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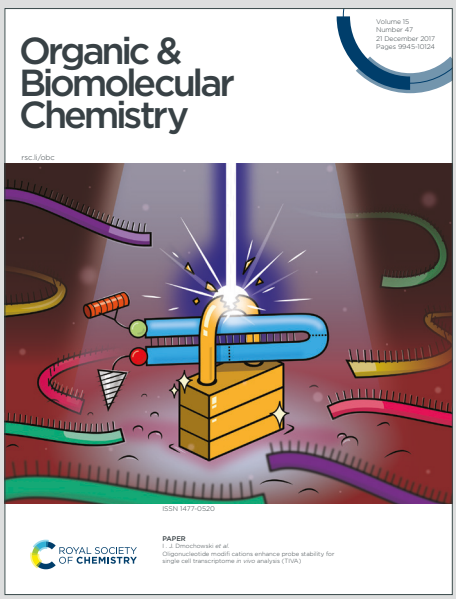

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ARTICLE

A new fluorescence probe for sensing of biothiols and screening of acetylcholinesterase inhibitors

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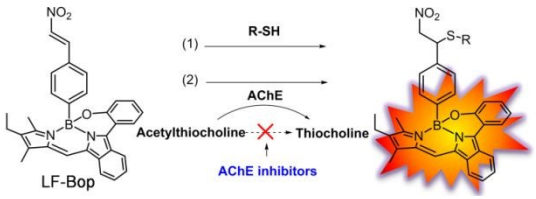
A new N_2O-type bodipy probe (LF-Bop) has been proposed for selective and sensitive detection of bio-relevant small molecular thiols. The detection is based on the Michael addition between thiol and nitrostyrene group in the probe, which decreases the quenching effect from nitro group thus recovering the deep-red fluorescence from bodipy structure. The result shows that LF-Bop is able to detect all tested free thiols through a fluorescence turn-on manner. The lowest limit of detection (LOD) toward glutathione is found down to nanomolar levels (220 nM). Based on this probe, we have developed a new fluorescence assay for the screening of acetylcholinesterase inhibitors. In total, 11 natural and synthetic alkaloids have been evaluated. Both experimental measurement and theoretical molecular docking reveal that natural berberine and its synthetic derivative dihydroberberine are both potential inhibitors of acetylcholinesterase.

Introduction,

Small molecular thiols are important to living organisms, for example, endogenous thiols such as L-cysteine (L-Cys) and glutathione (GSH) play critical roles in regulating cellular redox balance. The disruption of thiol mediated microenvironment balance will lead to the risks of various diseases.[1, 2] Besides, exogenous thiols such as N-acetylcysteine (NAC) are widely applied as antioxidants for the treatment of human health care issues, due to the reductive function of thiol groups.[3, 4] The importance of thiols has been attracting great efforts in the development of detection strategies. Fluorescence assays are most attractive among all techniques, because of their excellent sensitivity and simplicity.[5-8] There have been numerous fluorescence chemical probes for thiol quantification and imaging based on different reaction mechanisms.[9-14] Michael addition has been extensively used as the basis for probe construction, with the consideration of the intrinsic strong nucleophilicity of thiols. Under this technique aspect, a number of maleimide conjugated fluorescence probes have been reported for thiol sensing.[15-20] The fluorescence of those probes is quenched by photoinduced electron transfer (PET), since maleimide serves as a perfect PET acceptor.[21, 22] Thiol addition can greatly suppress PET, thus restoring the fluorescence.[21, 23] Conjugation with the strongly electron-deficient 2, 4-dinitrophenyl sulfonyl group is another important strategy for thiol-response probe design. The detection is based on the displacement of the nitro quenching groups induced by thiol's nucleophilic attacking.[24-27] Although great efforts have been made in this field, searching for much advanced fluorescence probes for sensing of bio-relevant thiols with promising sensitivity and selectivity is still desired.

