.57, 118.71, 114.99, 113.11, 52.63, 37.41, 26.86, 13.14, 13.10, 9.60, 1.02. HRMS (ESI) m/z calcd for $C_{27}H_{26}NO_2^+$ ($[M]^+$): 396.1959; found 396.1967.

Optical experiments

Prepare a 2 mM the probe stock solution (DMSO) and store at -20°C. The stock solution for BChE was prepared from Tris-HCl buffer at pH 7.30 and stored in the same conditions. All stock solutions were rewarmed to 37°C in a water bath before use. The probe concentration was fixed at 10 μM, and the concentration of BChE was adjusted to ensure that its d/p ratio in the Tris-HCl buffer (10 mM, pH 7.30) was within the range of 0-1.

Docking methods

The crystal structure of cholinesterase (PDB: 1p0i) was downloaded from the PDB database. AutoDock was used to remove water molecules and ligands, complete missing residues, optimize the hydrogen bond network, and add charges. The 3D conformation of the ligand was obtained from Chem3D 20.0. A genetic algorithm (AutoDock) was selected. A docking grid (Grid Box) was set around the entire crystal structure. Tools such as PyMOL were used to check key interactions.

Detection limit

The detection limit was determined from the fluorescence titration data, and calculated with the following equation:

$$\text{Detection limit} = \frac{3\sigma}{k}$$

Where σ is the standard deviation of blank measurement, in the absence of BChE, the fluorescence of probe was measured by eleven times, to get the standard deviation (σ), k is the slope between the fluorescence intensity versus BChE level.

Kinetic Assay

At 37°C, a fixed amount of BChE or AChE was added to a Tris-HCl buffer (10 mM, pH7.30) containing different concentrations of **YNP** (2 μM, 4 μM, 6 μM, 8 μM, and 10 μM, respectively), and changes in fluorescence intensity were recorded. K_m and V_{max} were determined using the Michaelis-Menten equation. K_{cat} was calculated using the equation $K_{cat} = V_{max}/[E]_t$. $[E]_t$ means total enzyme concentration.

Cell culture and cytotoxicity assays

HL-7702 cells and HepG2 cells were cultured in DMEM supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin and streptomycin. The cells were grown in incubator in atmosphere of 5% CO_2 at 37 °C.

Cell culture and fluorescence imaging

HL-7702 cells and HepG2 cells were stimulated with drugs, and used to detect changes in endogenous BChE level.

a. LPS was used at a dosage of 20 μg/mL to stimulate HL-7702 cells and HepG2 cells for different time (2 h, 4 h, 6 h, or 8 h), respectively.

b. HL-7702 cells and HepG2 cells, respectively, was simultaneously added to 20 μg/mL of LPS and 50 μM of tacrine for 8 h.

c. Different concentrations of Glucose (25 mM, 50 mM, 75 mM and 100 mM, respectively) was used to stimulate HL-7702 cells and HepG2 cells for 12 h, respectively.

d. 100 mM of glucose and 50 μM of tacrine were added to stimulate HL-7702 cells and HepG2 cells simultaneously for 12 h, respectively.

e. Hydrocortisone (10 μM, 20 μM, and 30 μM, respectively) was added to HepG2 and HL-

7702 cells and incubated for 24 h.

f. Hydrocortisone (30 μM) and Tacrine (50 μM) were added to HepG2 cells and HL-7702 cells for 24 h, respectively.

After processing the cells as described above, incubate them with 10 μM of **YNP** for 50 min. Subsequently, the cells were used for fluorescence imaging. Imaging was performed using an upright-inverted integrated fluorescence microscope (Echo Revolve).

Fluorescent imaging of zebrafish

Zebrafish were stimulated with drugs, and used to detect changes of BChE level.

a. LPS was used at a dosage of 20 μg/mL to stimulate zebrafish for different times (2 h, 4 h, 6 h, or 8 h).

b. Zebrafish was simultaneously added to 20 μg/mL of LPS and 50 μM of tacrine for 8 h.

c. Different concentration of Glucose (25 mM, 50 mM, 75 mM, and 100 mM, respectively) was used to stimulate zebrafish for 12 h.

d. 100 mM of glucose and 50 μM of tacrine were co-incubated with zebrafish for 12 h.

After processing the zebrafish as described above, incubate them with 10 μM of **YNP** for 50 min. Subsequently, the zebrafish were used for fluorescence imaging. Imaging was performed using an upright-inverted integrated fluorescence microscope (Echo Revolve).

Oral glucose tolerance test (OGTT) measurement

The mice were fasted for 6 h before the experiment, and blood was collected from the tail tip to measure the fasting (0 min) blood glucose level.[4, 5] Subsequently, a 20% glucose solution was administered by gavage at a dose of 2 g/kg, and timing was started immediately. Blood samples were then collected from the tail tip at 15 min, 30 min, 45 min, 60 min, 75 min, 90 min, 105 min, and 120 min post-gavage to measure the blood glucose levels at each time point. Finally, a blood glucose response curve was plotted with time on the x-axis and blood glucose levels on the y-axis to evaluate the mouse's ability to regulate glucose.

Fluorescent imaging of mice

All animal studies were conducted in strict accordance with relevant laws and were approved by the Institutional Animal Care and Use Committee of Guangxi University (protocol number: Gxu-2024-020). Kunlun mice (6–8 weeks old) were procured from the Experimental Animal Center of Guangxi Medical University (Nanning, China). Comfortable conditions were provided for the mice during the 2-month period, with the temperature maintained at about 25°C and 50% relative humidity.

Experimental conditions of type 2 diabetes mellitus (T2DM) mice:

a. Normal mice are raised under regular conditions (provided with tap water and standard chow) for 9 weeks.

b. The specific modeling conditions for T2DM mice: The mice were continuously fed a high-fat diet and tap water for 9 weeks. At week 8, the mice received a single intraperitoneal injection of alloxan onohydrate (dose: 110 mg/kg), followed by replacement of their tap water with a 20% glucose solution for 1 week.

c. The specific modeling conditions for treatment T2DM mice: Based on the experimental conditions established for T2DM mice, add 10 days of drug treatment (administration method: oral gavage; dose: 200 mg/kg/day metformin).

Prior to imaging, the tissues of mice were immersed in physiological saline containing **YNP** (100 μM). Fluorescence imaging was done at Guangxi University (Small Animal Imaging System, IVIS Lumina III, and USA). All fluorescence images were acquired at an excitation wavelength of 440 nm (460 nm or 480 nm) and an emission wavelength of 580 nm.

