

Article

Improved Affinity: A Customized Fluorescent Probe for the Rapid Detection of Butyrylcholinesterase

Wei Wang ^{1,2}, Xiao-Fei Chen ^{1,2}, Yi Zhang ³, Yang Ran ^{1,2}, Long Jin ^{1,2}, Shuai Li ^{4,*} and Bai-Ou Guan ^{1,2,*}

¹ Guangdong Provincial Key Laboratory of Optical Fiber Sensing and Communication, Institute of Photonics Technology, Jinan University, Guangzhou 510632, China; wangwei_0415@jnu.edu.cn (W.W.); cxf@stu2021.jnu.edu.cn (X.-F.C.); tranyang@jnu.edu.cn (Y.R.); tjinlong@jnu.edu.cn (L.J.)

² College of Physics & Optoelectronic Engineering, Jinan University, Guangzhou 510632, China

³ College of Life Science and Technology, Jinan University, Guangzhou 510632, China; tzhangyi@jnu.edu.cn

⁴ Special Needs Medical Center, The Eighth Affiliated Hospital of Sun Yat-sen University, Shenzhen 518033, China

* Correspondence: lish398@mail.sysu.edu.cn (S.L.); tguanbo@jnu.edu.cn (B.-O.G.); Tel.: +86-20-37337012 (B.-O.G.)

Abstract: This article presents the distinctive butyrylcholinesterase (BChE) fluorescent probe **P5**, designed via a targeting-site method. This method was proposed to enhance the affinity of the probe for BChE by targeting the peripheral anionic site (PAS) of BChE. By mimicking the natural substrate butyrylcholine, the structure of the probe was optimized by introducing a positive charge. Fluorescent probe **P5**, selected from a series of designed fluorescent probes **P1–P6**, exhibited excellent affinity and specificity towards BChE, enabling rapid detection within 5 min with a low detection limit of 16.7 ng/mL. Furthermore, this probe can distinguish between normal cells and Alzheimer's disease (AD) model cells, and demonstrated good imaging results in a P12 cell AD model. The results of this study indicate that this novel fluorescent probe could serve as a promising tool for the rapid detection of BChE and accurate AD diagnosis.

Keywords: butyrylcholinesterase; fluorescent probe; cell imaging



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1. Introduction

Butyrylcholinesterase (BChE) is a primary human cholinesterase (ChE). BChE is produced in the liver and widely distributed in the blood, organs, and tissues [1–4]. In medical diagnostics, serum BChE activity serves as a significant indicator for evaluating liver diseases such as acute hepatitis and liver cancer [5–9]. In addition, BChE is intricately linked to Alzheimer's disease (AD) [10–13]. Recent studies have indicated that during the progression of AD, the average activity of BChE in the brain can increase to up to 120% of normal levels [11]. Moreover, in the orbitofrontal cortex of some AD patients, BChE levels can surge up to nine times higher than those in the orbitofrontal cortex of healthy individuals [14–21]. Thus, the rapid and precise detection of BChE is of great significance in the diagnosis of AD.

To date, a variety of methods have been developed to detect BChE activity, such as enzyme-linked immunosorbent assays (ELISAs) based on enzyme cascade reactions [22] and the Ellman colorimetric assay [23,24]. However, signal detection with these indirect measurement methods, which require the addition of extra substrates, is susceptible to environmental influences, potentially resulting in false positives [25,26]. On the other hand, some direct measurement methods, such as isothermal titration calorimetry [27,28], ¹⁴C-substrate labeled radiometric assays [29,30], and surface-enhanced Raman spectroscopy [31], exhibit high accuracy. Nevertheless, their complex and time-consuming operations render them unsuitable for high-throughput analysis; additionally, their high equipment costs and poor stability have impeded their further application [32]. As an

effective means of detecting BChE, fluorescent probes can compensate for the challenges of traditional detection methods, combining the advantages of simple operation, good stability, high precision, and high sensitivity [33–35]. Recently, Yang's group designed and screened BChE recognition groups based on the size of the BChE substrate-binding pocket for the first time [36,37]. The optimized fluorescent probe demonstrated excellent BChE selectivity. Furthermore, Guo's group further enhanced the selectivity of the BChE fluorescent probe by introducing chlorine atoms to increase the steric hindrance [38]. However, due to the low affinity of these probes for the enzyme, the detection time was prolonged. Thus, determining how to further enhance the affinity of the probes for BChE remains a challenge. It is noteworthy that during the binding of butyrylcholine to BChE, the peripheral anionic site (PAS) of BChE, aspartate 70 (Asp70), facilitates the rapid entry of butyrylcholine into the catalytic active site (CAS) of BChE through electrostatic interactions [39,40]. Therefore, by designing fluorescent probes based on the size of the BChE substrate-binding pocket and the anionic site Asp70, monitoring sensitivity and response time are expected to improve.