Besides thiol detection and imaging, the probes with thiol-response functions are also useful in revealing the activities of thiol-manipulating enzymes. For example, glutathione reductase (GR) can covert GSSG back to GSH, which is important for living organisms to maintain their reductive microenvironment. Recently, thiol detection probes have been developed to monitor GR activities through fluorescent manners.[28, 29] Acetylcholinesterase (AChE) is another important enzyme in human body, particularly in the nervous system.[30] It's found that the reaction between AChE and acetylthiocholine can generate free thiocholine, which offers the opportunities for AChE activities evaluation using thiol-response probes.[31-33]



Scheme 1. Schematic show of LF-Bop based fluorescence assay for thiol detection and AChE inhibitors screening

Herein, we present a new fluorescence probe for thiol detection. The probe LF-Bop is made of an N_2O-type bodipy dye and an α,β-unsaturated nitro group as fluorescence quencher (Scheme 1). The strong electron withdrawing effect of nitro group in the conjugation

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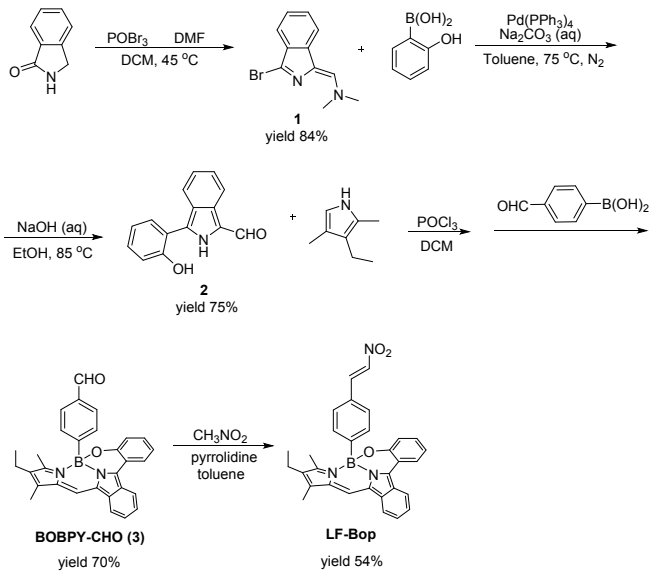
system is thought to induce fluorescence quenching probably through intramolecular charge transfer (ICT) process. Upon Michael addition by thiols, the conjugation of nitrostyrene is disrupted, thus leading to electron transferring effect decrease and fluorescence recovery. The detection of various endogenous and exogenous bio-relevant thiols, including 2-mercaptoethanol (BME), H_2S, L-Cys, D-Cys, GSH and NAC, have been evaluated by monitoring the deep-red fluorescence (peaked at 637 nm) changes. More importantly, with the good performance of LF-Bop on thiol detection, we developed an efficient fluorescence assay for AChE inhibitors screening.



Scheme 2. Synthetic route of probe LF-Bop

Results and discussion

The axial N_2O-type bodipy was designed as the fluorescence indicator mostly because of its promising red fluorescence emitting property, and meanwhile, the axial-substitution can prevent their possible aggregations through steric protection. Benzaldehyde capped bodipy-CHO (compound **3** in Scheme 2) was synthesized according to previously reported methods (Scheme S1).[34, 35] Bodipy-CHO was then reacted with nitromethane to form the final product LF-Bop using pyrrolidine as the catalyst (Scheme S2). The important intermediates and final product have been well characterized by NMR and LC-MS. Thiol detection property was firstly examined by adding GSH into LF-Bop solutions in a 96-well plate followed by fluorescence monitoring using a plate reader. As found in Fig. 1 and Fig. S1, GSH addition leads to fluorescence increases from LF-Bop solutions. The fluorescence intensity of the reaction solutions varies between different solvent ratios (PBS:DMSO). The reaction product is weak fluorescent in pure aqueous solutions and becomes strong fluorescent in organic solvents. Similar hydrophobicity sensitive properties have been reported on some other kinds of bodipy probes.[36, 37] The ratio of PBS (pH 7.4) to DMSO was finally fixed at 1:1 for the rest tests after screening. Fig. 1A shows that the fluorescence from LF-Bop/GSH solution reaches to an intensity plateau within 10 minutes, which indicates a reasonable reaction rate between LF-Bop and GSH. In contrast, LF-Bop alone without GSH only exhibits background level fluorescence. GSH addition does not affect the UV-vis spectrum of LF-Bop too much, as the main fluorogenic structure is maintained during the reaction (Fig. S2). LF-Bop behaves similarly in terms of NAC detection as illustrated in Fig. S2. The most important photo-physical parameters of LF-Bop including molar extinction coefficient, stokes shift and quantum yield were then measured in the presence or in the absence of thiols, the result was presented in Table S1.