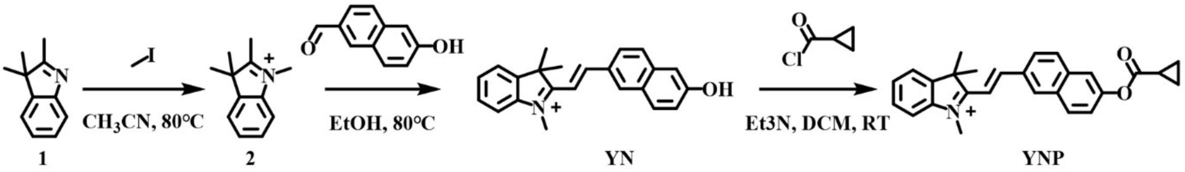
Measurement method for liver tissue homogenate

a. Diabetes model confirmation:
After modeling, the typical symptoms of diabetes - three more and one less (polydipsia, polyuria, and emasculation) - were observed in the mice. Fasting blood glucose levels were measured, and values greater than 11.0 mmol/L were defined as successful modeling.[6] Specifically, blood glucose remained above 11.1 mmol/L for three consecutive days.
b. Liver tissue homogenate preparation:
Liver tissue was collected from mice, and 7.5 mL of Tris-HCl buffer (10 mM, pH 7.30) was added to the tissue. The tissue was then homogenized and ground using an integrated ultrasonic cell disruptor (XM-650T). The homogenate was centrifuged, and the supernatant was collected. The supernatant was diluted 50-fold with Tris-HCl buffer prior to fluorescence detection.
c. Fluorescence detection:
Fluorescence spectroscopic analysis was performed on the diluted supernatant in Tris-HCl buffer (10 mM, pH 7.30).[7]

Statistical Analysis

Bilateral significance tests were employed to assess statistical significance. The Bonferroni correction method was used for mean comparisons. P values < 0.05 (*), P values < 0.01 (**), and P values < 0.001 (***) indicate statistically significant differences between groups, respectively, with increasing levels of significance.

B. Supplementary Scheme and Figures



Scheme S1 The synthetic route of the probe **YNP**.

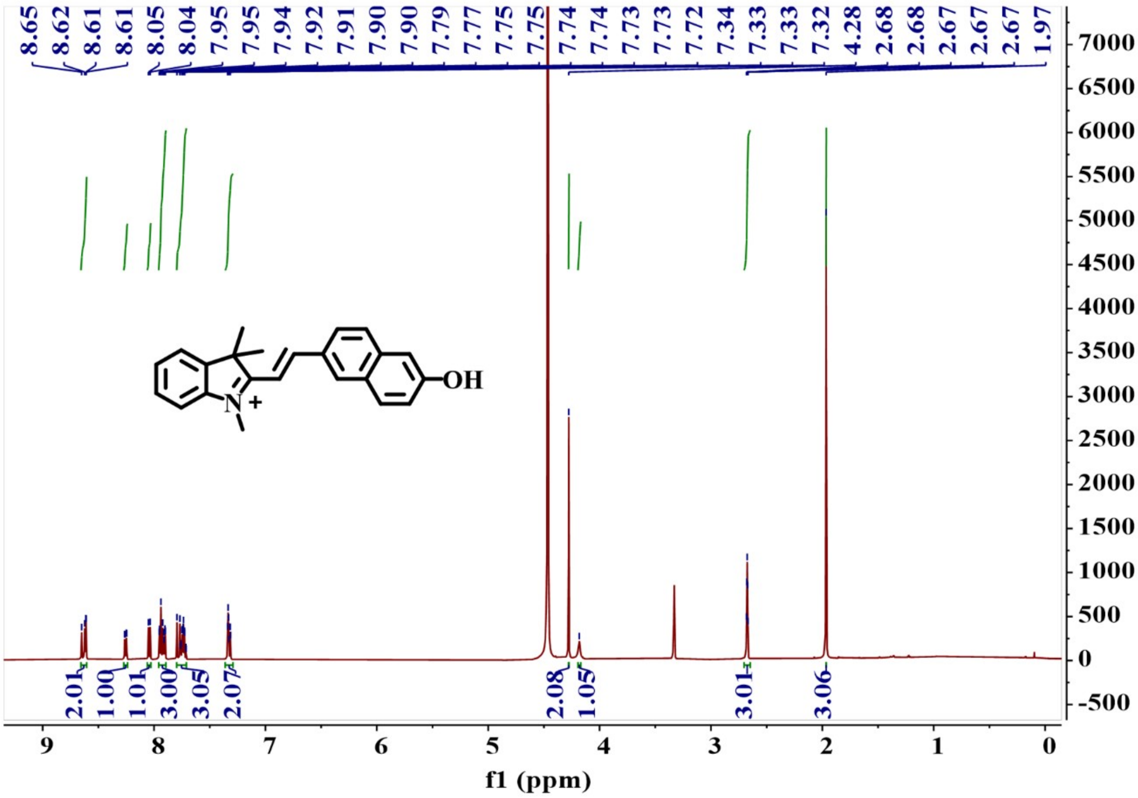


Fig. S1 ^{1}H NMR of compound **YN**.

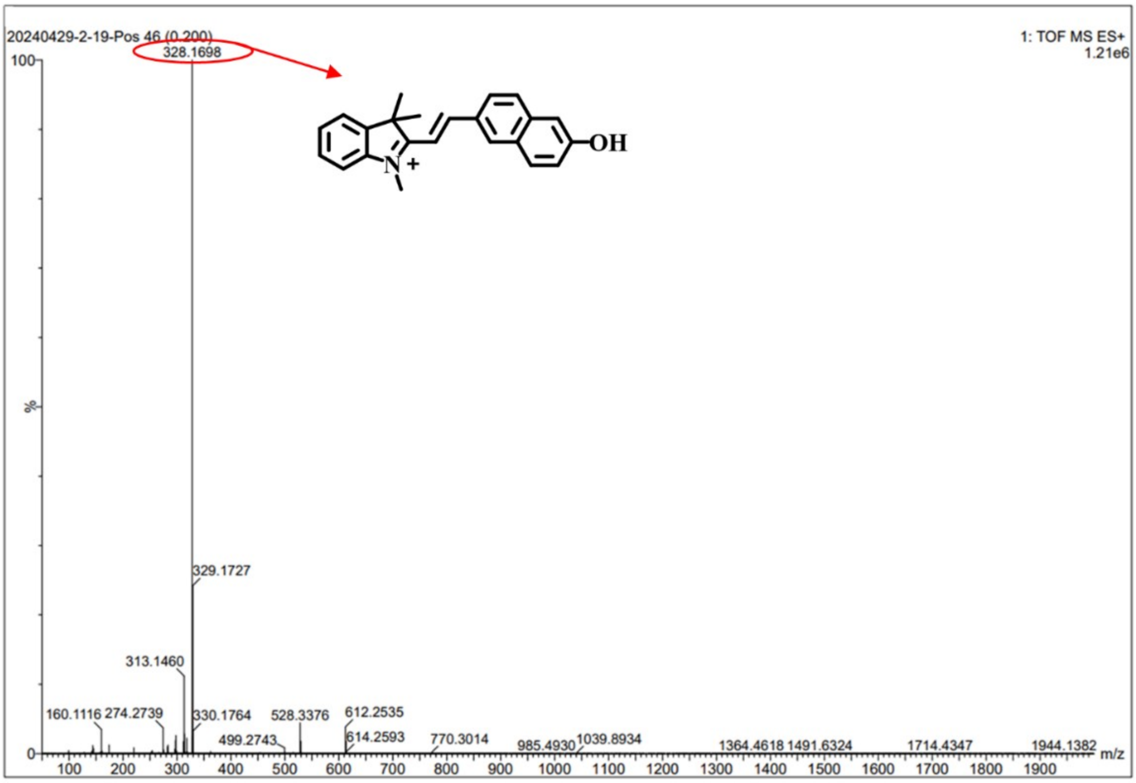


Fig. S2 HRMS of the probe **YN**.

