DOI: 10.1002/chem.201300980

Specifically Monitoring Butyrylcholinesterase by Supramolecular Tandem Assay

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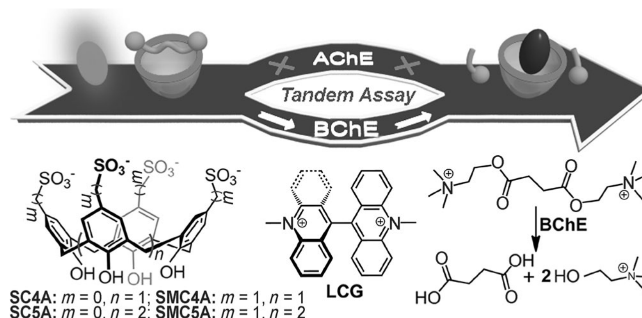
Cholinesterases (ChEs) are a family of serine enzymes that show three distinct activities: esterase, aryl acylamidase, and peptidase.[1] In all vertebrate species, two types of ChEs (with >65% homology), that is, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), ubiquitously coexist throughout the body.[2] AChE has a vital function in the termination of synaptic transmission by hydrolysis of the neurotransmitter acetylcholine, whereas BChE has previously been underestimated, because its "real" substrate(s) is still unknown.[3] New findings have shown that, significantly, BChE not only acts as a detoxification enzyme to scavenge anticholinesterase compounds, but also as an activator enzyme that converts prodrugs into their active forms.[4] Moreover, in Alzheimer's disease, BChE activity has been shown to increase, whereas AChE activity decreases, thus affording the potential for BChE activity to be used as a diagnostic biomarker of disease progression or as a target for future therapies.[5]

To date, ChE-activity assays and their corresponding inhibitor screenings have been successfully achieved by creating various sensing ensembles with either colorimetric or fluorometric outputs. Optical techniques allow for continuous and rapid assays, owing to their easily implemented procedures. The assays for ChEs can be classified into three types, according to their analytical strategies: 1) quantification of the thiols that are released by alkanoylthiocholines (pseudosubstrates) through enzymatic reactions;[6] 2) the detection of H_2O_2, which is produced by enzyme-involved cascade reactions;[7] and 3) enzyme-responsive self-assembly processes.[8] More recently, we collaborated with Nau and co-workers, who developed supramolecular tandem assays (STA)[9] and put forward a conceptually new substrate-selective enzyme-coupled tandem assay for AChE and the screening of inhibitors.[10] However, most current assays solely focus on AChE and, more notably, these reported approaches are almost helpless in conveniently discriminating between AChE and BChE by outputting qualitatively differentiated signals.[11] On account of the increasing biological and pharmacological significance of BChE, it is highly appealing to develop a sensitive, convenient, and continuous method for the specific assaying of BChE activity.

Herein, we report a robust and facile approach for the real-time, continuous monitoring of BChE activity and for the screening of inhibitors by using the STA principle. STA is defined as a new approach towards enzyme assaying that relies on the differential binding of a macrocyclic host to a fluorescent dye, the enzymatic substrate, and the corresponding product,[9e] thus representing a direct application of competitive binding titrations.[12] With the aim of setting up a STA for BChE, we employed succinylcholine (SuCh), a clinical neuromuscular relaxant, as a substrate that could be specifically degraded by BChE but not by AChE[13] and calixarene•lucigenin as the reporter pair. The calixarene hosts present the prerequisite differential affinities to SuCh and its enzymatic product, choline (Ch). Lucigenin (LCG) serves as a competitive dye that shows a high quantum yield, strong affinity to the calixarene, and a good corresponding fluorescence response.[10,14] Therefore, such a combinational system achieves the direct monitoring of BChE without the obstacle of AChE (Scheme 1).



Scheme 1. Schematic representation of a supramolecular tandem assay based on the calixarene•LCG reporter pair and their corresponding chemical structures. A substrate-selective assay for BChE was achieved with a "switch-off" fluorescence response.

Four kinds of sulfonatocalixarenes, that is, two *para*-sulfonatocalix[4,5]arenes (SC4A and SC5A) and two *para*-sulfonatomethylcalix[4,5]arenes (SMC4A and SMC5A), were preselected as candidate hosts, owing to their binding preference towards organic cations.[15] These four hosts all bind LCG with high stability constants (K_S, Table 1), thereby heavily quenching the fluorescence (see the Supporting

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.201300980.

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Table 1. Binding constants (K_S, $\times 10^5\,\text{M}^{-1}$) of calixarenes with the LCG dye, SuCh substrate, and Ch product in 10 mM phosphate buffer at pH 8.0.

Calixarene	LCG[a]	SuCh[b]	Ch[b]	Selectivity[c]
SC4A	92[d]	10.7±1.0	1.00±0.06	11:1
SC5A	53.4±2.6	2.54±0.28	0.14±0.01	18:1
SMC4A	2.20±0.03	1.51±0.37	0.13±0.01	12:1
SMC5A	16.1±0.3	1.83±0.15	0.13±0.02	15:1

[a] Determined from fluorescence titrations by assuming a 1:1 stoichiometry. [b] Determined from competitive titrations by assuming a 1:1 stoichiometry. The corresponding binding-constant fits are given in the Supporting Information. [c] Selectivity of the substrate/product. [d] Data are taken from the Supporting Information in Ref. [10].