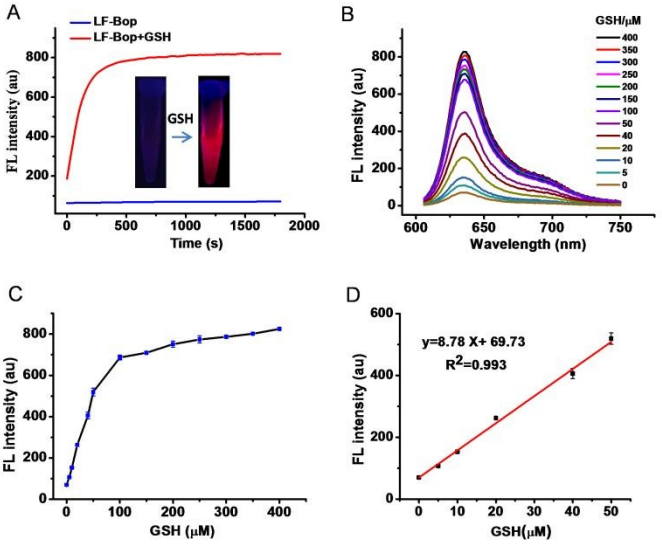


Fig. 1. A) Kinetic monitoring fluorescence intensity I_{637} of LF-Bop solution (5 μM) in the presence of GSH (200 μM) ; B) Fluoresces spectra of LF-Bop solution (5 μM) after reaction (10 min) with GSH at series concentrations (0-400 μM); C) I_{637} against the concentrations of GSH; D) Linear correlation when GSH between 0-50 μM. All measurements were performed in PBS (pH7.4):DMSO (1:1) solution, LF-Bop was excited at 581 nm.

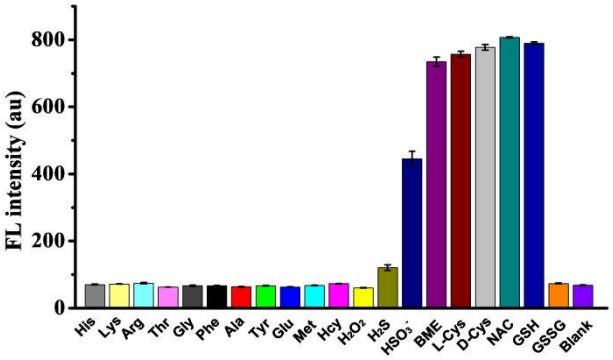


Fig. 2. LF-Bop selectively responds to thiols against other biorelevant amino acids as indicated by the increase of fluorescence (I_{637}, excited at 581 nm). LF-Bop (5 μM) was used for test, all analytes were used at 200 μM. NaHS (200 μM) was applied to generate H_2S.

Other kinds of bio-relevant thiols including BME, L-Cys, D-Cys and NAC have also been chosen to check the fluorescence responsiveness of LF-Bop. As illustrated in Fig. 2, LF-Bop can indeed detect those tested thiols as indicated by the increased fluorescence. However, all the tested bio-relevant amino acids at the same concentrations to GSH (200 μM) cannot lead to