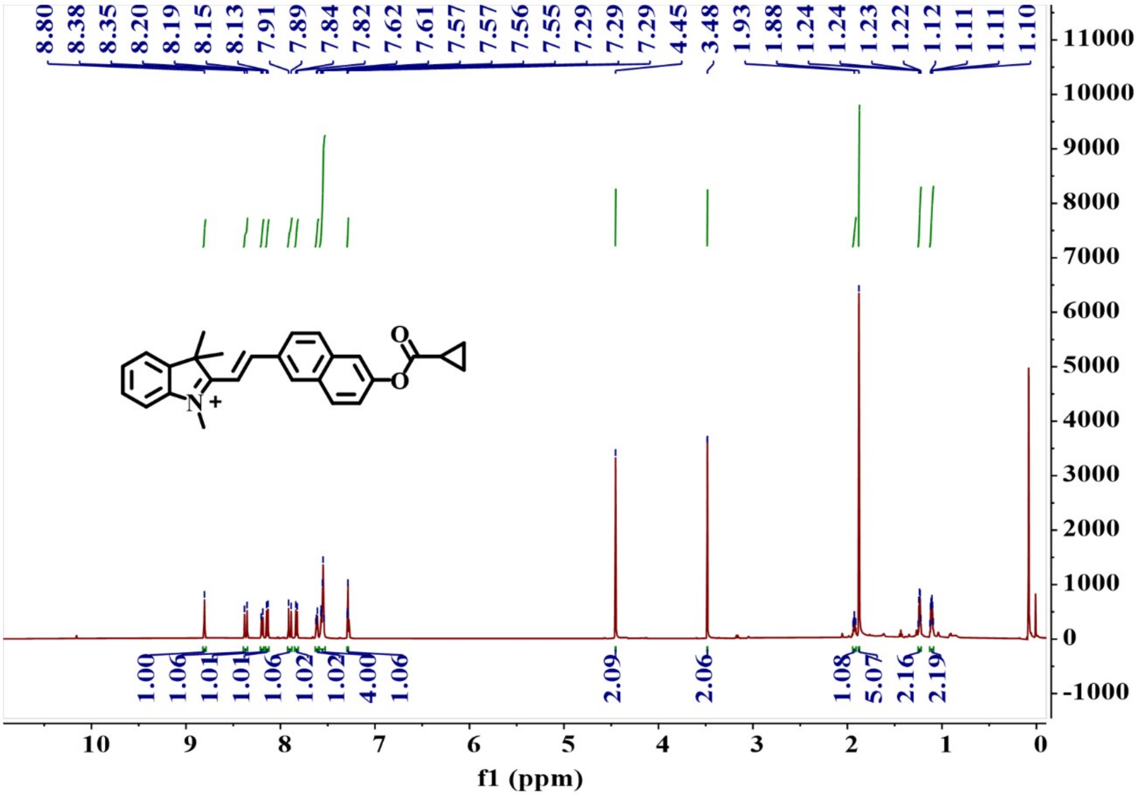


Fig. S3 ^{1}H NMR of the probe **YNP**.

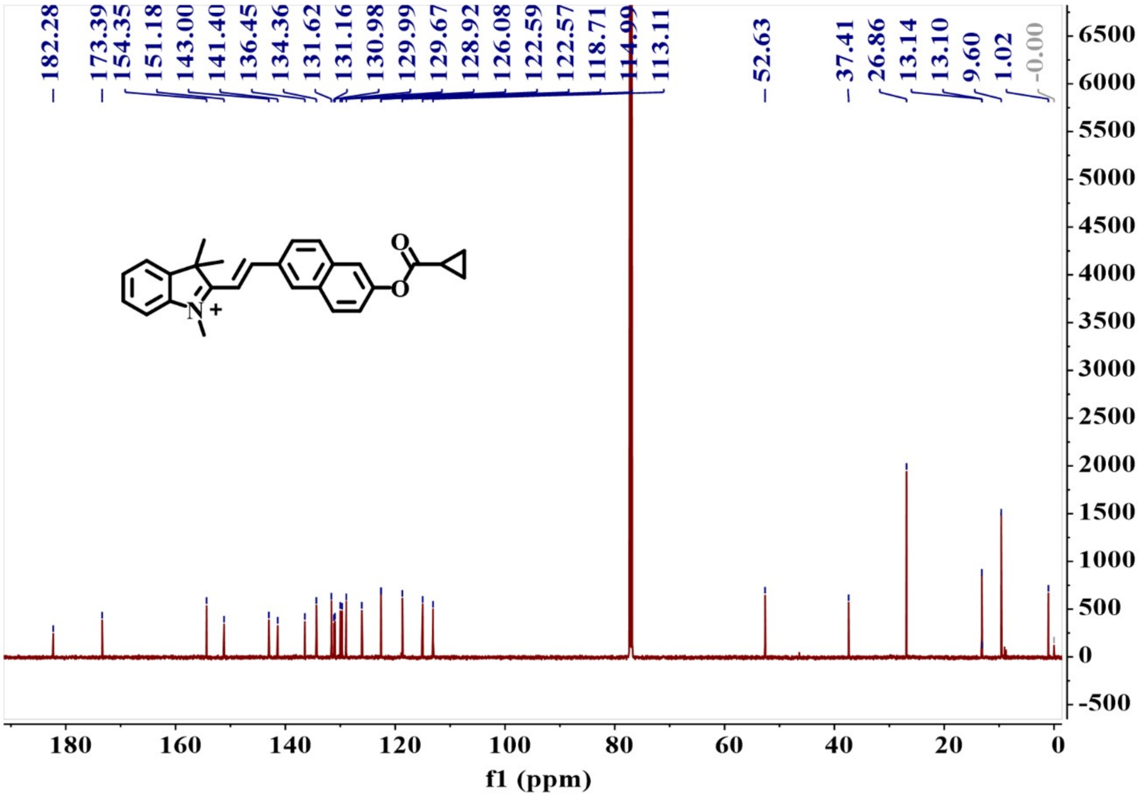


Fig. S4 ^{13}C NMR of the probe **YNP**.

