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# Sensitive detection of acetylcholine based on a novel boronate intramolecular charge transfer fluorescence probe

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ABSTRACT

A highly sensitive and selective fluorescence method for the detection of acetylcholine (ACh) based on enzyme-generated hydrogen peroxide ($H_2O_2$) and a new boronate intramolecular charge transfer (ICT) fluorescence probe, 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-*N*-butyl-1,8-naphthalimide (BN), was developed. This strategy involves the reaction of ACh with acetylcholinesterase (AChE) to produce choline, which is further oxidized by choline oxidase (ChOx) to obtain betaine and $H_2O_2$. The enzyme-generated $H_2O_2$ reacts with BN and results in hydrolytic deprotection of BN to generate fluorescent product (4-hydroxyl-*N*-butyl-1,8-naphthalimide, ON). Two consecutive linear response ranges allow determining ACh in a wide concentration range with a low detection limit of 2.7 nM (signal/noise = 3). Compared with other fluorescent probes based on the mechanism of nonspecific oxidation, this reported boronate probe has the advantage of no interference from other biologically relevant reactive oxygen species (ROS) on the detection of ACh. This study provides a new method for the detection of ACh with high selectivity and sensitivity.



Acetylcholine (ACh)[1] is one of the most important neurotransmitters contained in the central and peripheral nervous systems and is widely involved in controlling nerve conduction in the body [1]. Acetylcholine comes from choline in the presence of choline acetyltransferase and acetyl-coenzyme A and has a significant influence on human memory and sleep [1]. The metabolic abnormalities of ACh in the brain may cause neuropsychiatric disorders such as Huntington's disease, Alzheimer's disease, schizophrenia, and Parkinson's disease [1,2]. Therefore, highly sensitive and selective detection of ACh is very necessary and of great importance.

A number of analytical techniques have been reported for the detection of ACh. Direct analytical techniques include mass spectrometry (MS), gas chromatography (GC), high-performance liquid chromatography (HPLC), and liquid chromatography coupled with mass spectrometry (LC/MS) [3,4]. Although these techniques are sensitive and reliable, most of them require tedious sample pretreatment, complex separation processes, and skilled operators [1,3]. The enzyme-based method is an indirect detection technique of ACh with high sensitivity. Acetylcholinesterase (AChE) and choline oxidase (ChOx) could catalyze their substrates to produce electrochemically active product (hydrogen peroxide, $H_2O_2$) or spectral (absorbance and fluorescence) sensing products. Therefore, the enzyme-based detection method usually depends on electrochemical and optical techniques. However, electrochemical detection of ACh by measuring its electrochemical response (enzyme-generated $H_2O_2$) is not sensitive enough [5], and the complex pretreatment of electrode is needed. Alternatively, optical detection methods, such as colorimetric Ellman assays and fluorescence assays, are widely used due to the simple operations and easy pretreatment processes. Colorimetric assays always depend on the use of peroxidase- or oxidase-like enzymes, such as horseradish peroxidase (HRP) and various of nanoparticles (NPs), and also need various substrates, including 3,3,5,5-tetrame-thylbenzidine (TMB), *o*-phenylenediame (OPD), pyrogallol, and 2,2′-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS) [1]. The detection can be performed through the color change by the naked eye or ultraviolet–visible (UV–vis) absorption. Colorimetric assays are simple but not sensitive enough [6]. Nowadays, more and more attention is paid to fluorescence analysis for high sensitivity [7].

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[1] *Abbreviations used:* ACh, acetylcholine; AChE, acetylcholinesterase; ChOx, choline oxidase; $H_2O_2$, hydrogen peroxide; HRP, horseradish peroxidase; UV–vis, ultraviolet–visible; ROS, reactive oxygen species; Amplex Red, 10-acetyl-3,7-dihydroxyphenoxazine; ICT, intramolecular charge transfer; BN, 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-*N*-butyl-1,8-naphthalimide; ON, 4-hydroxyl-*N*-butyl-1,8-naphthalimide; PBS, phosphate buffer solution; FTIR, Fourier transform infrared.

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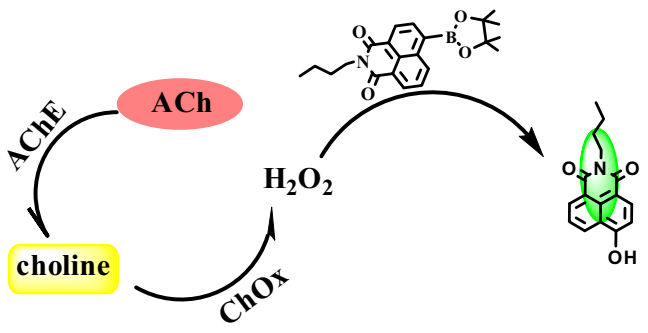
Up to now, there have been few reports about the detection of ACh directly using fluorescence probe. Based on the principle of indirect detection, it should be useful for the detection of ACh through detecting enzyme-generated $H_2O_2$ by using $H_2O_2$-sensitive fluorescent probes. There are several fluorescent probes that have been developed for measuring $H_2O_2$, superoxide, and peroxynitrite in biological systems or detecting enzyme-generated $H_2O_2$ [8,9]. Unfortunately, the assays based on dichlorodihydrofluorescein diacetate (DCFH–DA), hydroethidine (HE), and Mito-SOX always have high fluorescence background produced by their reaction with reactive oxygen species (ROS) [7,9–11]. To the best of our knowledge, it is reported that only the 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red) ACh/AChE Assay Kit provides ultrasensitive measurement of ACh based on enzyme-generated $H_2O_2$ [10]. However, HRP is an indispensable factor in the catalyzing oxidation of Amplex Red in the presence of $H_2O_2$. In addition, the maximum absorption band and fluorescence emission peak of the oxidation product of Amplex Red are extraordinarily close (absorbance at 571 and emission at 585 nm) [9,10], which may cause spectral interferences. There are some fluorescence approaches for detection of ACh based on nanoparticles such as C-dots [2] and Au/Ag nanoparticles [1]. These methods are highly sensitive and selective; however, they always suffer from potential toxicity and chemical instability.