fluorescence enhancement of LF-Bop. The result implies that although both thiol and amine groups are all strong nucleophiles, the Michael addition acceptor nitrostyrene prefers thiols rather than amines. H_2S is a small gaseous signalling biothiol, which also has strong tendency to undergo Michael addition.[35, 38] Fig.2 and Fig. S3 illustrate obviously that H_2S can react with LF-Bop, thus leading to fluorescence increase. However, it is less reactive than other tested thiols. Bisulfite ion (HSO_3^-) can also react with LF-Bop, as it has been previously proven to have strong reactivity toward Michael acceptors.[39] The pH effect on thiol detection by LF-Bop was then checked. Under the tested conditions from pH 6.5 to pH 10.5, LF-Bop exhibited promising abilities for thiol detection. Acidic pHs affected the detection greatly, probably induced by the decreased reactivity of Michael addition under acidic conditions (Fig. S4). The fluorescence intensity of the probe is susceptible to basic pHs, pH increasing from 10.5 to 12.5 greatly increases the background fluorescence of LF-Bop in the absence of thiols (Fig. S5).

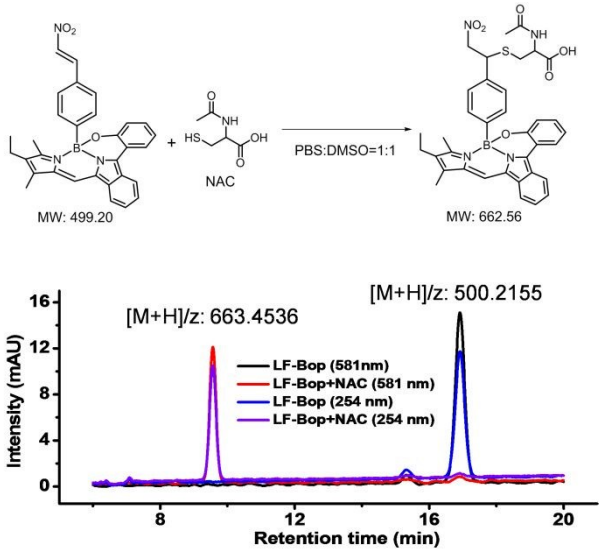


Fig. 3. HPLC and HRMS characterization of the thiol addition reaction on LF-Bop. LF-Bop (10 μM) was mixed with 400 μM NAC in PBS (pH 7.4)/DMSO solution for 30 min before HPLC analysis. UV/VIS monitor was set at 254 nm and 581 nm.

To look into the reaction, the solution of LF-Bop and NAC was further analyzed by high performance liquid chromatography (HPLC) and high resolution mass (HRMS). As found in Fig. 3, a new product with retention time at 9.5 min formed when mixing LF-Bop with NAC. The HRMS result further confirmed that the product was indeed formed from the addition reaction between LF-Bop and NAC (see ESI). It should be noted that LF-Bop itself has reasonable long-term stability when stored in pure DMSO and DMSO/PBS mixtures in the fridge, as indicated by the HPLC analysis in Fig. S6.

With the good result of thiol detection, we further explored the possibility to use LF-Bop for AChE's inhibitors screening. As we know, AChE is an important enzyme in human body, whose function is mainly involved in rapid hydrolysis of the neurotransmitter, acetylcholine (ACh).[40] Owing to its critical roles in acetylcholine-mediated neurotransmission, AChE is an interesting target for drug development against neurological disorders such as alzheimer's disease (AD). Up to date, the discovery of AChE inhibitors has resulted to several approved drugs (e.g. tacrine, donepezil, rivastigmine and galantamine) for AD treatment.[41, 42] AChE inhibitor screening is currently still attracting great attentions in drug discovery. It has been previously proven that AChE persists great hydrolysis activities toward acetylthiocholine with the generation of thiocholine,[43, 44] which offers the potentials for inhibitor screening by using thiol–response fluorescence assays.