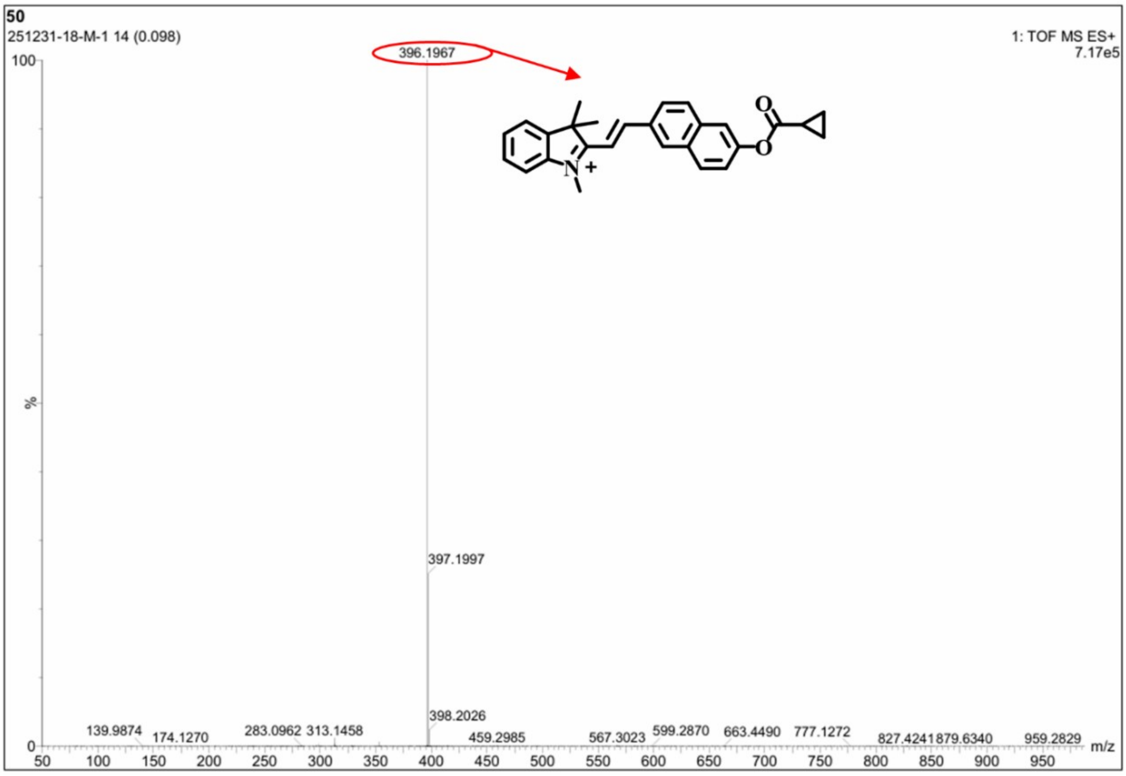


Fig. S5 HRMS of the probe **YNP**.

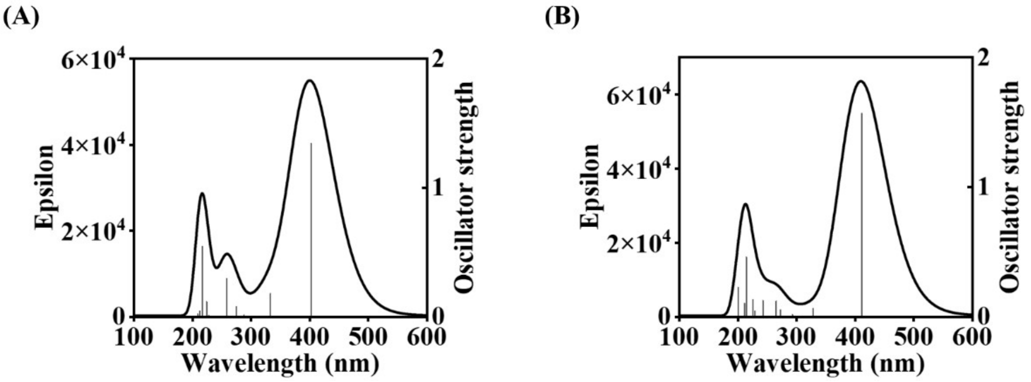


Fig. S6 Theoretical calculation of (A) the probe **YNP** and (B) compound **YN** based on TD-DFT calculations at the CAM-B3LYP/6-31g(d,p) scrf=(smd, solvent=water) em=gd3 TD(nstates=12).

Table S1 Computational chemistry associated with the probe **YNP**.

compound	Electronic Transition	TDDFT//CAM-B3LYP/6-31g(d,p) scrf=(smd, solvent=water) em=gd3 TD(nstates=12)	
		Wavelength (nm)	Osc. Str (f)
YNP	$S_0 \rightarrow S_1$	402.19	1.3423
	$S_0 \rightarrow S_2$	332.85	0.1810
	$S_0 \rightarrow S_3$	287.75	0.0152

Table S2 Computational chemistry associated with compound **YN**.

compound	Electronic Transition	TDDFT//CAM-B3LYP/6-31g(d,p) scrf=(smd, solvent=water) em=gd3 TD(nstates=12)	
		Wavelength (nm)	Osc. Str (f)
YN	$S_0 \rightarrow S_1$	411.06	1.5678
	$S_0 \rightarrow S_2$	327.71	0.0646
	$S_0 \rightarrow S_3$	292.67	0.0169

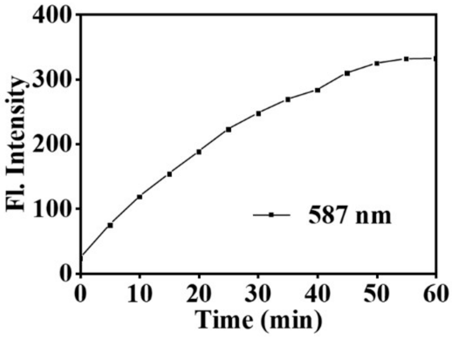


Fig. S7 Time-depend fluorescence responses of **YNP** (10 μM) towards BChE (258.6 U/L) in the Tris-HCl buffer (10 mM, pH = 7.30).

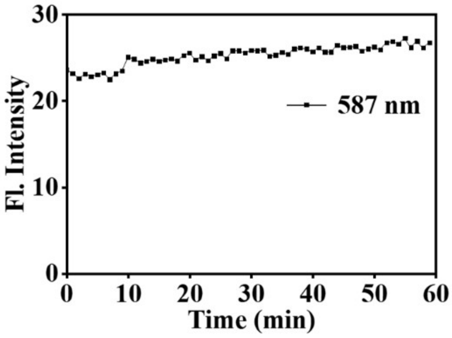


Fig. S8 The photo-stability of **YNP** (10 μM) in the Tris-HCl buffer (10 mM, pH 7.30).

