An anionic cyanine probe with enhanced ROS-stability for butylcholinesterase detection in non-alcoholic fatty liver disease

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CONTENTS

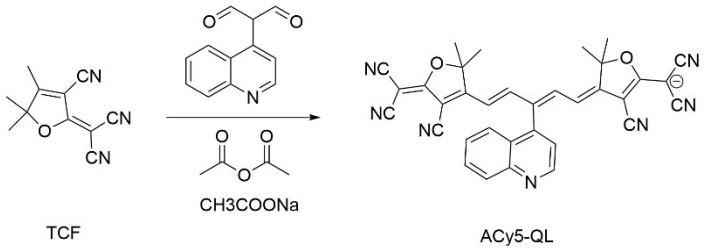
1. Materials and General Experimental Methods

1.1. Apparatus and reagents

All reagents and solvents were obtained from commercial suppliers and were used without further purification. The ^{1}H-NMR spectra of all compounds were recorded using a 400 MHz NMR spectrometer (Bruker AVANCE III HD, USA), and the ^{13}C-NMR spectra were recorded using a 600 MHz NMR spectrometer (AVANCE III HD). High resolution mass spectrometry (HRMS) was collected by Brooke microTOF-Q II mass spectrometer (USA). The UV absorption spectrum was measured using a PerkinElmer UV Visible spectrophotometer (UH4150, China). The fluorescence emission spectrum was measured using a fluorescence spectrometer (F-4600 FL spectrophotometer, Japan). Confocal fluorescence imaging was performed using an LSM880 (Germany) microscope with an objective lens (× 40). Cell Counting Kit-8 assay was carried out to evaluate the cyto-toxic effect of the probe on a Multiskan FC microplate reader. The model of the small animal live imaging system is Series III (America).

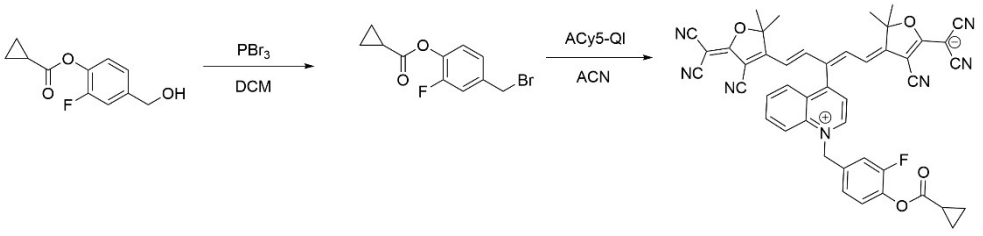
Butylcholinesterase was obtained from McLean. The high sugar DMEM culture medium is from Shanghai Yuanpei Biotechnology Co., Ltd. Trypsin/EDTA digestive solution is from Tianjin Haoyang Biological Products Technology Co., Ltd., and high-quality Fetal bovine serum is from Beijing Labgic Technology Co., Ltd. MTT (3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) and 4% paraformaldehyde fixative were purchased from Biyun Tian. Zebrafish was purchased from Nanjing Yishu Lihua Biotechnology Co., Ltd. Female KM mice aged 7 to 8 weeks were purchased from Jinan Pengyue Experimental Animal Breeding Co., Ltd.

1.2. Synthesis and characterization of ACy5-QL-BChE



Synthesis of TCF: This compound was synthesized using previous literature.[1]

Synthesis of ACy5-QL: TCF (6.00 g, 12.05 mmol), quinoline aldehyde (3.04 g, 13.68 mmol), and sodium acetate (224.6 mg, 2.74 mmol) were added to a round-bottom flask, followed by the addition of 40 mL of acetic anhydride to dissolve the reactants. The reaction mixture was stirred at 65 °C for 2–3 h. Upon completion, indicated by the appearance of a new green spot on TLC, the reaction mixture was extracted with dichloromethane (DCM). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, and concentrated. The crude product was purified by column chromatography to afford ACy5-QL (2.2g, 60.66% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.01 (d, J = 4.0 Hz, 1H), δ 8.12 (q, J = 3.0 Hz, 3H), δ 7.61 (t, J = 4.0 Hz, 1H), δ 7.38 (d, J = 4.0 Hz, 1H), δ 6.1 (d, J = 16.0 Hz, 1H), δ 5.75 (s, 1H), δ 5.19 (d, J = 16.0 Hz, 2H), δ 1.76 (s, 12H).^{13}C NMR (600 MHz, DMSO-d_6) δ 176.53, 171.96, 170.07, 151.04, 149.33, 148.58, 142.79, 130.32, 130.22, 129.68, 127.78, 126.61, 125.58, 123.45, 115.49, 114.63, 106.53, 96.04, 55.38, 45.77, 27.06, 26.98, 26.48, 22.98.



Compound B Compound C ACy5-QL-BChE

Synthesis of compound B: This compound was synthesized using previous literature.[2]

Synthesis of compound C: compound B (100.00 mg, 0.28 mmol) was dissolved in anhydrous DCM (20 mL), then phosphorus tribromide (38.20 mg, 0.14 mmol) was slowly added dropwise under ice-cooling, and the mixture was stirred at 0 °C for 4–6 h. Upon completion (monitored by TLC), the appearance of a new spot was observed. The reaction mixture was extracted with dichloromethane (DCM). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure to obtain crude product for the next reaction.

Synthesis of ACy5-QL-BChE: ACy5-QL (100.00 mg, 0.20 mmol) and compound C (83.41 mg, 0.59 mmol) were dissolved in acetonitrile (10 mL). The reaction mixture was stirred at 75 °C overnight. Upon completion (monitored by TLC), a new spot was observed. The reaction mixture was extracted with DCM. The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, and concentrated. The crude product was purified by column chromatography to afford ACy5-QL-BChE(88.00 mg, 85.48% yield).^{1}H NMR (400 MHz, DMSO-d_6) δ 9.85 (d, *J* = 4.0 Hz, 1H), δ 8.55 (d, *J* = 9.0 Hz, 1H), δ 8.27 (q, *J* = 7.6 Hz, 2H), δ 8.15 (s, 1H), δ 8.09 (t, *J* = 7.3 Hz, 2H), δ 7.98 (t, *J* = 8.3 Hz, 1H), δ 7.62 (d, *J* = 13.0 Hz, 1H), δ 7.19 (t, *J* = 8.3 Hz, 1H), δ 6.85 (d, *J* = 7.2 Hz, 1H), δ 6.48 (s, 2H), δ 5.75 (s, 2H), 1.97-1.90(m, 1H), δ 1.74 (s, 12H), δ 1.09 (t, *J* = 4.5 Hz, 2H), δ 1.00 (t, *J* = 3.2 Hz, 2H).^{13}C NMR (600 MHz, DMSO-d_6) δ 176.81, 172.36, 170.91, 156.44, 154.91, 153.26, 151.15, 148.38, 138.32, 138.09, 138.01, 136.73, 134.24, 134.19, 131.27, 129.10,128.58, 125.42,125.32, 123.17, 120.54, 116.43, 116.30, 115.22, 114.41, 105.90, 96.35, 59.61, 55.38, 46.66, 26.92, 12.58, 9.93.