With these considerations, we then tested the possibilities to use LF-Bop for the evaluation of AChE activity. Since enzymes cannot tolerate high concentrations of DMSO, the enzyme reaction was performed in pure aqueous solutions (PBS pH7.4) in the presence of LF-Bop (10 μM) and acetylthiocholine iodide (400 μM). The reaction was performed at 37 °C for 30 min, equal amount of DMSO was added into the reaction mixture before fluorescence measurement. To check the possible influence caused by changing the sequence of reagents addition, kinetic measurement and selectivity study have been re-performed. It's found that changing the sequence of reagents addition did not affect the detection and selectivity (Fig. S7). As shown in Fig. 4B, AChE catalyzed reaction indeed leads to fluorescence enhancement of LF-Bop. The increase of fluorescence intensity indicates an AChE concentration dependent manner. In contrast, AChE itself does not result to fluorescence changes greatly even at a high concentration (400 mU) (Fig. S8). The result encouraged us to further explore this assay for inhibitor screening. In a typical test, potential inhibitors at designed concentrations were co-incubated with AChE in the presence of acetylthiocholine and LF-Bop. As proposed in Fig 4A, effective inhibition will lead to decreased thiocholine and fluorescence when compared to the assay without inhibitor. These fluorescence changes were then applied for quantitatively analyzing of inhibition effect (Equation 2).

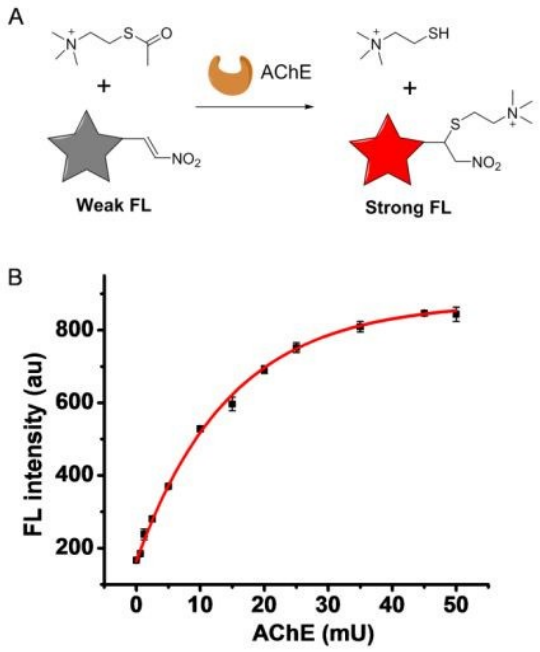
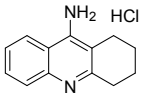
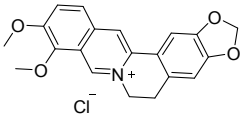
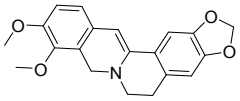
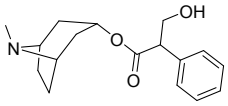
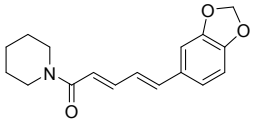
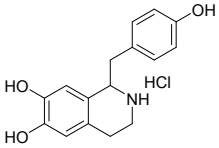
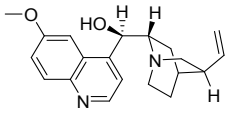
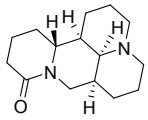
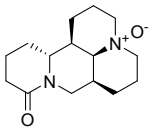
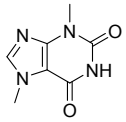
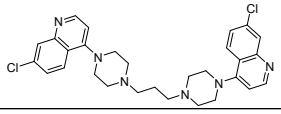


Fig. 4. A) Schematic show of the procedure to use LF-Bop for AChE activity evaluation; B) LF-Bop (10 μM) fluorescence turn on assay for monitoring AChE activity under different concentrations (0-50mU) in the presence of acetylthiocholine iodide (400 μM). The reaction was performed in PBS 7.4

buffer, before fluorescence measurement, DMSO was added to reach PBS:DMSO=1:1. I_{637} was recorded under 581 nm excitation.

Table 1. Tested compounds for AChE inhibitor screening.