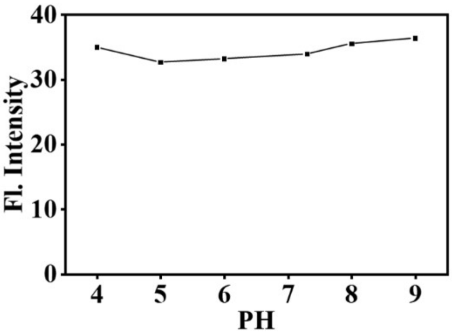


Fig. S9 Fluorescence spectra of **YNP** at different pH (4.00, 5.00, 6.00, 7.30, 8.00 and 9.00, respectively) conditions.

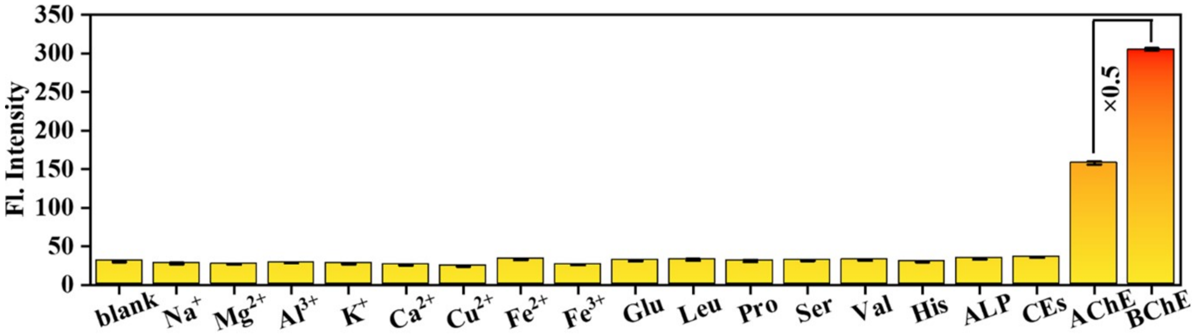


Fig. S10 The fluorescence intensity of probe **YNP** (10 μM) in response to analytes in the Tris-HCl buffer. Concentration of the analytes: 258.6 U/L (0.49 μg /ml) for BChE, 25 μg/mL for Alkaline phosphatases (ALP), 258.6 U/L (1293 μg /ml) for AChE, 1 mM for other analytes (Na^+, Mg^{2+}, Al^{3+}, K^+, Ca^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Glutamic acid (Glu), Leucine (Leu), Proline (Pro), Serine (Ser), Valine (Val), Histidine (His), carboxylesterase (CEs).).

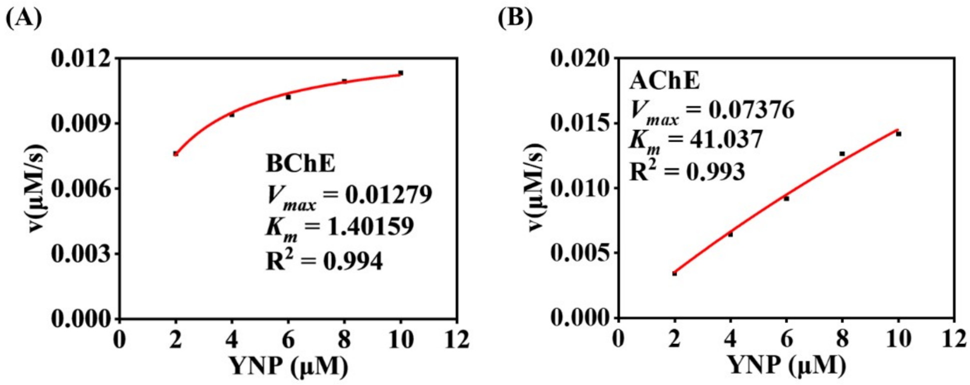


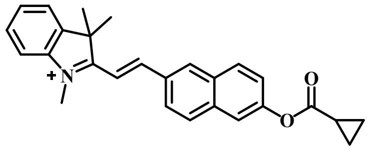
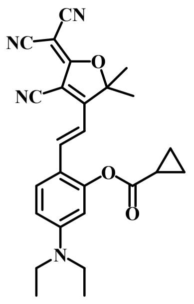
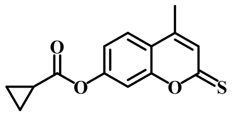
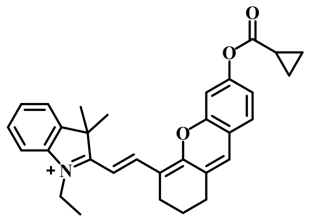
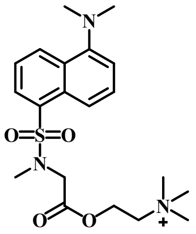
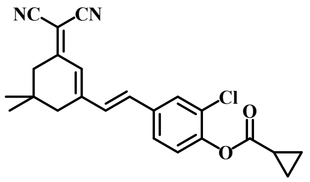
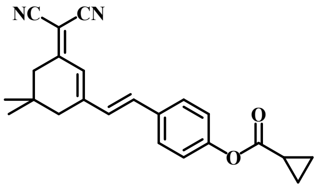
Fig. S11 The parameters of enzyme kinetics were calculation, including to K_m, V_{max}, K_{cat}, and K_{cat}/K_m. The fluorescence responses of BChE or AChE towards the different concentrations of **YNP** (2 μM, 4 μM, 6 μM, 8 μM, and 10 μM, repectively) in the Tris-HCl buffer (10 mM, pH7.30). (A) The fitting curves of BChE at different concentrations of **YNP**. (B) The fitting curves of AChE at different concentrations of **YNP**.