Because of the limitations of currently available $H_2O_2$-responsive probes, many researchers are focusing their studies on boronate probes for their reaction with $H_2O_2$ [7,9,12]. So far, boronate probes, fluorescein peroxyfluor 1 (PF1) probes, and peroxyxanthone 1 (PX1) probes have been synthesized and used to detect $H_2O_2$. The interaction of $H_2O_2$ and boronate probes is ambiphilic and nucleophilic reactivity which is beneficial to hydrolytic deprotection of the boronates. In comparison with other probes taking the mechanism of nonspecific oxidation, specific interaction of $H_2O_2$ with boronates will achieve higher selectivity than other biologically relevant ROS probes [7,13].

Based on the advantages of boronate probes, a novel intramolecular charge transfer (ICT) boronate probe, 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-*N*-butyl-1,8-naphthalimide (BN) is designed and synthesized for the selective detection of ACh with significant spectral shift (455/555 nm). BN contained 1,8-naphthalimide as the fluorophore and boronic ester as the recognition unit. Because of the structure of BN, it has a high quantum yield at 76.24% (stated in Section S1 of the online supplementary material). The novel boronate probe is highly sensitive and selective toward enzyme-generated $H_2O_2$ that is produced from the enzyme catalytic reaction of ACh. When $H_2O_2$ coexists with BN under appropriate conditions, BN is transferred to a highly fluorescent product, 4-hydroxyl-*N*-butyl-1,8-naphthalimide (ON) by the hydrolytic deprotection of the boronates. The novel detection strategy is based on the fluorescence change depending on enzyme-generated $H_2O_2$, as shown in Scheme 1. This method is sensitive and does not require a complex pretreatment process or enzyme immobilization.




**Scheme 1.** Schematic representation of the sensing strategy using 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-*N*-butyl-1,8-naphthalimide to detect ACh.

## Materials and methods

### *Materials and apparatus*

Acetylcholine, amino acetic acid, alanine, dopamine, glutamic acid, proline, tryptophane, arginine, and aspartate were purchased from Wako Pure Chemical Industries (Osaka, Japan). Acetonitrile was obtained from Sinopharm Chemical Reagent. Acetylcholinesterase and choline oxidase were obtained from Sigma–Aldrich. All other chemicals used here were of analytical grade. The detection buffer was phosphate buffer solution (PBS, pH 7.5). Milli-Q ultrapure water ($\geqslant$18 MΩ cm, Millipore) was used throughout. Unless otherwise noted, solvents were purified by distillation. Human serum samples were provided by the Hospital of Hunan Normal University (Changsha, China).

A Bruker AVB-500 spectrometer and an API 4000 QTRAP LC/MS/MS System with ESI Ion Source (AB Sciex, USA) were used to characterize BN. Fourier transform infrared (FTIR) spectra were collected on a Nicolet Nexus 670 FTIR instrument (Nicolet Instrument, USA). UV–vis and fluorescence spectra were recorded on a UV-2450 spectrophotometer (Shimazu, Japan) and an F-4500 fluorescence spectrophotometer (Hitachi, Japan), respectively.

### *Synthesis of 4-bromo-N-butyl-1,8-naphthalimide*

The synthesis of 4-bromo-*N*-butyl-1,8-naphthalimide was similar to that reported by Zhengneng and coworkers [14]. 4-Bromo-1,8-naphthalic anhydride (486.4 mg, 2 mmol) and butylamine (204.8 mg, 2.8 mmol) were dissolved in 50 ml of ethanol, and the resulting solution was stirred at 80 °C for 12 h. After being cooled to room temperature, the precipitated solid was filtered and recrystallized in ethanol to give a light yellow solid (386 mg, 65%) [15].

### *Synthesis of 4-boronate-N-butyl-1,8-naphthalimide*

A nitrogen-flushed three-neck round-bottom flask was charged with 4-bromo-*N*-butyl-1,8-naphthalimide (99.7 mg, 0.3 mM), bis(pinacolato)diboron (91.4 mg, 0.36 mM), sodium acetate (73.8 mg, 0.9 mM), bis(triphenylphosphine)palladium(II) chloride (11.0 mg, 0.015 mM), CuI (6.0 mg, 0.03 mM), and triphenylphosphine (19.0 mg, 0.075 mM). 1,2-Dimethoxyethane (210 ml) was then added, and the mixture was bubbled with nitrogen for 15 min. After stirring at 60 °C for 24 h, the reaction mixture was cooled to room temperature and then poured into icewater (200 ml). It was then extracted with methylene chloride (20 ml), and the combined organic layer was dried over anhydrous magnesium sulfate. Then, the organic solvent was removed by rotary evaporation, and the residue was passed through a flash silica gel column with mineral ether as the eluent to give white crystals (73.51 mg, yield 65%) [16].

### *Fluorescent detection of ACh*

The experiments were repeated three times to ensure the accuracy of the measurements. The fluorescent measurements for the detection of ACh were carried out as follows [12]. First, 150 µl of PBS containing 3 U/ml AChE, 1 U/ml ChOx, 0.01 to 200 µM ACh, and 36.5 µM BN was incubated under gentle shaking at 37 °C for 30 min [1]. Subsequently, fluorescence spectra were recorded at