No.	Name	Structure	IC_{50}
1	Tacrine hydrochloride		3.08 μM
2	Berberine hydrochloride		7.15 μM
3	Dihydroberberine		13.69 μM
4	Atropine		>160 μM
5	Piperine		>160 μM
6	Higenamine hydrochloride		>160 μM
7	Quinine		>160 μM
8	Matrine		>160 μM
9	Oxymatrine		>160 μM
10	Theobromine		>160 μM
11	Piperaquine		>160 μM

Tacrine, a well-known inhibitor of AChE was used as the positive control.[45] Altogether, 11 kinds of natural alkaloids and synthetic molecules have been tested for their inhibition activities, the result was listed in Table 1 and Fig. S9. Among all, only berberine (BBR), dihydroberberine (DB) and the positive control tacrine exhibited excellent inhibition to AChE with IC_{50} at 7.15 μM, 13.69 μM and 3.08 μM respectively (Fig. 5). Some other alkaloids can partially inhibit AChE at high concentrations, but cannot reach to their IC_{50} even up to 160 μM. BBR is actually an actively studied inhibitor of AChE,[46] whereas, there is rare information regarding the inhibition effect of DB to AChE (Scheme S3). Our result shows that DB has reduced inhibition effect than BBR, which is also supported by computational molecular docking result. As shown in Fig. 6A and 6B, both BBR and DB can bind with AChE mostly through hydrogen bonds, pi-alkyl interaction and so son. Total-score values from the calculation based on the software Sybyl-X2.1.1 are used to evaluate the molecular docking effect, where larger numbers indicate greater potentials. As listed in Table S2, Total-scores for BBR and DB are 11 and 10 respectively, which indicates a relative weaker potential for DB docking with AChE. The same trend is found in case of C-scores, where a value much closer to 5 implies better activity. BBR and DB have similar binding pockets close to the surface of AChE, both are different from tacrine's binding pocket (Fig. S10). The detailed mechanism of AChE inhibition caused by BBR and DB is still unclear. Nevertheless, although DB exhibits lower inhibition effect, it still holds great potentials to inhibit AChE in biological environments since DB is usually found to have better biocompatibility and higher membrane penetrability than BBR.[47, 48]

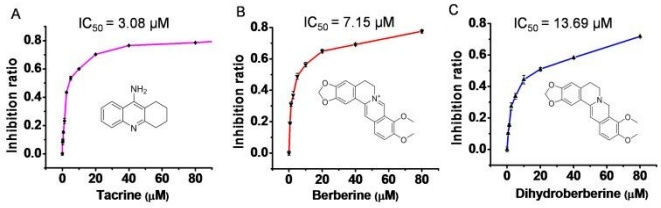


Fig. 5. Inhibition evaluation based on LF-Bop/AChE/acetylthiocholine fluorescence assay. The inhibition ratio was plotted as the function of concentrations for A) tacrine, B) berberine, C) dihydroberberine. 10 μM LF-Bop, 40 mU AChE and 400 μM acetylthiocholine in PBS buffer were used for test. The reaction was performed at 37 °C for 30 min, equal amount of DMSO was added into the reaction mixture before fluorescence measurement.

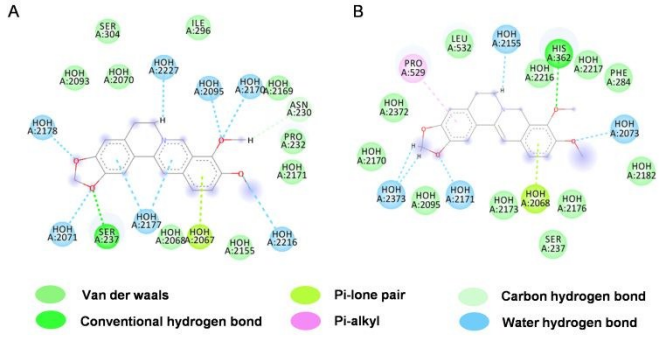


Fig. 6. A) Interaction between AChE and berberine from molecular docking; B) Interaction between AChE and dihydroberberine from molecular docking.