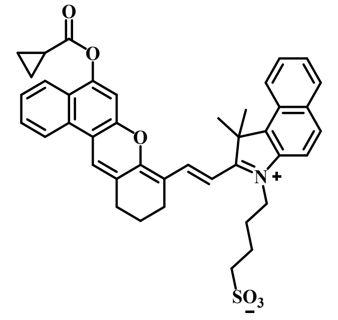
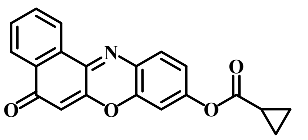
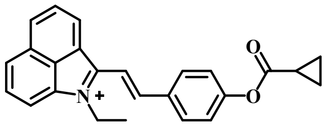
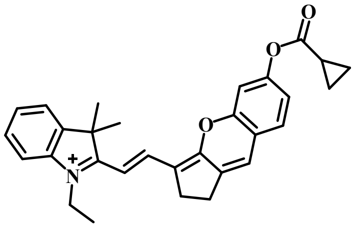
Table S3 Parameters for Enzyme Kinetics

enzyme	K_m (μM)	V_{max} (μM·s^{-1})	K_{cat} (s^{-1})	K_{cat}/K_m (s^{-1}·μM^{-1})
BChE	1.40159	0.01279	281.38	200.76
AChE	41.037	0.07376	10.33	0.25

M_w of BChE: 440 KDa；M_w of AChE: 280 KDa; $C_{BChE} = 0.02$ mg·mL^{-1}; $C_{AChE} = 2$ mg·mL^{-1}

Table S4 Comparison of **YNP** with other detection of BChE probes

probe	Response mechanism	K_m/K_{cat}	LOD	Animal model	Reference
	ICT	200.76 $s^{-1}\cdot\mu M^{-1}$	2.14 U/L	T2DM mouse model	This work
	ICT	/	39.24 ng mL^{-1}	NAFLD's mouse model	*J. Mater. Chem. B*, **2022**,10, 4254-4260
	ICT	/	8.28 U L^{-1}	Alzheimer's disease model	*Anal. Chem.* **2023**, 95, 46, 16868–16876
	/	617.86 $\mu M^{-1}\cdot min^{-1}$	/	Alzheimer's disease model	*ACS. Sens.* **2018**, 3, 2118
	/	/	1.2 U L^{-1}	/	*Chem.Commun.* **2019**, 55, 14574
	ICT	/	0.08 U mL^{-1}	tumor-bearing mouse model	*Analytica Chimica Acta.* **2022**, 1235, 340540
	/	/	3.93 U L^{-1}	/	*Analytica Chimica Acta.* **2023**, 1282, 341932

	/	7.648 μM^{-1} min^{-1}	0.96 U/L	acute liver injury mouse model	*Talanta.* **2025**, 286, 127478
	ICT	/	4.92 U/L	liver injury mouse model and Diabetic mouse model	*Sensors and Actuators B: Chemical.* **2023**, 394, 134432,
	/	/	0.077 μg/mL	/	*Dyes and Pigments.* **2022**, 197, 109874
	/	/	0.12 ng/mL	Tumor-Bearing Mice Models and AD Mice Models	*Analytical Chemistry* **2022** 94, 13498-13506

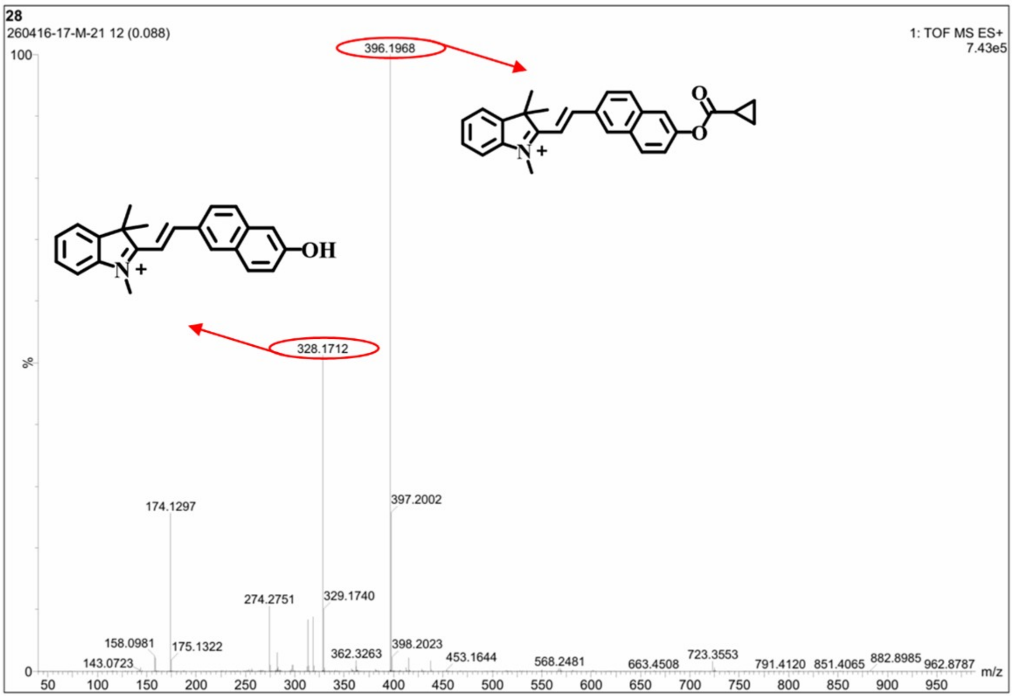


Fig. S12 Response mechanism between **YNP** and BChE determined by HRMS.

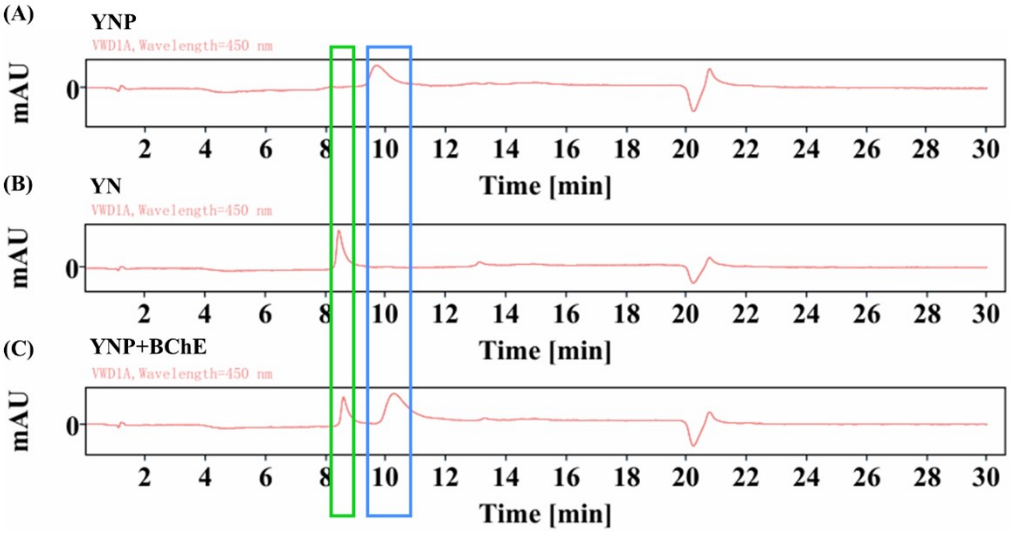


Fig. S13 High performance liquid chromatography (HPLC) analysis of the probe **YNP** (A), compound **YN** (B) , and reaction product of **YNP** + 258.6 U/L BChE (C). The HPLC mobile phase was as follows: solvent A (CH_3CN), solvent B (MeOH), A/B = 7/3. Column: C18 column (Shimadzu, 5 µm, 250 mm × 4.6 mm), temperature: 37 °C, detection wavelength: 450 nm, flow rate of mobile phase: 1 mL/min. The peaks inside the green box represent compound **YN**. The peaks inside the blue box represent **YNP**.