1.3. In vitro experimental method

ACy5-QL-BChE was dissolved in DMSO to obtain a 10 mM stock solution, storing at -20 °C. In selective study, stock solutions of all analytes were prepared in PBS buffer (pH 7.4, 10 mM). All stock solutions and BChE were diluted in PBS buffer to desired concentrations for spectral analysis. ACy5-QL-BChE (10 μM) was incubated with various analytes in PBS buffer (pH 7.4, 10 mM) at 37 ℃ for 1 h. The concentrations of all analytes were as follows: Ca^{2+}, Co^{3+}, Cu^{2+} Fe^{3+}, H_2O_2, K^+, Mg^{2+}, S^{2-}, $S_2O_3^{2-}$, SO_3^{2-}, HSO_3^-, NO_2^-, GSH, D-Cys, L-Cys (100 μM); ALP, Gal, GUS, POD, XOD, AChE, BChE (1 U/mL). For all the measurements, the excitation wavelength of ACy5-QL-BChE was 700 nm. The excitation slit widths, emission slit widths and excitation voltage was 5.0 nm, 5.0 nm, and 1000 V, respectively.

1.4. Determination of the limit of detection

The detection limit was determined from the fluorescence titration assay. The fluorescence intensity of ACy5-QL-BChE (10 μM) was measured after incubation with various concentrations of BChE. The standard deviation of blank measurement was calculated by the fluorescence intensity of ACy5-QL-BChE in the absence of BChE. To gain the slop, the fluorescent intensity at

762 nm was plotted as BChE concentration. The detection limit of ACy5-QL-BChE toward BChE was calculated by the following equation:

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

Where σ was the standard deviation of blank measurement. k was the slope of the plot of fluorescence intensity (760 nm) against BChE concentration.

1.5. Enzyme kinetic study

Use Lineweaver Burke to evaluate the reaction rate of ACy5-QL-BChE with BChE and calculate the relevant kinetic constants. The reaction rate between ACy5-QL-BChE and BChE was calculated using the Lineweaver Burk equation:

$$\frac{1}{V} = \frac{K_m}{V_{max}[ACy5 - QL - BChE]} + \frac{1}{V_{max}}$$

Among them, [ACy5-QL-BChE] represents the probe concentration, V represents the reaction rate in the presence of different [ACy5-QL-BChE], Km and Vmax are the Michaelis constant and maximum initial reaction rate, respectively.

2. Cell imaging

2.1. Cell culture and cytotoxicity assays

HepG2 cells were utilized in this work. HepG2 cells were cultured in DMEM supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin and streptomycin. Cells were grown in incubator in atmosphere of 5% CO_2 at 37 °C. Cellular fluorescence imaging in LSM880 (Germany) confocal laser scanning microscope. The excitation wavelength is set to 640 nm, and the fluorescence emission wavelength is collected at 663-738 nm.

Inoculate HepG2 cells into a 96 well plate at a density of 1×10^5 cells per well, with a volume of 200 μL, and culture in a cell culture incubator at 37 ℃ and 5% CO_2 atmosphere for 24 h. After covering a single layer of cells at the bottom of each well, wash the cells three times with PBS and replace them with fresh culture medium containing different concentrations of ACy5-QL-BChE (0, 1, 5, 10, 20 and 30 μM). After 24 h of treatment, remove the culture medium and wash the cells three times with PBS. Add 20 μL of 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyl-2-tetrazolium bromide (thiazole blue, MTT, 5 mg/mL in PBS), Incubate for 4 h. After carefully suctioning out the excess MTT solution, dissolve the purple crystalline formaldehyde in 150 μL DMSO. Measure the absorbance of each well at 450 nm to indirectly reflect the survival rate of live cells. Measure the absorbance of each well at 450 nm to indirectly reflect the survival rate of live cells. The formula for calculating cell viability is:

$$\text{Cell viability (\%)} = (OD_{unknown} - OD_{blank}) / (OD_{control} - OD_{blank}) \times 100\%$$

The subscript unknown represents the absorbance of each well containing different concentrations of ACy5-QL-BChE, blank represents the OD value of DMSO without cells and fluorescent dyes, and control represents cells in the culture medium without fluorescent dyes.

2.2. OA-induced NAFLD in cells

The HepG2 cells were divided into two groups. The first group were pretreated with 500 μM OA for different times (0, 0.5, 1, and 2 h); the second group were pretreated with different concentrations OA (0, 50, 100, 250, 500 μM) for 1 h. And then two groups were treated with ACy5-QL-BChE (20 μM) for 1 h.

2.3. Endogenous tracking ability of ACy5-QL-BChE in NAFLD model

HepG2 cells were pretreated with 500 μM OA for 1 h, then treated with iso-OMPA for 2 h,

and finally treated with ACy5-QL-BChE (20 μM) for 1 h.

2.4. Monitoring the content of BChE in cells

HepG2 cells were pretreated with different concentrations of H_2O_2 for 0.5 h, followed by treatment with ACy5-QL-BChE (20 μM) for 1 h.

2.5. Drug therapy for NAFLD in cells

HepG2 cells were pretreated with 500 μM OA for 1 h, then treated with different concentrations of vitamin E (0, 10, 25, 50 μM) for 24 h, and finally treated with ACy5-QL-BChE (20 μM) for 1 h.

Each experiment was repeated three times. Wash the cells three times with PBS before imaging. λ_{ex} = 640 nm, λ_{em} = 663 - 738 nm.

3. Zebrafish imaging experiments

3.1. DXM-induced NAFLD in zebrafish

Three-day-old zebrafish were transferred to laser confocal petri dishes for fluorescence imaging. One culture dish was used as the control group, while other culture dishes were incubated with different concentrations of DXM (10, 20, 30 μM) for 0.5 h, followed by incubation with ACy5-QL-BChE for 1 h. After probe insertion, zebrafish were gently washed with PBS and placed on a fixative for confocal imaging using a 4× objective lens.

3.2. Endogenous tracking ability of ACy5-QL-BChE in zebrafish

Three-day-old zebrafish were transferred to a laser confocal culture dish for fluorescence imaging. One culture dish was used as the control group and incubated with DXM (20 μM) for 0.5 h. The other culture dishes were incubated with DXM (20 μM) for 0.5 h, followed by iso-OMPA for 2 h, and finally pretreated with ACy5-QL-BChE (20 μM) for 1 h. After probe insertion, zebrafish were gently washed with PBS and placed on a fixative, and confocal imaging was performed using a 4× objective lens.

4. In Vivo Imaging

All live animal operations were in accord with institutional animal use and care regulations, according to protocol No. SYXK (Xiang) 2020-0012, approved by Laboratory Animal Center of Hunan. A high-fat diet (HFD) was used to model NAFLD in mice. The mouse model was induced by feeding HFD chow and intraperitoneal injection of 100 mg/kg DXM. The control group received equivalent volumes of saline injections and standard chow. The induction lasted for seven days. Prior to in vivo imaging, all mouse abdomens were depilated to ensure liver leakage for imaging purposes. Mice in both the control and model groups were injected with the same dose of probe (1mg/kg) before imaging. After that, the important tissues of each mouse, including the heart, liver, spleen, lungs, and kidneys were dissected, followed by H&E staining and biosafety analysis. Each experiment is repeated three times to ensure the accuracy of the results. λ_{ex} = 700 nm, λ_{em} = 790 nm.