Experimental

Synthesis of Bodipy-CHO

Compound **1**, **2** and BOBPY-CHO (**3**) were synthesized by referring

previous study.[34, 35] Briefly, to synthesize the intermediate **3**, 2, 4-dimethyl-3-ethylpyrrole (2 mmol) was dissolved in dichloromethane (5 mL), $POCl_3$ (1 mmol) was then added at 0 °C, followed by the dropwise addition of compound **2** (1 mmol) in dichloromethane (5 mL). The reaction mixture was then stirred at room temperature for 4 h. 4-formylphenylboronic acid (10 mmol) in THF was added to the reaction mixture. Then the reaction was further stirred at room temperature and monitored by TLC. After the reaction completed, the solvent was removed and the crude product was purified by silica gel column chromatography to give BOBPY-CHO (yield 70%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.72 (s, 1H), 8.01 (d, *J* = 8.2 Hz, 1H), 7.88 – 7.86 (m, 1H), 7.81 (d, *J* = 8.1 Hz, 1H), 7.45 (d, *J* = 8.1 Hz, 2H), 7.41 – 7.33 (m, 3H), 7.30 – 7.17 (m, 4H), 6.92 – 6.88 (m, 1H), 2.37 (s, 3H), 2.30 – 2.28 (m, 2H), 2.19 (s, 3H), 0.97 – 0.93 (m, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.83, 156.67, 150.86, 143.42, 135.35, 134.89, 133.81, 133.36, 132.07, 131.96, 130.63, 128.82, 128.68, 126.43, 125.74, 125.68, 123.52, 120.20, 120.09, 119.71, 118.95, 116.09, 17.49, 14.86, 13.06, 9.65. Mass spectrometry (ESI-HRMS, m/z): $[M]^+$ calcd. for $[C30H25BN2O2]^+$ 456.2009; found 456.1982.

Synthesis of probe LF-Bop

A mixture of compound **3** (0.7 mmol), nitromethane (5.39 mmol) and toluene (3 mL) was stirred at room temperature for 5 minutes. After that, pyrrolidine (0.25 mmol) was added and the mixture was stirred overnight. After reaction completed, distilled water was added and extracted with ethyl acetate. The organic layer was separated and dried with Na_2SO_4. After concentrating under vacuum, the crude product was purified by silica gel column (dichloromethane/ethyl acetate/petroleum ether=1:1:50) to get LF-Bop (yield 54%). ^{1}H NMR (400 MHz, *d6*-DMSO) δ 8.33 (d, *J* = 8.3 Hz, 1H), 8.24 (d, *J* = 8.1 Hz, 1H), 8.15 (d, *J* = 7.0 Hz, 2H), 8.00 – 7.88 (m, 2H), 7.64 – 7.60 (m, 1H), 7.52 – 7.43 (m, 4H), 7.29 (d, *J* = 8.1 Hz, 1H), 7.15 (d, *J* = 8.0 Hz, 2H), 7.05 – 7.02 (m, 1H), 5.75 (s, 1H), 2.42 (s, 3H), 2.35 (d, *J* = 7.6 Hz, 2H), 2.29 (s, 3H), 1.01 – 0.97 (m, 3H). ^{13}C NMR (100 MHz, *d6*-DMSO) δ 156.48, 150.72, 142.71, 140.18, 137.48, 135.35, 134.81, 134.12, 132.21, 132.10, 130.51, 129.71, 129.22, 128.89, 128.09, 127.24, 126.62, 125.88, 124.05, 121.05, 120.92, 120.33, 118.74, 118.61, 17.29, 15.20, 13.27, 9.77. Mass spectrometry (ESI-HRMS, m/z): $[M+H]^+$ calcd. for $[C31H27BN3O3]^+$ 500.2145; found 500.2155.