In this study, to enhance the affinity of the fluorescent probe for BChE, we systematically optimized the probe structure. By introducing a positive charge, we increased the targeted interaction between the probe and the peripheral anionic site Asp70 of BChE, thereby improving the affinity of the probe for the enzyme. The probe employs cyclopropylformyl as the recognition unit. Due to the weakening of the intramolecular charge transfer (ICT) effect by cyclopropylformyl, the fluorescence intensity of the probe itself is negligible. With the existence of BChE, the cyclopropylformyl group is removed, and the strong electron-donating ability of the phenolic hydroxyl group restores the ICT effect, leading to fluorescence enhancement. The well-designed probe **P5** maintains an excellent detection limit and selectivity, while significantly reducing the detection time, which is of paramount importance. Thus, this work holds great potential for guiding the design of future BChE probes.

2. Materials and Methods

2.1. General Steps for Spectral Measurement

All spectral measurements were carried out following the described procedure. Probes **P1–P6** and **FL** were dissolved in DMSO to prepare 1 mM stock solutions of probes **P1–P6** and **FL**, which were stored at 4 °C. An amount of 1 mg of BChE was dissolved in 1 mL of distilled water to prepare a 1 mg/mL stock solution of BChE. In a 5 mL centrifuge tube, 10 µL of the probe stock solution was diluted in 1.94 mL of PBS buffer (0.1 M, pH = 7.4) and incubated with 50 µL of the corresponding concentration of BChE at 37 °C for a specified time. Subsequently, the solution was transferred to a quartz cuvette, and both the absorbance spectra and fluorescence spectra were recorded separately.

2.2. Cytotoxicity Experiment

The cytotoxic effect of probe **P5** on PC12 cells was determined by the Cell Counting Kit-8 (CCK-8) method. PC12 cells were seeded in DMEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin, and 1% streptomycin, and maintained in a humidified incubator at 37 °C with 5% CO₂. The solid powder of probe **P5** was dissolved in dimethyl sulfoxide to prepare a 1 mM stock solution, which was sterilized by filtration through a 0.22 µm filter. The cells were then treated with various concentrations of probe **P5** (0, 0.001, 0.01, 0.1, 1, 2, 4, 5, 10 µM) for 24 h. Untreated cells were used as controls and received the equal volume of culture medium. The cells were washed three times with PBS and the supernatant was removed. An amount of 110 µL of culture medium containing 10% CCK-8 was added to each dish and incubated for 2 h. The absorbance at 450 nm was measured using a microplate reader.

2.3. Fluorescence Imaging of Probe **P5** in PC12 Cells

In the fluorescence imaging experiment, cells were seeded in a 12-dish plate at a density of 4×10^4 cells per dish and incubated for 24 h in a humidified incubator at 37 °C

and 5% CO₂. For the first group, PC12 cells were cultured with probe **P5** (1 μ M) in DMEM for 1 h, followed by washing with PBS three times before imaging. In the second group, PC12 cells were cultured in a medium containing 20 mM glutamate for 12 h to establish an AD cell model. Subsequently, the cells were incubated with probe **P5** (1 μ M) in DMEM for 1 h, washed three times with PBS, and imaged. In the third group, PC12 cells were initially cultured in a medium containing 20 mM glutamate for 12 h to establish an AD cell model. They were then incubated in a medium containing 50 μ M tacrine for 2 h. Finally, the cells were treated with probe **P5** (1 μ M) in DMEM for 1 h, washed three times with PBS, and imaged. For fluorescence confocal imaging, probe fluorescence imaging was obtained using the excitation wavelength of 520 nm and the emission wavelength of 560–600 nm (red channel). In the statistical analysis, the average fluorescence intensity of the three groups of fluorescence images was calculated by ImageJ, followed by statistical significance analysis of the average fluorescence intensity using GraphPad Prism 9.

3. Results and Discussion

3.1. Probe Design and Synthesis

To rationally design a probe, the structure and catalytic mechanism of BChE were analyzed. Previous studies have highlighted that BChE has a striking structural similarities to acetylcholinesterase (AChE), both of which are characterized by a catalytic site comprising amino acids Ser-His-Glu [40]. However, subtle differences in the chemical composition of the substrate-binding pocket between the two enzymes are manifested by the replacement of Phe295, Phe297, and Tyr124 in AChE with Leu286, Val288, and Gln119 in BChE [41–43]. This results in the substrate-binding pocket of BChE being larger than that of AChE, enabling BChE to bind larger substrates [44–46], as illustrated in Figure 1.

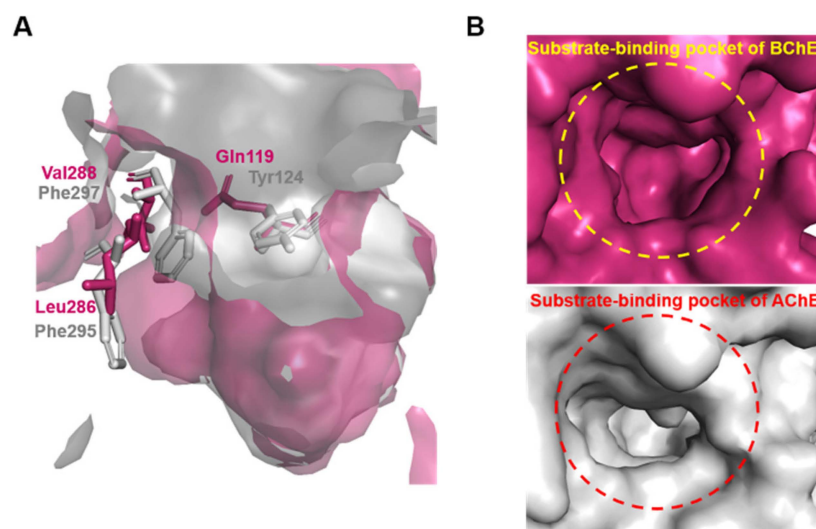


Figure 1. (A) Schematic representation of the spatial positions of key amino acids in the substrate-binding pockets of BChE and AChE. (B) Schematic representation of the simulated substrate-binding pocket space of BChE (PDB code: 1p0m) and AChE (PDB code: 4ey4).