5. Supplementary Figures

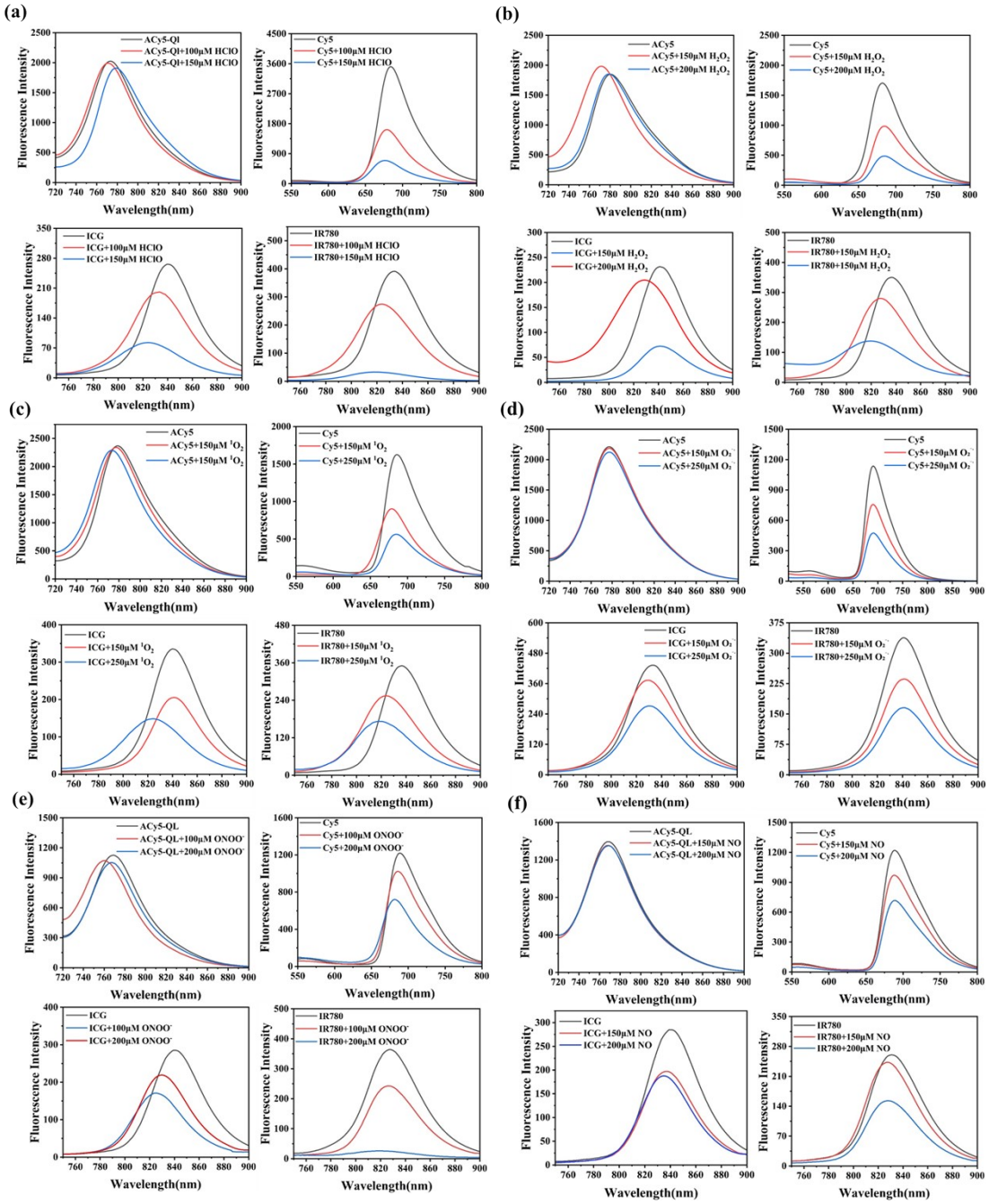


Fig. S1 The fluorescence spectra of ACy5-QL, Cy5, ICG and IR780 in the presence of various concentrations of HClO (a), H_2O_2 (b), $^1O_2$ (c), $O_2^{-\cdot}$ (d), $ONOO^-$ (e) and NO (f).

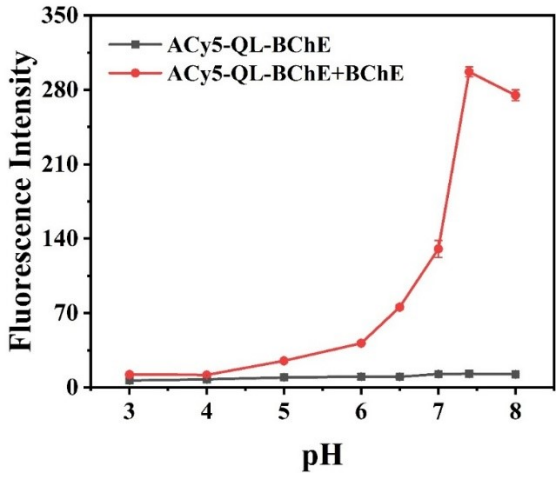


Fig. S2 Fluorescence spectra of ACy5-QL-BChE and BChE before and after reaction in different pH buffer solutions.

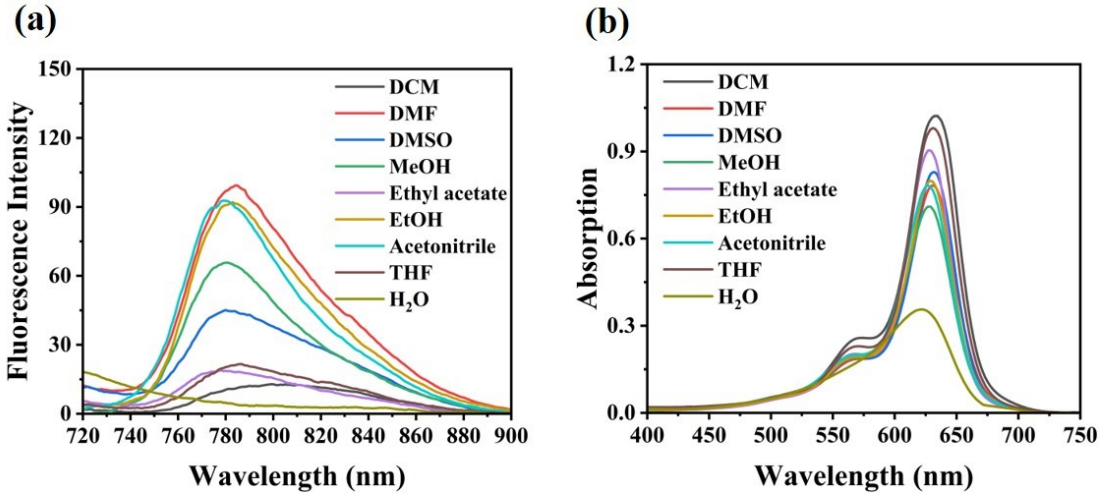


Fig. S3 Solvent-dependent absorption and fluorescence spectra of ACy5-QL-BChE.