Synthesis of dihydroberberine

Sodium borohydride (54 mg, 1.43 mmol) was dissolved in 5% sodium hydroxide aqueous solution, the solution was then added to the mixture of berberine chloride (450 mg, 1.34 mmol) and potassium carbonate (550 mg, 4.0 mmol) in methanol (18 mL). The reaction mixture is stirred at rt for 15 min. The yellow solution became green, the product was collected by filtration and washed with water and then ethanol/water (30% v/v). The product was further purified by recrystallization against ethanol.[49] ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.08 (s, 1H), 6.65 (s, 2H), 6.49 (s, 1H), 5.86 (d, *J* = 6.5 Hz, 3H), 4.24 (s, 2H), 3.76 (d, *J* = 2.1 Hz, 6H), 3.06 – 3.03 (m, 2H), 2.80 – 2.77 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 150.42, 147.29, 146.70, 144.51, 141.66, 128.77, 128.55, 124.55, 122.15, 118.83, 111.48, 107.87, 103.80, 101.03, 96.35, 60.75, 55.97, 49.36, 49.06, 29.85. ESI $[M+H]^+$ m/z calcd. for C20H20NO4 337.1, found 338.7 $[M+H]^+$.

Thiol detection

Detection was based on 96-well plate and the use of a Varioskan LUX plate reader. In a typical test, LF-Bop (5 μM) was mixed with the analytes at designed concentrations in PBS (pH 7.4):DMSO (1:1) solutions. The thiol-addition reaction was performed at room temperature for 10 min before measurement. The solutions were excited at 581 nm, the fluorescence spectra or the intensity at 637 nm was recorded. To prepare PBS:DMSO solutions with different pHs, PBS buffer with pH at 5.5, 6.5, 7.5, 8.5, 9.5, and 10.5 were separately mixed with DMSO at the vol/vol ratio of 1:1.

The lowest limit of detection (LOD) for GSH is estimated by a well established method (S/N=3).[50] Briefly,

$$LOD = 3\sigma/B \qquad \text{Equation 1}$$

where σ is the standard deviation obtained from three individual measured fluorescence intensity I_{637} in the absence of thiols. *B* is the slope from the linear fitting of the titration curve.

AChE activity evaluation and inhibitor screening

Since enzymes cannot tolerate high concentrations of DMSO, the enzyme reaction was performed in pure aqueous solutions (PBS 7.4) in the presence of LF-Bop (10 μM) and acetylthiocholine iodide (400 μM). The reaction was performed at 37 °C for 30 min, equal amount of DMSO was added into the reaction mixture before fluorescence measurement. For inhibitor screening, the reaction solution contains LF-Bop (10 μM) and AChE 40 mU.

$$\text{Inhibition ratio} = 1-(I_w-I_{blank})/(I_{w/o}-I_{blank}) \qquad \text{Equation 2}$$

Where, I_w stands for the fluorescence intensity I_{637} in the presence of inhibitors, $I_{w/o}$ stands for I_{637} in the absence of inhibitors, I_{blank} means the background I_{637} from LF-Bop/AChE solution without the substrate acetylthiocholine iodide.

Computational molecular docking

The crystal structure of AChE (E.C. 3.1.1.7, PDB ID: 1GQR) was found from the open source protein data bank (PDB) https://www.rcsb.org/. The crystal structure was then open with Sybyl-X2.1.1, the intrinsic ligand, surrounding water molecules and ions were removed before docking. Molecular geometry was optimized with MMFF94 force field. Multi-channel surface was set as the protomol-generation mode, the fully automatic flexible molecular docking (Surflex) was performed. Result analysis could be found in the supporting information.

Conclusions

In summary, we have synthesized a novel deep-red N_2O-type bodipy (LF-Bop) for rapid and convenient sensing of bio-relevant thiols. A nitrostyrene group is incorporated into the probe to induce fluorescence quenching, which also serves as an acceptor for thiol–Michael-addition. The deep-red emission of LF-Bop peaked at 637 nm makes it promising for analysis in biological environments due to the limited autofluorescence of biomolecules in deep-red region. The time to fully recover the fluorescence is less than 10 min, indicating a fast reaction rate. Moreover, we show the evidence that nitrostyrene is a promising Michael-addition acceptor for thiol probe design. On this basis, a fluorescence assay has been developed for the screening of AChE inhibitors, which provides the

evidence that both the natural berberine and the synthetic dihydroberberine are great inhibitors to AChE.

Conflicts of interest

There are no conflicts to declare.

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