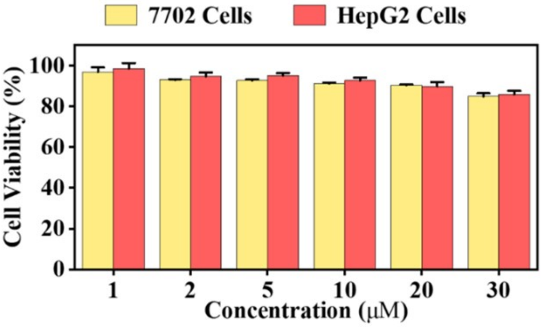


Fig. S14 Viability of HepG2 cells and HL-7702 cells after 24 h incubation with different concentrations of **YNP**, respectively.

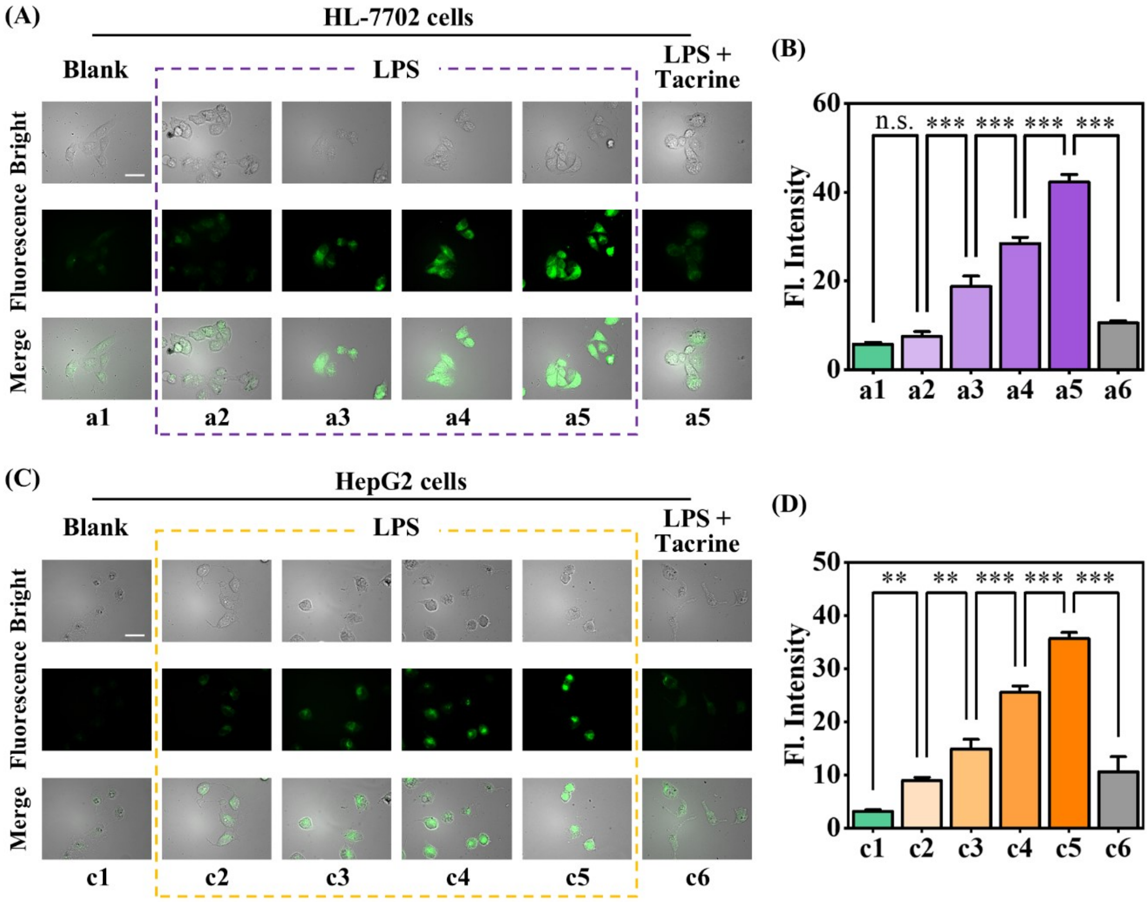


Fig. S15 (A) Fluorescence images of HL-7702 cells with/without LPS after the addition of **YNP**. (B) Quantification of fluorescence intensity in A, values were mean ± SD (n = 3). (C) Fluorescence images of HepG2 cells with/without LPS after the addition of **YNP** (10 μM) for 50 min. (D) Quantification of fluorescence intensity in C, values were mean ± SD (n = 3). Experimental conditions: a1 and c1: The cells were incubated with **YNP** for 50 min; a2-a5 and c2-c5: The cells were incubated with LPS (20 μg/mL) for different times (2 h (a2, c2), 4 h (a3, c3), 6 h (a4, c4) and 8 h (a5, c5), respectively), and then treated with **YNP** for 50 min; a6 and c6: The cells were incubated with both LPS (20 μg/mL) and tacrine (50 μM) for 8 h, and then incubated with **YNP** for 50 min. (Scale bar: 20 μm.) (The upright-inverted integrated fluorescence microscope utilizes the FITC mode, which features an excitation wavelength of 470±40 nm and an emission wavelength of 525±50 nm.). (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$)