On the other hand, the affinities of larger substrates for BChE vary, as shown by the lower affinity of uncharged nitrobenzyl butyrate for BChE compared with that of butyrylcholine [47]. This difference is attributed to the presence of the PAS predominantly composed of Asp70 surrounding the substrate-binding pocket of BChE, as shown in Figure 2A. During BChE catalysis of the natural substrate butyrylcholine, butyrylcholine initially interacts electrostatically with the amino acid Asp70 in the PAS through the nitrogen present on the choline cation. This interaction enables the rapid entry of butyrylcholine into the substrate-binding pocket for efficient binding with the enzyme's CAS [48,49]. The PAS plays a pivotal role in guiding substrate binding to the enzyme [47,50].

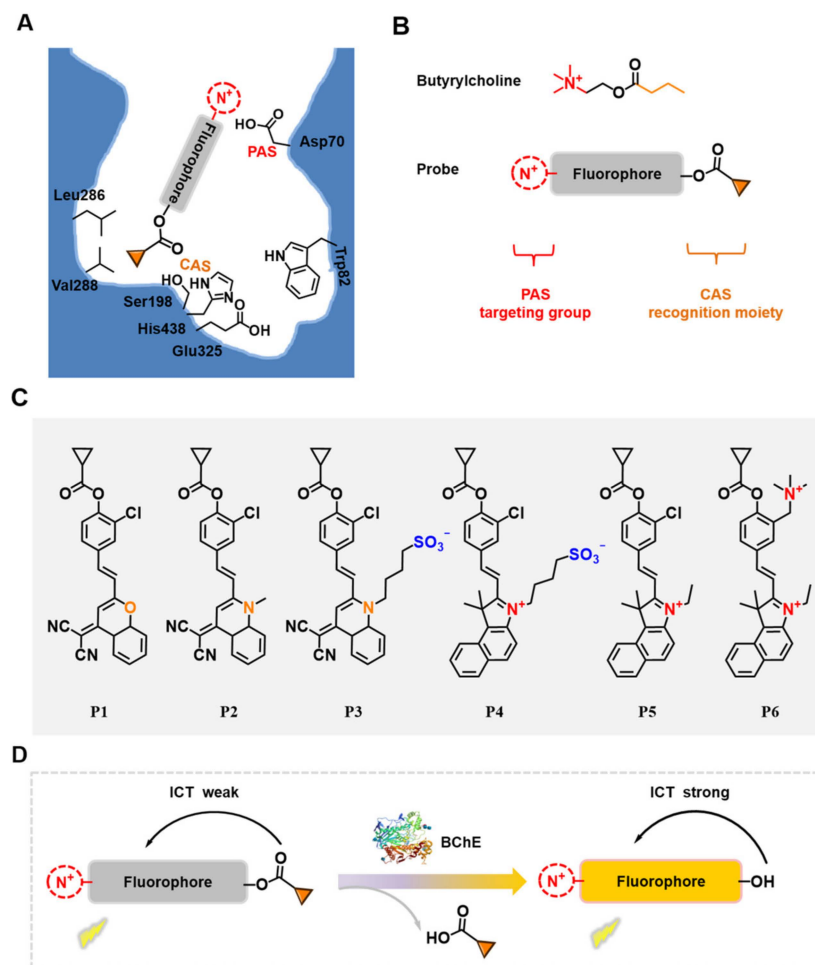


Figure 2. Design strategy of the BChE probe. (A) Schematic representation of the simulated spatial positions of the CAS and PAS in the substrate-binding pocket of BChE. (B) Comparison of the structure of butyrylcholine with the structure of the probe. (C) Chemical structures of fluorescent probes P1–P6. (D) Principle of the fluorescent response of the probe to BChE.