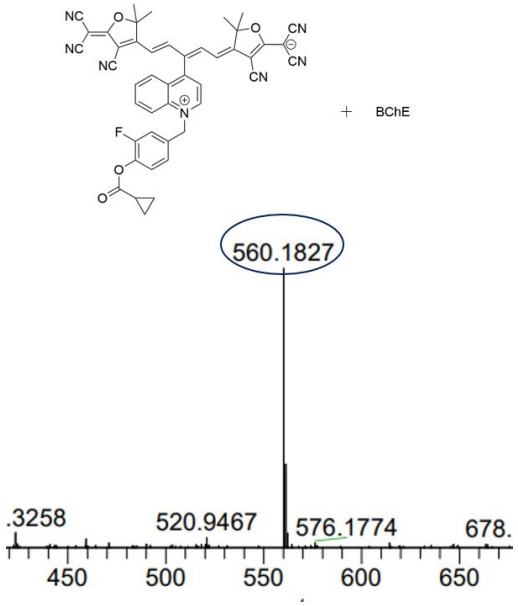
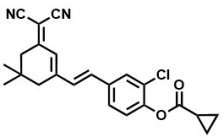
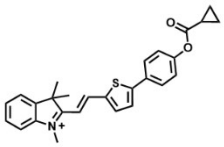
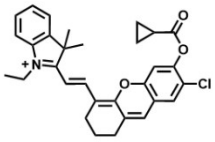
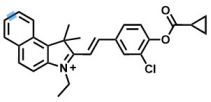
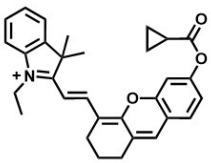
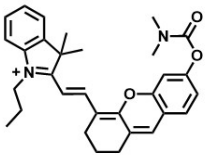
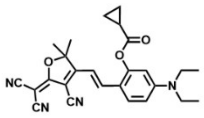
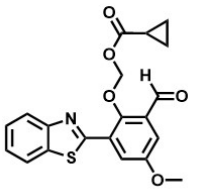
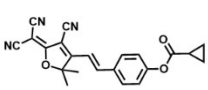
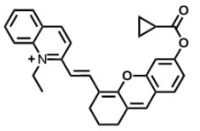
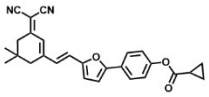
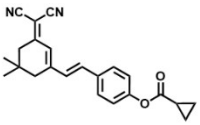
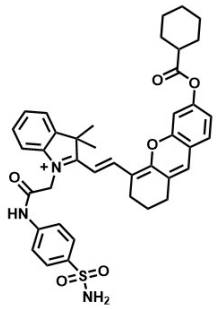
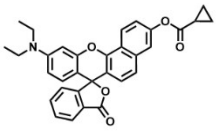
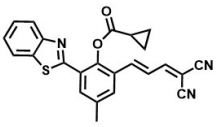
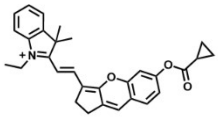
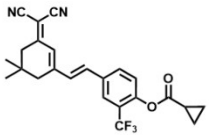
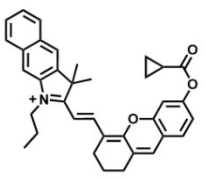
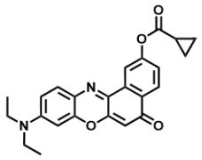
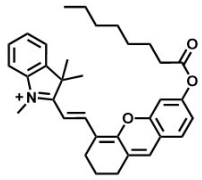
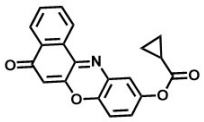
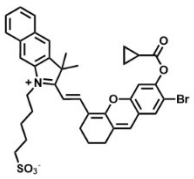
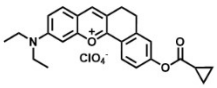
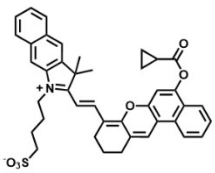
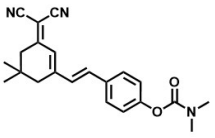
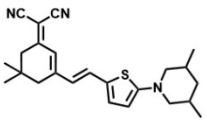
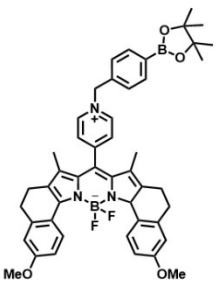


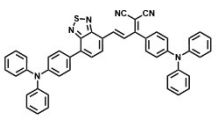
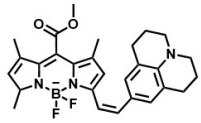
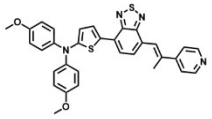
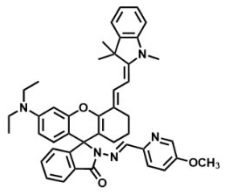
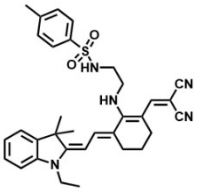
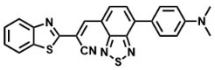
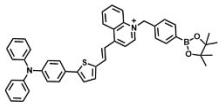
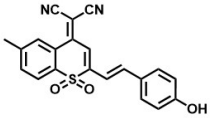
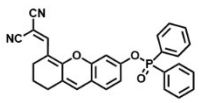
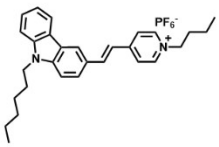
Fig. S4 HRMS spectrum of the reaction between ACy5-QL-BChE and BChE.

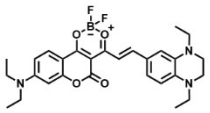
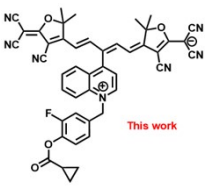
Table S1 Comparison of probe performance for detecting analytes in BChE and NAFLD models

Compound	λ_{em}	Analyte	SNR	Detection limit	ROS resistance
	665nm	BChE	/	0.08U/mL	/
	640nm	BChE	/	0.0086U/mL	/
	708nm	BChE	3	3.75U/L	/
	584nm	BChE	/	16.7 ng/mL	/
	705nm	BChE	/	0.083U/mL	H_2O_2, ·OH
	676nm	BChE	/	0.14 U/mL	H_2O_2, HClO, ·OH, $ONOO^-$
	626nm	BChE	/	39.24 ng/mL	/
	614nm	BChE	/	7.54×10^{-4} U/mL	/
	562nm	BChE	/	0.12 μg/mL	H_2O_2,·OH

	775nm	BChE	/	0.032 U/L	/
	820nm	BChE	/	0.05 U/L	HClO,·OH
	689nm	BChE	/	3.93 U/L	/
	710nm	BChE	/	2.2 mU/mL	/
	671nm	BChE	/	7.2×10^{-3} U/mL	/
	660nm	BChE	/	0.2 mU/mL	H_2O_2,$ONOO^-$,$^1O_2$, ·OH, HClO
	715nm	BChE	/	0.12 ng/mL	H_2O_2, ·OH
	650nm	BChE	/	0.0144 U/mL	H_2O_2,HClO,$^1O_2$,·OH, $O_2^{\cdot-}$
	720nm	BChE	/	2.08×10^{-7} U/mL	H_2O_2