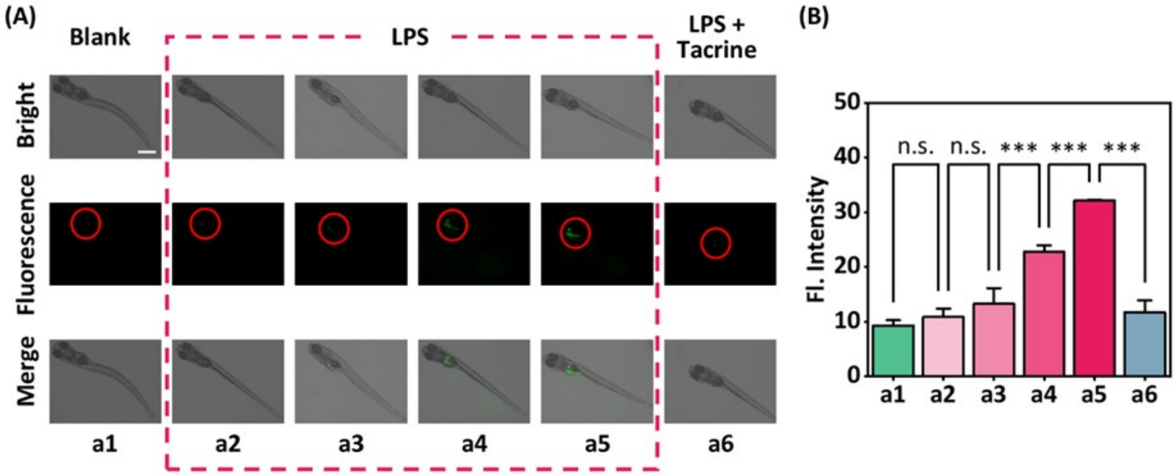


Fig. S16 (A) Fluorescence images of zebrafish with/without LPS after the addition of **YNP**. a1: Zebrafish were added to **YNP** (10 μM) for 50 min; a2-a5: The zebrafish were incubated with LPS (20 μg/mL) for different times (2 h (a2), 4 h (a3), 6 h (a4), and 8 h (a5), respectively), and subsequently added to **YNP** (10 μM) for 50 min; a6: The zebrafish was incubated with both LPS (20 μg/mL) and tacrine (50 μM) for 8 h, and then incubated with **YNP** for 50 min. The red circles indicate the locations of fluorescent signal detection in zebrafish. (B) Quantification of fluorescence intensity in **A**, values were mean ± SD (n = 3). (Scale bar: 500 μm). (The upright-inverted integrated fluorescence microscope utilizes the FITC mode, which features an excitation wavelength of 470±40 nm and an emission wavelength of 525±50 nm.). (* $p \le 0.05$; ** $p \le 0.01$; *** $p \le 0.001$)

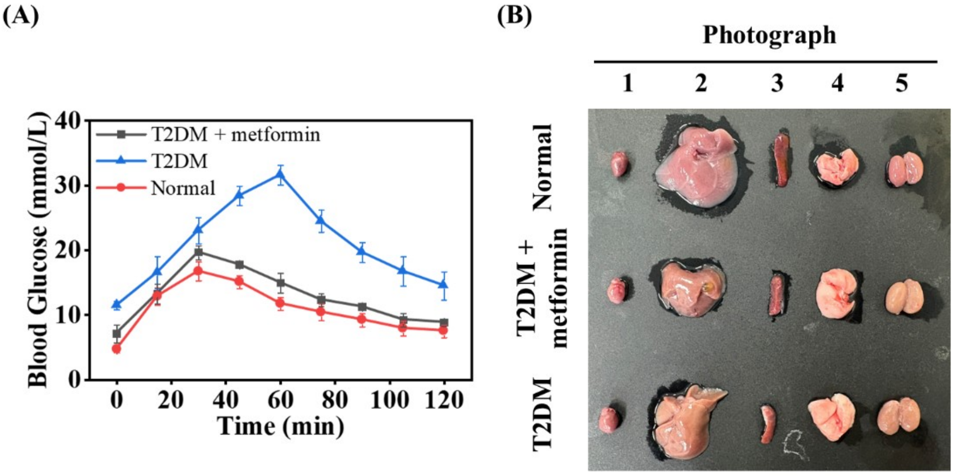


Fig. S17 (A) Oral glucose tolerance test (OGTT) in the mice. Blood glucose levels were measured at the indicated time points (0 min, 15 min, 30 min, 45 min, 60 min, 75 min, 90 min, 105 min, and 120 min, respectively) after oral glucose administration (2 g/kg). Values are presented as mean ± SD (n = 3 per group). (B) Photograph of the tissues of mice (I: The normal mice; II: The treatment mice with T2DM; III: The T2DM mice). 1: heart; 2: liver; 3: spleen; 4: lung; 5: kidney.

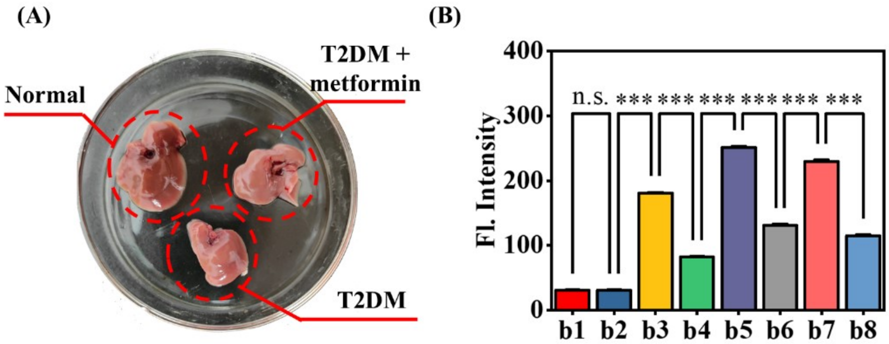


Fig. S18 Fluorescence spectroscopic analysis of the supernatant from liver tissue homogenate. (A) Photograph of the liver tissues of mice. B. Fluorescence intensity of the probe **YNP** in the presence or absence of supernatant. (b1) **YNP**; (b2) **YNP** + propranolol (100 µM); (b3) **YNP** + 10 ul extract from liver tissue of normal mice; (b4) **YNP** + 10 uL extract from liver tissue of normal mice + propranolol (100 µM); (b5) **YNP** + 10 uL extract from liver tissue of the treatment mice with T2DM; (b6) **YNP** + 10 uL extract from liver tissue of the treatment mice with T2DM + propranolol (100 µM); (b7) **YNP** + 10 uL extract from liver tissue of the mice with T2DM, and (b8) **YNP** + 10 uL extract from liver tissue of the mice with T2DM + propranolol (100 µM). Values were mean ± SD (n = 3). (*** $p \leq 0.001$)

References

1. A. Samanta, K. K. Maiti, K. S. Soh, X. Liao, M. Vendrell, U. Dinish, S. W. Yun, R. Bhuvaneswari, H. Kim and S. Rautela, *Angewandte Chemie-International Edition*, 2011, **50**, 6089.
2. Z.-M. Yang, Q.-Y. Mo, J.-M. He, D.-L. Mo, J. Li, H. Chen, S.-L. Zhao and J.-K. Qin, *ACS sensors*, 2020, **5**, 943-951.
3. S.-Y. Liu, H. Xiong, J.-Q. Yang, S.-H. Yang, Y. Li, W.-C. Yang and G.-F. Yang, *ACS sensors*, 2018, **3**, 2118-2128.
4. S. Virtue and A. Vidal-Puig, *Nature metabolism*, 2021, **3**, 883-886.
5. Y. Liu, Q. Miao, Y. Liu and M. Jiang, *International Journal of Biological Macromolecules*, 2024, **279**, 135422.
6. L. Yue, H. Huang, W. Song and W. Lin, *Chemical Engineering Journal*, 2022, **441**, 135981.
7. Y. Jin, X. Tian, L. Jin, Y. Cui, T. Liu, Z. Yu, X. Huo, J. Cui, C. Sun and C. Wang, *Analytical chemistry*, 2018, **90**, 3276-3283.