The size of the enzyme's substrate-binding pocket and the targeted binding of the probe to the enzyme's PAS are two crucial factors to consider when designing fluorescent probes for BChE [38]. Based on these factors, a strategy for designing a fluorescent probe targeting the PAS of BChE is proposed. The approximate spatial positions of BChE's CAS and PAS in the substrate-binding pocket are shown in Figure 2A. To target the PAS site, the chemical structure of the substrate butyrylcholine was analyzed, as shown in Figure 2B. When designing the structure of the probe, the existing CAS recognition group, cyclopropyl methyl ester, was utilized. A positive charge resembling a quaternary ammonium cation and a larger cyclopropyl methyl ester recognition group were introduced into the structure of the fluorophore to construct the fluorescent probe. A positive charge was employed to facilitate the probe's ability to target the PAS. Finally, different fluorescent probes with distinct charges were designed and synthesized by introducing diverse substituents. These probes included the neutral P1 and P2, negatively charged P3 with a sulfonic acid group, zero-net-charge P4 with indole and sulfonic acid groups, positively charged P5 with an indole group, and positively charged P6 with indole and trimethylammonium groups. The aim of this study was to explore the impact of charge on the binding of the probes to BChE. The chemical structures of the probes are depicted in Figure 2C. The fluorescent probe detection mechanism involves the ICT effect [51]. The introduction of cyclopropyl methyl ester weakens the ICT effect of the fluorophore, leading to reduced fluorescence intensity. Upon binding of the fluorescent probe to BChE, the cyclopropyl methyl ester is

hydrolyzed and detached, leading to ICT-induced fluorescence enhancement, as shown in Figure 2D. The synthesis scheme of the probe can be found in the Supporting Information. All the structures were characterized by nuclear magnetic resonance (^1H NMR and ^{13}C NMR) spectroscopy and high-resolution mass spectrometry (HRMS). The characterization data are provided in the Supporting Information.

3.2. Optical Responses of the Probes to BChE

Initially, the optical responses of several probes to BChE were investigated. Taking probe **P5** as an example (Figure 3), the maximum absorption wavelength of probe **P5** is 370 nm. Following 60 min of incubation with 50 $\mu\text{g}/\text{mL}$ BChE, the absorption wavelength of probe **P5** shifted to 520 nm, and the solution color changed from pale yellow to a visually discernible pink hue. At an excitation wavelength of 520 nm, the intensity of the fluorescence emission of probe **P5** in the absence of BChE can be disregarded ($\Phi = 0.0032$). Conversely, probe **P5** exhibited a 30-fold increase in fluorescence intensity at 584 nm ($\Phi = 0.0352$) in the presence of BChE, indicating that this enhancement in fluorescence is attributable to the fluorophore release during the reaction between probe **P5** and BChE (Figure S1). The spectral data related to the calculation of quantum yield are shown in Figure S2. The fluorescence response of the other probes to BChE is depicted in Figures 3C and S3. The fluorescence intensities of the neutral molecules probes **P1** and **P2** as well as the negatively charged **P3** remained nearly unchanged over 60 min. However, probes **P4**, **P5**, and **P6** with positive charges in their structures showed significant fluorescence enhancement for BChE. Furthermore, probe **P5** with only one net positive charge in the structure responded more strongly than probes **P4** and **P6**.

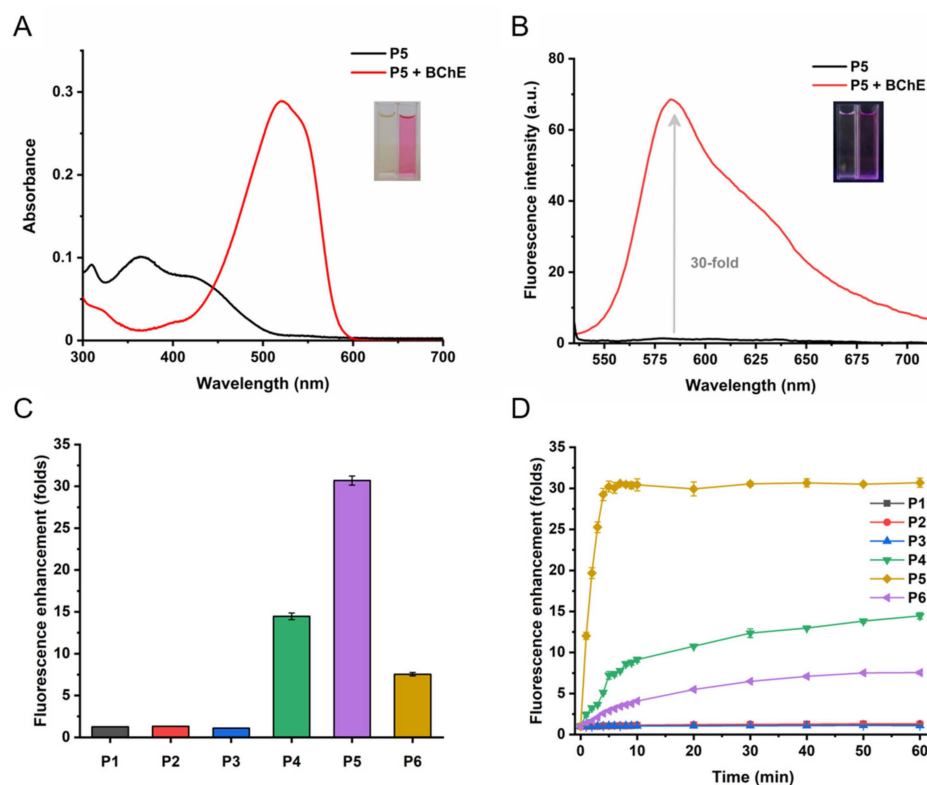


Figure 3. Absorption spectra (A) and fluorescence spectra (B) of probe **P5** (5 μM) with 50 $\mu\text{g}/\text{mL}$ BChE before (black line) and after reaction (red line) (inset: color changes in the solution before and after the reaction under white light or UV lamp (365 nm)), $\lambda_{\text{ex}} = 520$ nm. (C) Comparison of the fluorescence enhancement of probes **P1**–**P6** (5 μM) after 60 min of incubation with 50 $\mu\text{g}/\text{mL}$ BChE. (D) Fluorescence enhancement of probes **P1**–**P6** (5 μM) during the reaction with 50 $\mu\text{g}/\text{mL}$ BChE at 37 $^{\circ}\text{C}$ in 0.1 M PBS (pH 7.4) from 0 to 60 min.