	690nm	BChE	/	0.024 U/L	/
	705nm	BChE	/	28.9 ng/mL	/
	642nm	BChE	/	0.056 U/L	/
	758nm	BChE	/	2.64 U/L	H_2O_2, HClO, $^{\cdot}OH$
	628nm	BChE	/	29 ng/mL	/
	762nm	BChE	/	2.64 U/L	H_2O_2, HClO, $^{\cdot}OH$, $^1O_2$
	650nm	BChE	/	0.0117 U/mL	H_2O_2, HClO
	700nm	Viscosity	/	0.57cP	HClO
	661nm	$ONOO^-$	/	98 nM	HClO,H_2O_2,$^{\cdot}OH$

	600-675nm	Polarity	/	/	$HClO$,H_2O_2
	858nm	CE	/	0.37 μg/mL	$HClO$,H_2O_2
	620-720nm	Polarity	/	/	$HClO$,H_2O_2
	742nm	CO	/	/	$HClO$,NO
	626nm	Viscosity	/	/	$HClO$,NO,$ONOO^-$,$\cdot OH$,$O_2^{\cdot-}$
	700-810nm	Polarity	/	/	H_2O_2, $HClO$, OH^-, $ONOO^-$
	657nm	$ONOO^-$	/	/	ClO^-,$\cdot OH$
	760nm	Viscosity	/	/	H_2O_2
	640nm	$ONOO^-$	/	2.3 nM	$HClO$, H_2O_2, $\cdot OH$, $O_2^{\cdot-}$, $^1O_2$, NO
	600nm	Viscosity	/	/	$HClO$, H_2O_2

	700-850nm	Polarity	/	/	HClO, H_2O_2, $ONOO^-$
	762nm	BChE	13	0.02U/mL	HClO, H_2O_2, $ONOO^-$, $O_2^{\cdot-}$, $^1O_2$, NO

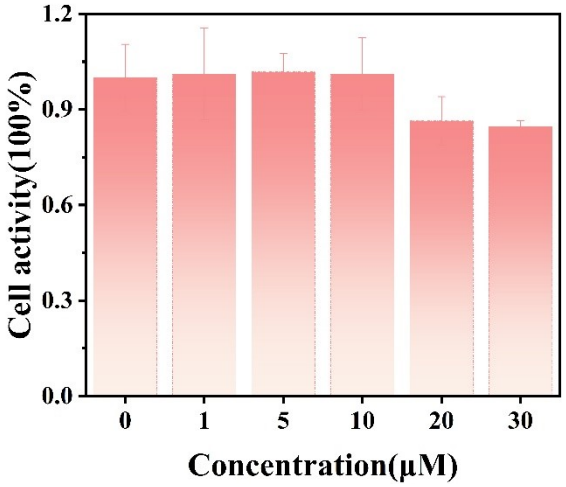


Fig. S5 Cytotoxicity of ACy5-QL-BChE in HepG2 cells through MTT.

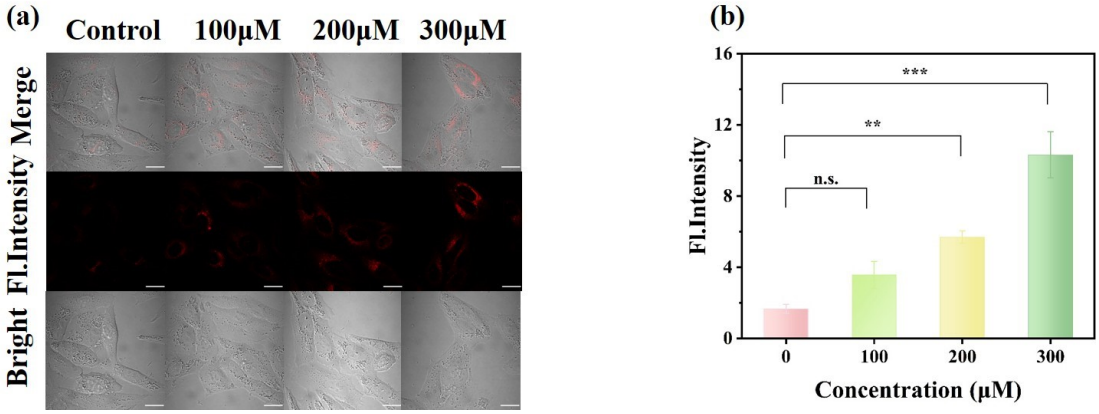


Fig. S6 Fluorescence image of BChE levels in HepG2 cells stimulated by H_2O_2. Relative fluorescent intensity of each group. λ_{ex} = 640 nm, λ_{em} = 663-738 nm. Scale bar: 20 μm. Statistical significance: n.s. P > 0.05, **P < 0.01, ***P < 0.001. means ± s.d., n = 3.

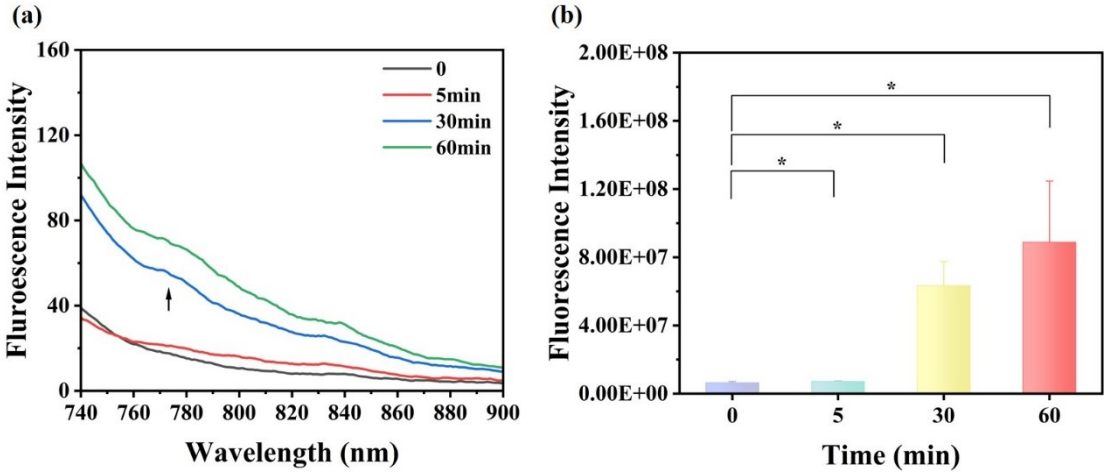


Fig. S7 Kinetic assay in live cells. (a) Fluorescence spectroscopy analysis of cell suspensions incubated with probe ACy5-QL at different time points. (b) Fluorescence intensity of cell suspensions acquired using a small animal live imaging system. Statistical significance: *P < 0.05. means ± s.d., n = 3.

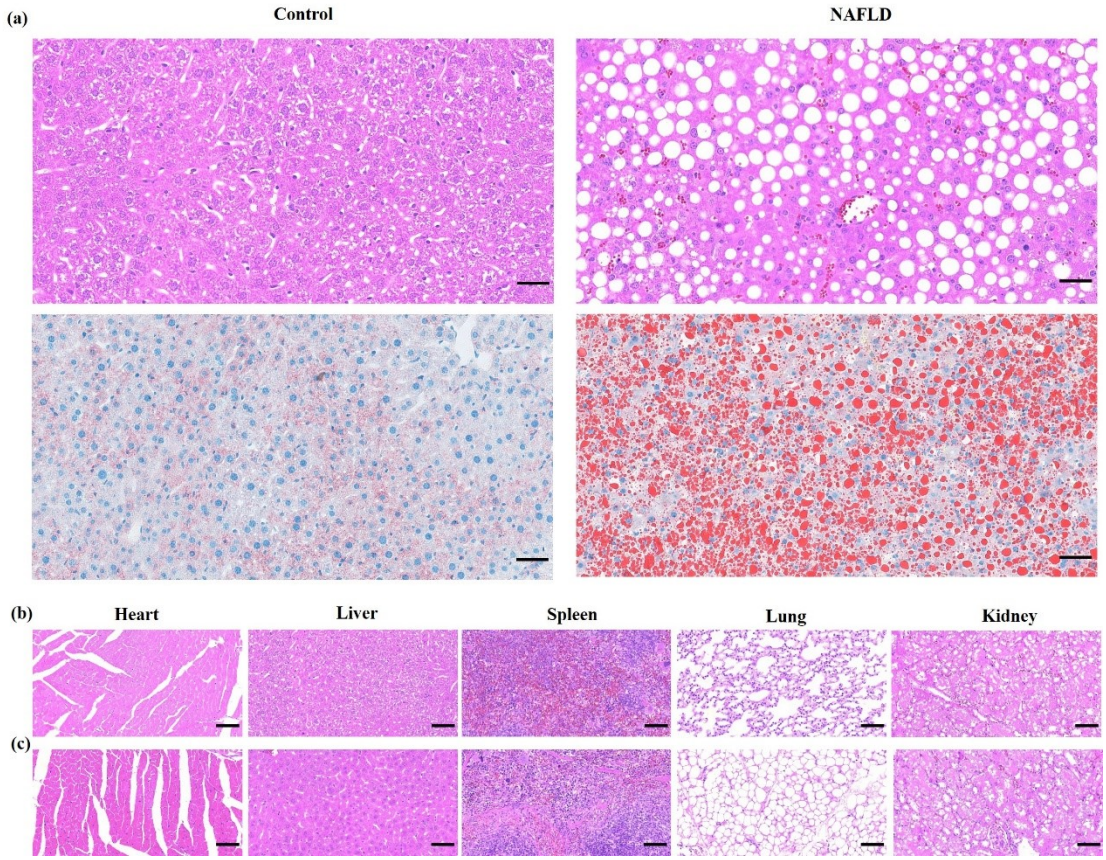


Fig. S8 (a) H&E and Oil red O staining of liver tissues in the control and NAFLD group. (b,c) The biocompatibility testing of probe in various organs. Scale: 50 μm.

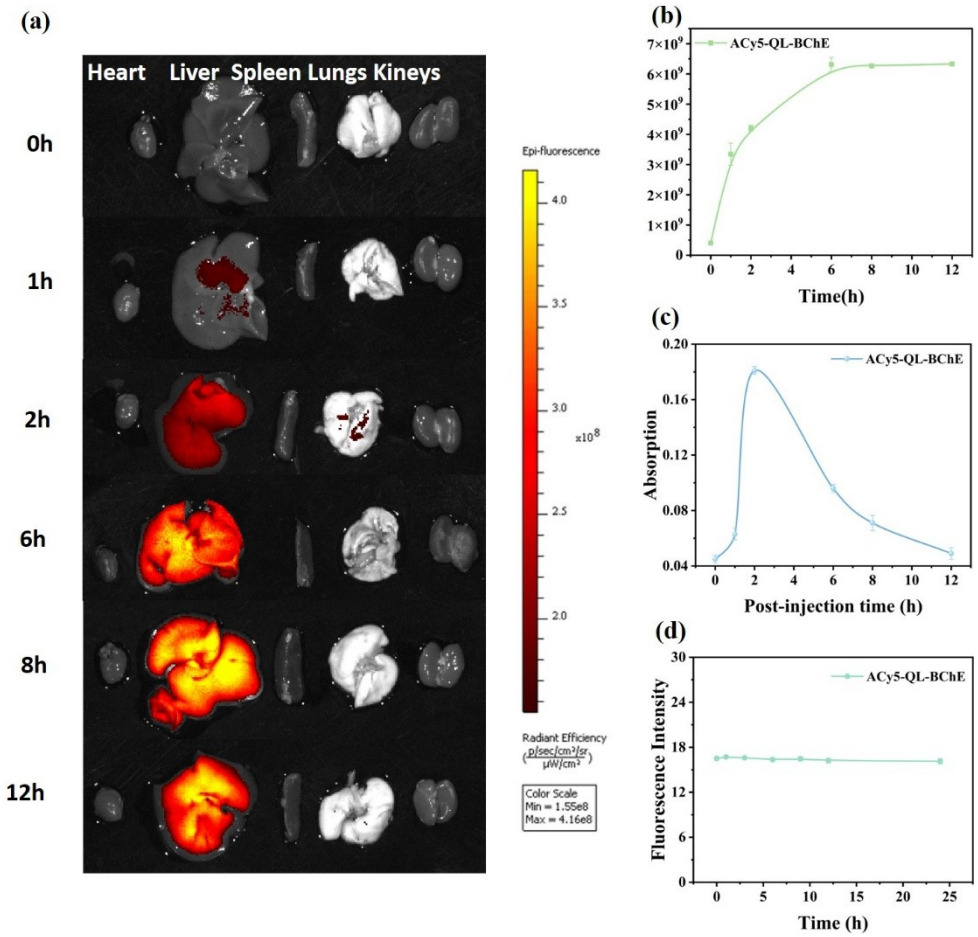


Fig. S9 Characteristics of probe ACy5-QL-BChE. (a) The in vitro biological distribution of major organs (Heart, Liver, Spleen, Lungs and Kidneys) at a specified time point after injection; (b) Fluorescence intensities of isolated liver from mice and rats in (a); (c) UV-vis absorption of probes in mouse plasma at different time points after injection; (d) Fluorescence intensity after incubation with mouse serum (pretreated with 200 μM iso-OMPA for 2 h) at 37°C for different durations;. means ± s.d. deviation (n=3 at each time point).