The response rates of probes **P1–P6** to BChE are presented in Figure 3D. Under identical detection conditions, the detection rate of probe **P4** was slightly slower than that of probe **P5**, which may be attributable to the introduction of the negative charge in probe **P4**. The introduction of a negative charge leads to repulsion between the probe and the PAS. This influences the binding of the probe to the peripheral anionic amino acid Asp70, which has a negative charge, thus prolonging the time for the probe to access the catalytic active site of BChE [50]. At the same time, the detection rate of probe **P6** also exhibited relative deceleration compared to probe **P5**, possibly because it contains the choline cation. Previous studies have indicated that the substrate-binding pocket of BChE contains the acetylcholine binding site tryptophan 82 (Trp82), which is also known as the inner anionic site [52–54]. Trp82 can engage in cation- π interactions with the choline cation of butyrylcholine [40]. Compared with probe **P5**, probe **P6** features an additional choline cation. Although the two positive charges carried by probe **P6** may accelerate the binding of the probe to the PAS, the positively charged trimethylammonium in probe **P6** may induce cation- π interactions with Trp82 after the hydrolysis of probe **P6** by BChE [40,49]. This interaction potentially obstructs the detachment of the fluorophore from the enzyme's substrate-binding pocket, subsequently delaying the binding of the next probe to BChE and resulting in a decrease in the detection rate of probe **P6**. In summary, probe **P5** exhibits the fastest response rate to BChE, while the introduction of more positive or negative charges is unfavorable for accelerating the probe binding to BChE.

Subsequently, we explored the specificities of probes **P1–P6** for BChE and its main interferents, AChE and carboxylesterase (CE). As shown in Figure 4, probes **P1**, **P2**, and **P3** exhibited nearly no selectivity towards BChE, AChE, or CE. However, probes **P4** and **P5** could effectively differentiate between BChE and AChE, which may be ascribed to the targeting effect of the positive charge toward the anion of BChE and the weak steric hindrance effect of the adjacent chlorine atom [38]. The relatively poor selectivity of probe **P6** may be due to the sensitivity of probe **P6** to AChE anions that resulted from the introduction of more positive charges into the probe **P6** structure. Overall, probe **P5** stands out for its remarkable performance in terms of detection rate and specificity for BChE compared to probes **P1–P4** and **P6**. Within the activity range of BChE (pH = 6–8), the fluorescence response of probe **P5** remained unaffected by pH as shown in Figure S4. Additionally, the impact of temperature on the response of probe **P5** to BChE was minimal (Figure S5). As the concentration of BChE increased, the absorption spectrum of probe **P5** red-shifted from 370 nm to 520 nm, as shown in Figure S6. The isothermal point of probe **P5** before and after the reaction was about 445 nm, which indicated that probe **P5** had the potential to detect BChE through absorption spectroscopy. As seen in Figures 5A and S7, the fluorescence intensity of probe **P5** increased with the addition of BChE, and reached saturation as the BChE concentration approached 60 $\mu\text{g/mL}$. Even at lower BChE concentrations of 0–10 $\mu\text{g/mL}$, fluorescence was observed. The fluorescence intensity of probe **P5** showed a good linear relationship with the BChE concentration in the range of 0–1 $\mu\text{g/mL}$, with a detection limit of 16.7 ng/mL, as shown in Figure 5B. The linear detection range of probe **P5** was lower than the physiological concentration range, which was suitable for detecting physiological BChE. The detection limit and reaction time of probe **P5** were superior to most of the previously reported probes (Table S1). This indicated that our strategy for designing the fluorescent probe targeting the PAS of BChE can effectively enhance the detection performance of the probe. Physiologically common ions and amino acids also had minimal impacts on the fluorescence of the probe, as shown in Figure 6.

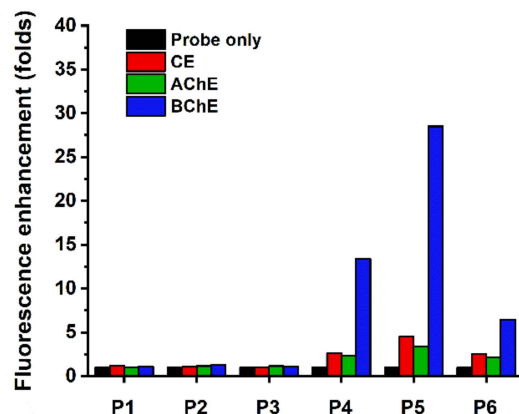


Figure 4. Enhancement of the fluorescence intensities of probes **P1–P6** (5 μ M) determined after incubation with 50 μ g/mL CE, AChE, and BChE at 37 $^{\circ}$ C in 0.1 M PBS (pH 7.4) for 1 h.

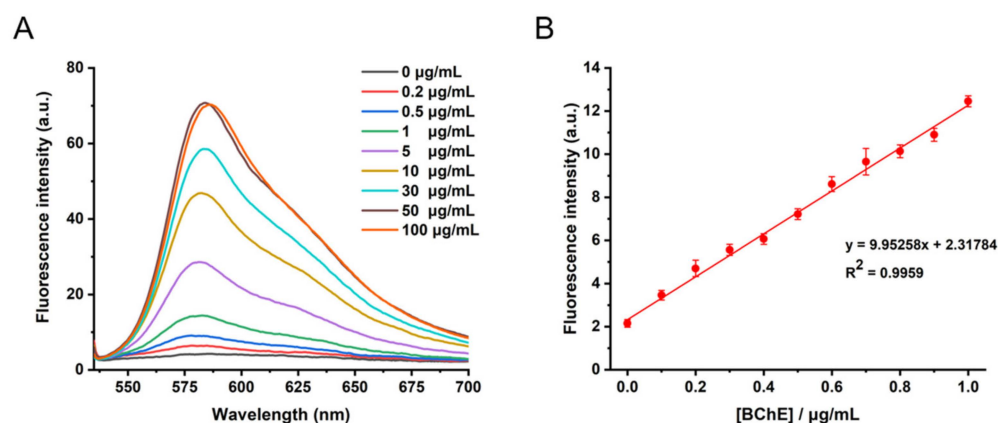


Figure 5. (A) Fluorescence spectra of probe **P5** (5 μ M) with BChE (0–100 μ g/mL) after 10 min of reaction in 0.1 M PBS (pH 7.4) at 37 $^{\circ}$ C. (B) The fitted curve of the fluorescence intensity of probe **P5** (5 μ M) at 584 nm with BChE concentrations ranging from 0 to 1 μ g/mL.

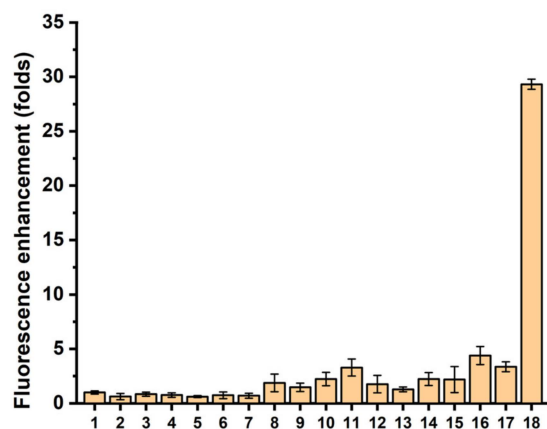


Figure 6. Probe **P5** (5 μ M) was subjected to incubation with various analytes (100 μ M) in 0.1 M PBS (pH 7.4) at 37 $^{\circ}$ C for 1 h to determine the magnitude of fluorescence enhancement: 1. **P5** only; 2. K^+ ; 3. Na^+ ; 4. Mg^{2+} ; 5. Zn^{2+} ; 6. Ca^{2+} ; 7. Fe^{3+} ; 8. Ala; 9. Phe; 10. Arg; 11. Cys; 12. Glu; 13. Tyr; 14. His; 15. Gly; 16. CE (50 μ g/mL); 17. AChE (50 μ g/mL); 18. BChE (50 μ g/mL). λ_{ex} = 520 nm.

To verify that the fluorescence signal originates from the hydrolysis of **P5** catalyzed by BChE, fluorescence titration was performed on probe **P5** with BChE treated with tacrine (a commercial BChE inhibitor). As shown in Figure S8, the fluorescence intensity of probe **P5**

treated with pre-inhibited BChE significantly decreased. The data allowed the calculation an IC_{50} constant of 52.45 nM for the efficiency of tacrine inhibition, which closely aligns with the value of 45.8 nM reported in the literature [55].

3.3. Fluorescence Imaging of Probe P5 in AD Model Cells

To evaluate the potential cytotoxicity of probe P5, the viability of PC12 cells incubated with different concentrations of probe P5 for 24 h was assessed by the CCK-8 method. As shown in Figure 7A, the significant decrease in the viability of PC12 cells at P5 concentrations exceeding 5 μ M indicated the high cytotoxicity of this dose. However, P5 exhibited lower toxicity toward PC12 cells within the range of 0–1 μ M. Moreover, the fluorescence intensity of 1 μ M probe P5 also maintained a good linear relationship with the BChE concentration in the range of 0–1 μ g/mL (Figure S9). The detection limit of 1 μ M probe P5 was 21.8 ng/mL, which was close to the previously measured value of 16.7 ng/mL. The impact of the concentration of probe P5 on the detection of BChE was minimal. Therefore, subsequent cell imaging was carried out using 1 μ M probe P5.