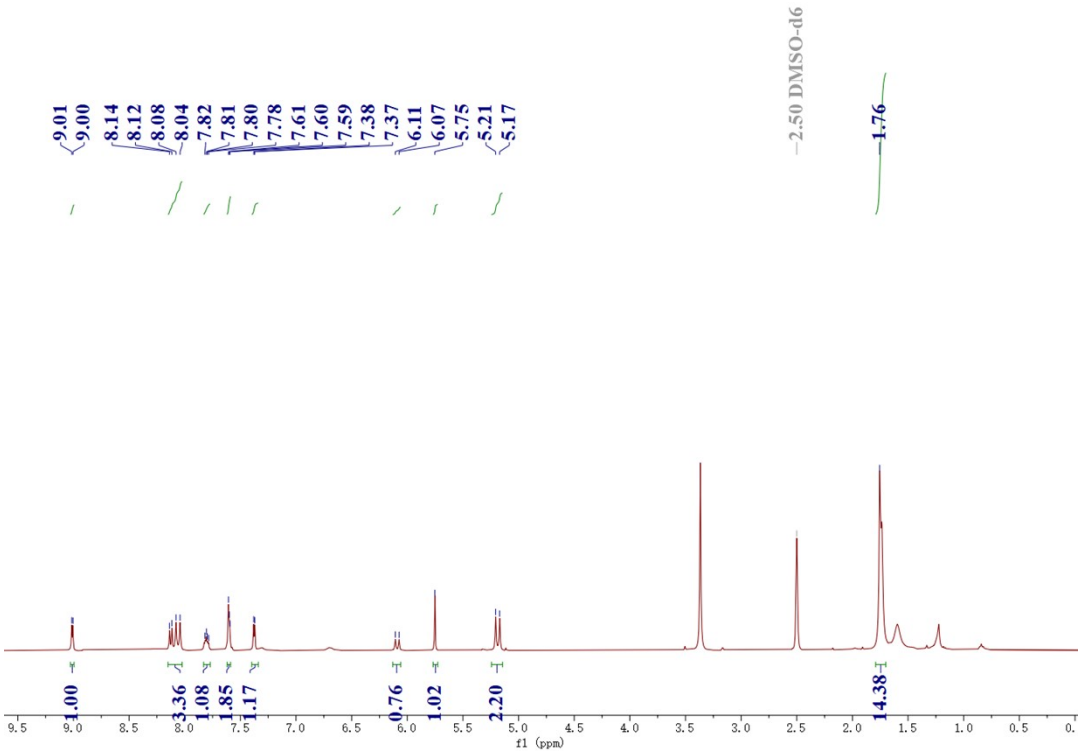


Fig. S10 ^{1}H-NMR spectrum of ACy5-QL in DMSO-d6 (400 MHz)

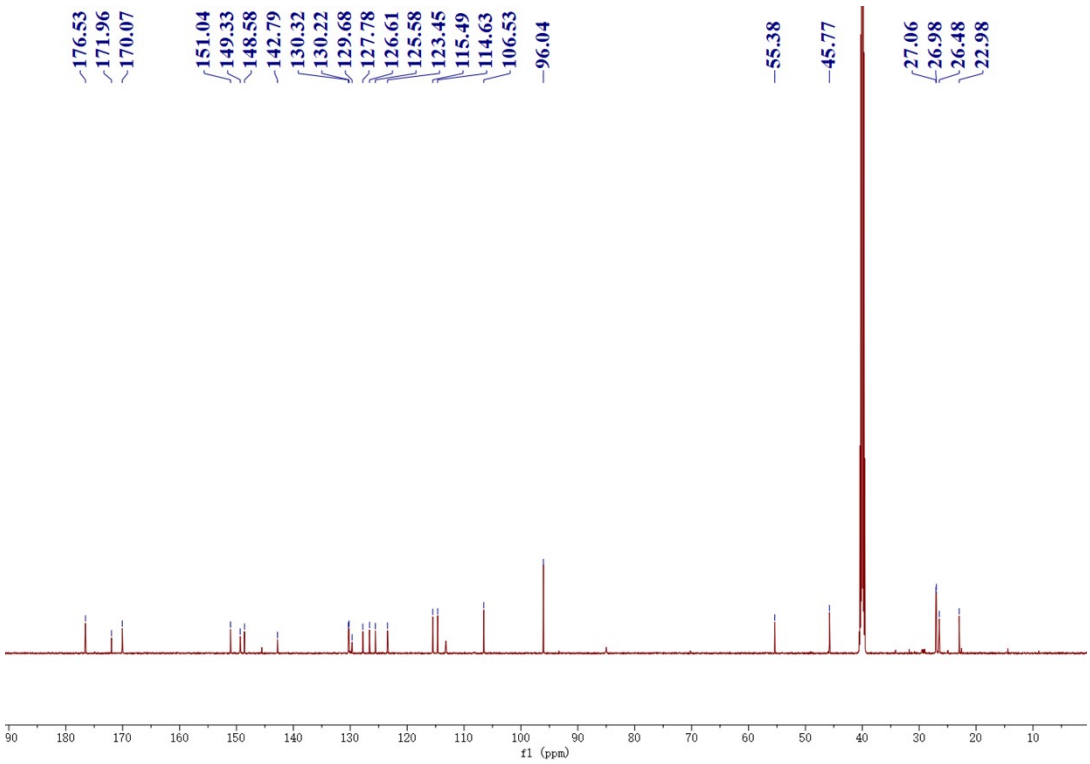


Fig. S11 ^{13}C-NMR spectrum of ACy5-QL in DMSO-d6 (600 MHz)

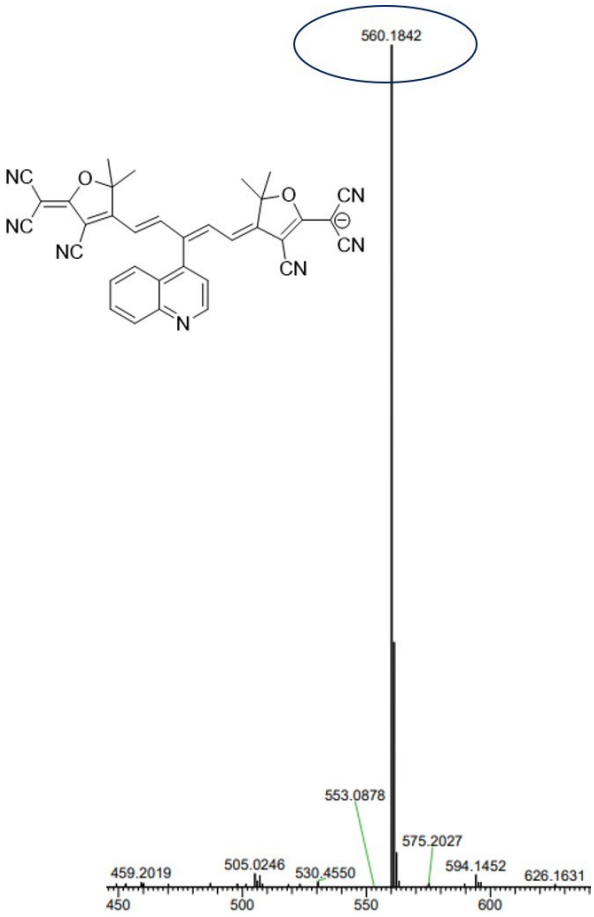


Fig. S12 HRMS spectrum of ACy5-QL.