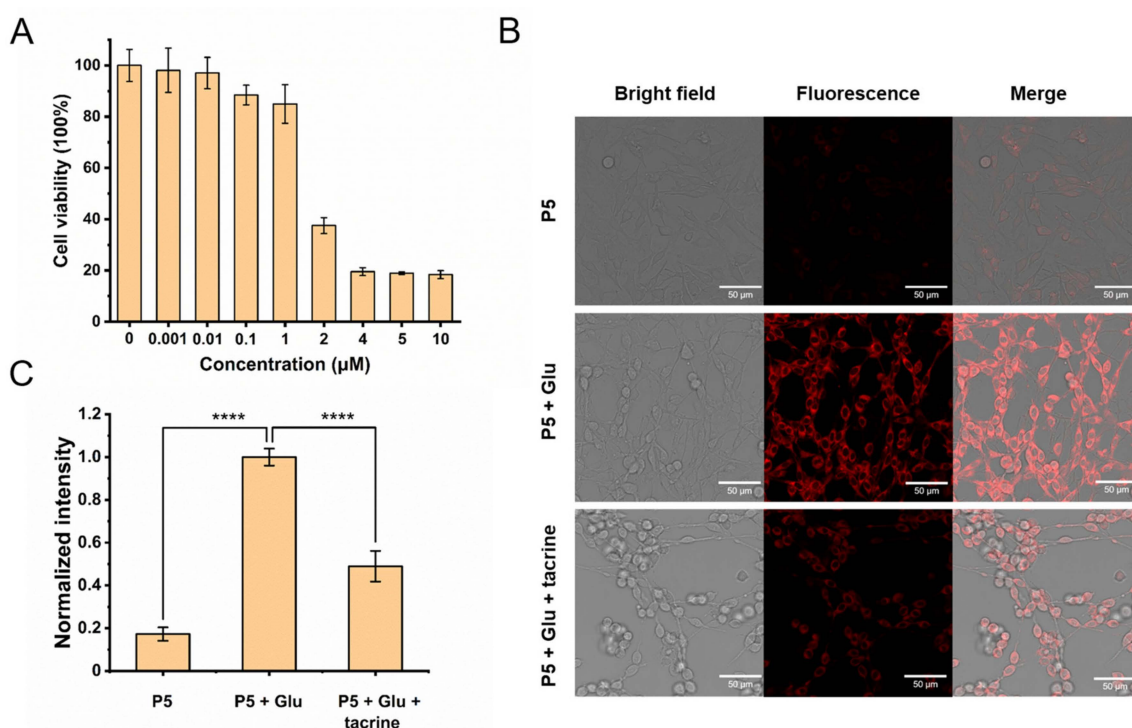


Figure 7. (A) Evaluation of the viability of PC12 cells incubated with different concentrations of probe P5 by the CCK-8 method. (B) Fluorescence images of live cells. Top row (P5): PC12 cells co-incubated with probe P5 for 1 h. Middle row (P5 + Glu): PC12 cells treated with Glu and then co-incubated with probe P5 for 1 h. Bottom row (P5 + Glu + tacrine): PC12 cells pretreated with Glu and then with tacrine, followed by coinubation with probe P5 for 1 h. $\lambda_{ex} = 520$ nm, $\lambda_{em} = 560$ –600 nm, scale bar: 50 μ m. (C) Bar graph representing the average fluorescence intensity of the indicated cells. **** Indicates a significant difference ($p < 0.0001$), $n = 4$.

To demonstrate the ability of probe P5 to rapidly detect BChE in AD, we investigated the imaging ability of P5 in AD model cells. Previous research has indicated that high concentrations of glutamate (Glu) or $A\beta_{1-42}$ can be utilized for establishing AD cell models, resulting in an increase in the level of BChE within the cells [41,56]. As depicted in Figure 7B,C, the AD model cells established by stimulating PC12 cells with glutamate (20 mM) for 12 h exhibited approximately 5-fold greater fluorescence than normal PC12 cells. This finding clearly demonstrates that P5 can differentiate between normal cells and AD model cells. Moreover, when the AD model cells treated with the BChE inhibitor

tacrine were further incubated with **P5**, the fluorescence attenuation was comparable to that of the untreated AD model cells. This indicates the efficacy of **P5** in detecting changes in the activity of BChE within AD model cells.