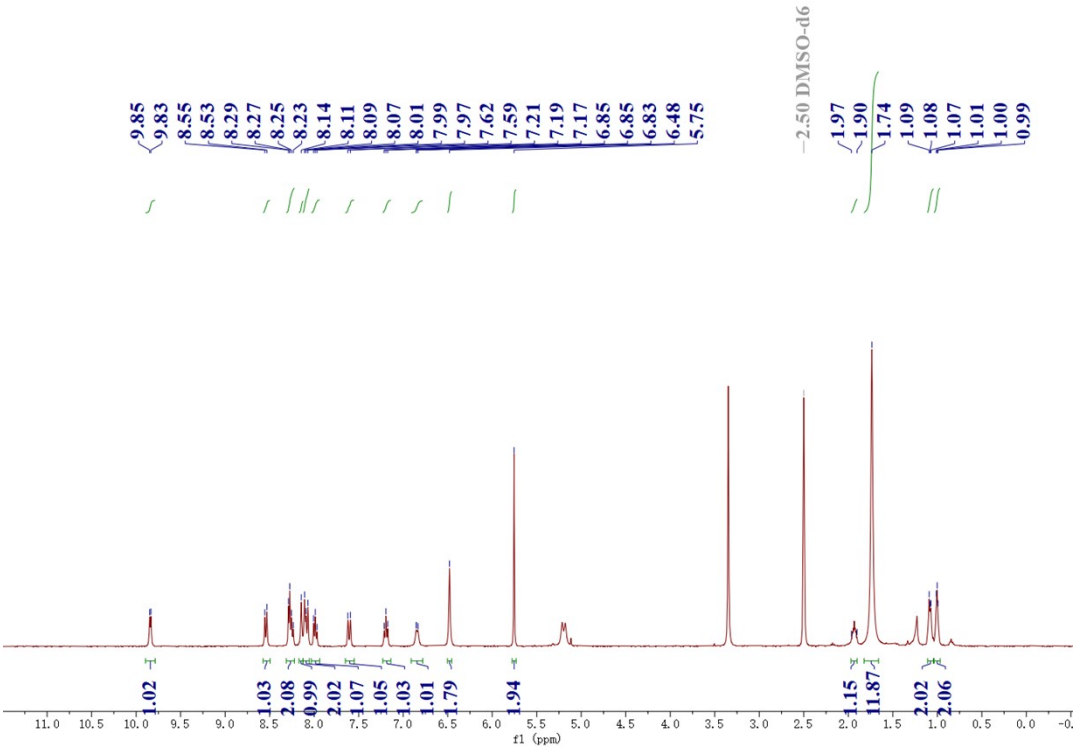


Fig. S13 ^{1}H-NMR spectrum of ACy5-QL-BChE in DMSO-d6 (400 MHz)

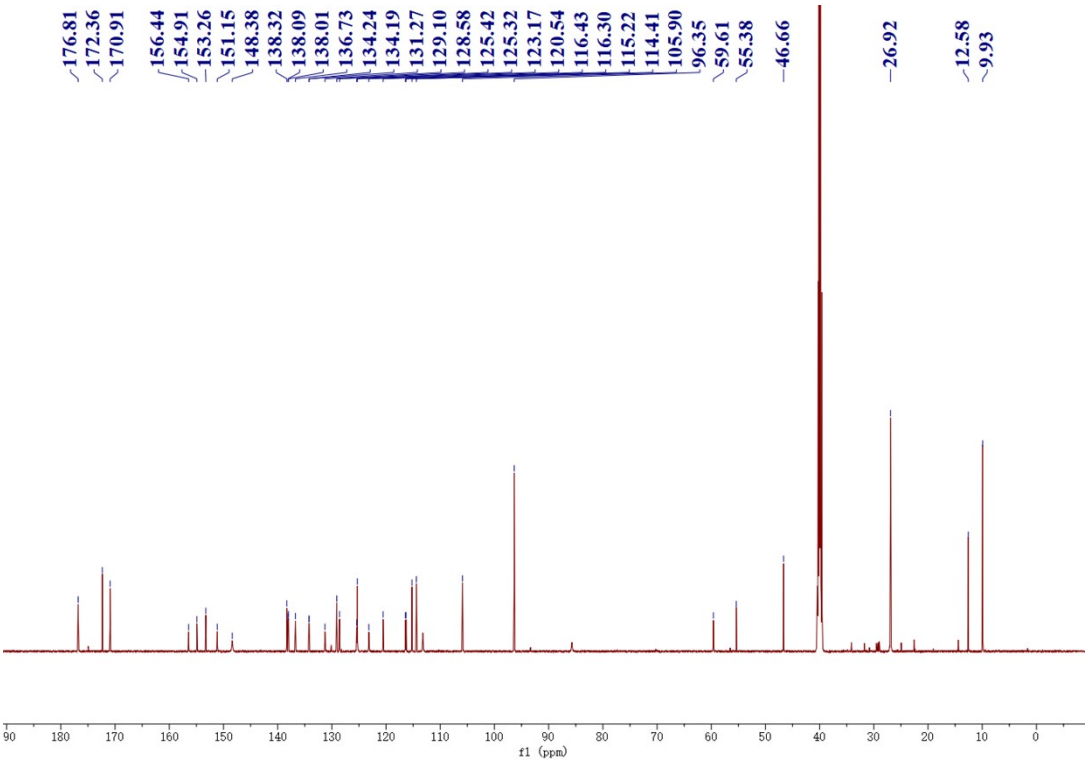


Fig. S14 ^{13}C-NMR spectrum of ACy5-QL-BChE in DMSO-d6 (600 MHz)

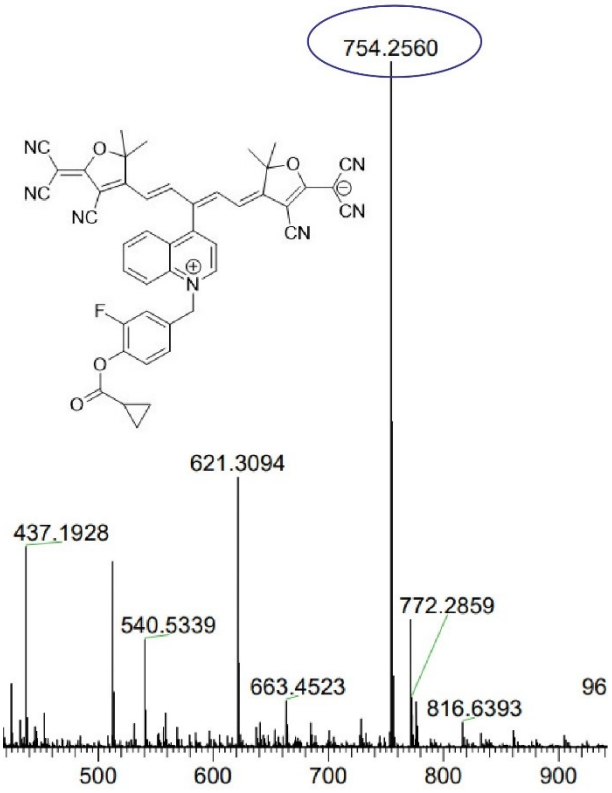


Fig. S15 HRMS spectrum of ACy5-QL-BChE.

6. References

1 E. S. Kim, J.-M. Lee, J.-Y. Kwak, H. W. Lee, I.-J. Lee and H. M. Kim, *Anal. Chem.*, 2024, **96**, 8467–8473.

2 C. M. Levinn, A. K. Steiger and M. D. Pluth, *ACS Chem. Biol.*, 2019, **14**, 170–175.