4. Conclusions

In conclusion, a series of small-molecule fluorescent probes, **P1–P6**, were designed and synthesized through progressive optimization of the probe structure. Specifically, the optimized probe **P5** targeted the PAS of BChE, enabling rapid BChE detection within 5 min with a low detection limit of 16.7 ng/mL. Probe **P5** also demonstrated high selectivity toward BChE, along with excellent water solubility and pH and temperature stability. Satisfyingly, probe **P5** was successfully applied to image BChE in AD model cells, indicating its great potential for future AD diagnosis. In summary, our current work demonstrates a high-affinity-based method for optimizing the structure of BChE fluorescent probes, which holds great importance for the subsequent development of BChE fluorescent probes.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/chemosensors12060100/s1>, Figure S1: (A) The chemical structural changes of probe **P5** before and after response with BChE. Following the incubation of probe **P5** (5 μ M) with BChE (50 μ g/mL) reaction at 37 $^{\circ}$ C in 0.1 M PBS buffer (pH = 7.4) for 10 min, absorption spectra (B) and fluorescence spectra (C) of the reaction mixture (red line) were compared with the fluorophore **FL** (blue line). λ_{ex} = 520 nm; Figure S2: Absorption spectra (A) and fluorescence spectra (B) of only probe **P5** (gray line; 5 μ M), probe **P5** (5 μ M) after reacting with 50 μ g/mL BChE (red line), and Rhodamine B (black line; 5 μ M); Figure S3: Fluorescent response of probes **P1–P6** (5 μ M) and BChE (50 μ g/mL) within 60 min in 0.1 M PBS buffer (pH = 7.4) at 37 $^{\circ}$ C. (A) Probe **P1**, (B) Probe **P2**, (C) Probe **P3**, (D) Probe **P4**, (E) Probe **P5**, (F) Probe **P6**; Figure S4: Fluorescence intensity at 584 nm of the reaction between probe **P5** (5 μ M) and BChE (50 μ g/mL) in 0.1 M PBS buffer at pH 4.5–9, before and after a 10-min incubation at 37 $^{\circ}$ C. λ_{ex} = 520 nm; Figure S5: Fluorescence intensity at 584 nm of the reaction between probe **P5** (5 μ M) and BChE (50 μ g/mL) in 0.1 M PBS buffer at pH 7.4, before and after a 10-min incubation at temperatures of 20–40 $^{\circ}$ C. λ_{ex} = 520 nm; Figure S6: Absorption spectra of probe **P5** (5 μ M) with BChE (0–100 μ g/mL) after 10 min of reaction in 0.1 M PBS (pH 7.4) at 37 $^{\circ}$ C; Figure S7: Fluorescence intensity curve at 584 nm of the reaction between probe **P5** (5 μ M) and BChE (0–100 μ g/mL) after the 10-min reaction in 0.1 M PBS buffer (pH 7.4) at 37 $^{\circ}$ C; Figure S8: (A) Inhibition efficiency curve of tacrine towards BChE, with an IC_{50} value of 52.45 nM. (B) Fluorescence spectra of the probe **P5** incubated with BChE (50 μ g/mL) pre-treated with different concentrations of tacrine (0–200 nM) at 37 $^{\circ}$ C in 0.1 M PBS buffer (pH = 7.4) for 10 min; Figure S9: (A) Fluorescence spectra of probe **P5** (1 μ M) with BChE (0–1 μ g/mL) after 10 min of reaction in 0.1 M PBS (pH 7.4) at 37 $^{\circ}$ C. (B) The fitted curve of the fluorescence intensity of probe **P5** (1 μ M) at 584 nm with BChE min concentrations ranging from 0 to 1 μ g/mL; Table S1: Reported fluorescence probes for BChE; Scheme S1: Synthetic route for probe **P1**; Scheme S2: Synthetic route for probe **P2**; Scheme S3: Synthetic route for probe **P3**; Scheme S4: Synthetic route for probe **P4**; Scheme S5: Synthetic route for probe **P5**; Scheme S6: Synthetic route for probe **P6**; Figure S10: 1H NMR spectra of compound **2**; Figure S11: ^{13}C NMR spectra of compound **2**; Figure S12: HRMS spectra of compound **2**; Figure S13: 1H NMR spectra of compound **P1**; Figure S14: ^{13}C NMR spectra of compound **P1**; Figure S15: HRMS spectra of compound **P1**; Figure S16: 1H NMR spectra of compound **4**; Figure S17: ^{13}C NMR spectra of compound **4**; Figure S18: HRMS spectra of compound **4**; Figure S19: 1H NMR spectra of compound **P2**; Figure S20: ^{13}C NMR spectra of compound **P2**; Figure S21: HRMS spectra of compound **P2**; Figure S22: 1H NMR spectra of compound **6**; Figure S23: ^{13}C NMR spectra of compound **6**; Figure S24: HRMS spectra of compound **6**; Figure S25: 1H NMR spectra of compound **P3**; Figure S26: ^{13}C NMR spectra of compound **P3**; Figure S27: HRMS spectra of compound **P3**; Figure S28: 1H NMR spectra of compound **8**; Figure S29: ^{13}C NMR spectra of compound **8**; Figure S30: HRMS spectra of compound **8**; Figure S31: 1H NMR spectra of compound **P4**; Figure S32: ^{13}C NMR spectra of compound **P4**; Figure S33: HRMS spectra of compound **P4**; Figure S34: 1H NMR spectra of compound **FL**; Figure S35: ^{13}C NMR spectra of compound **FL**; Figure S36: HRMS spectra of compound **FL**; Figure S37: 1H NMR spectra of compound **P5**; Figure S38: ^{13}C NMR spectra of compound **P5**; Figure S39: HRMS spectra of compound **P5**; Figure S40: 1H NMR spectra of compound **11**; Figure S41: ^{13}C NMR spectra of compound **11**; Figure S42: HRMS spectra of compound **11**; Figure S43: 1H NMR spectra of compound

P6; Figure S44: ^{13}C NMR spectra of compound **P6**; Figure S45: HRMS spectra of compound **P6**. References [33,36–38,57–72] are cited in the supplementary materials.

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