Information, Figure S1), which is highly desirable for the reporter pair of STA from a sensitivity and economic, as well as an interference point of view, in particular for potential applications in high-throughput screening for drug discovery.[14] Next, we measured the K_S values of these calixarenes with SuCh (substrate) and Ch (product) by performing competitive binding titrations (Table 1).[16] The macrocyclic hosts play a satisfactory role in differentiating between SuCh and Ch. A factor of 10 difference between the selectivities of the substrate and the product is generally sufficient for ensuring a sizable fluorescence response through the tandem assay working principle.[9c] In the structure of SuCh, there are two identical quaternary ammonium groups, which are expected to form a 2:1 host/guest complex. However, the titration data could be well-fitted by computer simulation to a 1:1 model. Such a 1:1 binding stoichiometry was further validated by NMR experiments (Figure 1). This result is because the ditopic SuCh is not large enough to span two calixarene cavities.[17]

In the following assay of BChE activity, we mainly focused on the SC4A•LCG reporter pair as a prototype of the calixarene•LCG complexes. This pair outperformed the others in several respects: First, SC4A showed the highest binding constants for LCG, SuCh, and Ch and, hence, the employed host, dye, and substrate concentrations could be lower. Second, both SC4A and LCG were commercially available and could be readily employed in future sensing applications. Third, SC4A has been demonstrated to only show low cellular and in vivo toxicity, rapidity of clearance, and almost no metabolism,[18] which pave the way for its potential biological application.

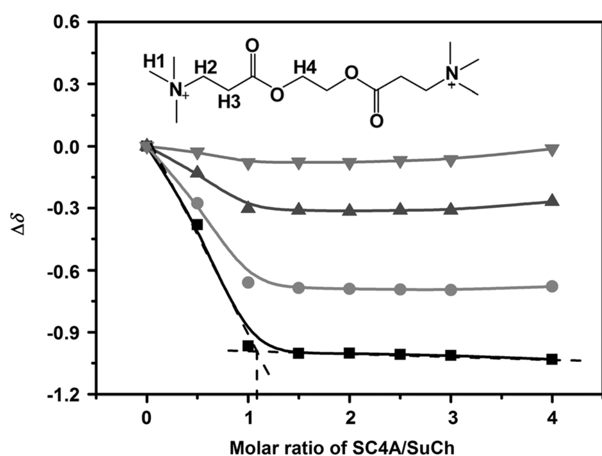

Figure 1. Plots of the $\Delta\delta$ values (in ppm) of the protons of SuCh (2.0 mM) versus [SC4A] in D_2O at 298.15 K (400 MHz). ■=H1; ●=H2; ▲=H3; and ▼=H4.

Figure 2 shows that the enzymatic conversion of SuCh (substrate) into Ch (product) can be easily monitored by using the SC4A•LCG reporter pair because this substrate/product pair shows a good fluorescence differentiation (see the Supporting Information, Figure S7). The competitive titration results allow us to implement convenient tandem assays for BChE. Notably, the proposed tandem assay for BChE needs to be qualified as a substrate-selective one,[9c] because SuCh acts as a strong competitor, whereas choline is a weak one. Therefore, a "switch-off" fluorescence response was expected when the assay was performed at a fixed concentration of substrate (e.g., 10 μM; Figure 2, arrow).

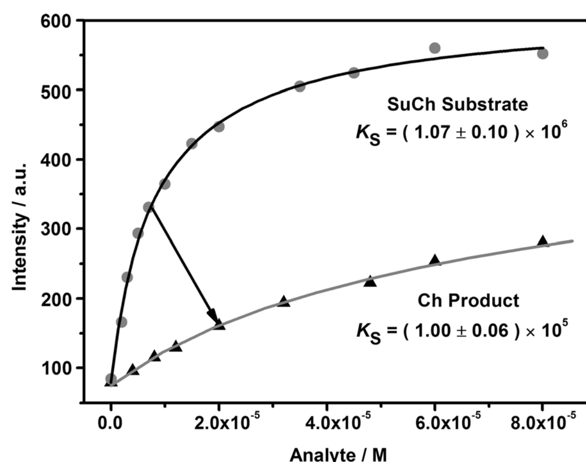

Figure 2. Competitive fluorescence titrations of SuCh and its enzymatic product (Ch) in the presence of LCG (0.5 μM) and SC4A (0.9 μM) in 10 mM phosphate buffer at pH 8.0 (λ_{ex}=360 nm, λ_{em}=504 nm). The binding constant of SC4A with SuCh was also measured by using ITC, thus giving a similar K_S value of $9.78\times10^5\,\text{M}^{-1}$ (see the Supporting Information, Figure S6).

In fact, the BChE assay worked exactly as projected (Figure 3a) and showed the expected dependence of the enzyme kinetics on the substrate concentration (the concentration of BChE was chosen to afford conversion within 20 min; Figure 3b). A tentative fitting of the data according to the Michaelis–Menten model gave a K_M value of (4.1±0.8) μM (Figure 3c), which agreed well with the literature value.[19] This result suggests that this tandem assay may, despite its complexity, even be suitable for the determination of enzyme kinetics. As shown in Figure 3b, the final plateau region also depends on the substrate concentration (0.4–5 μM) because larger amounts of the more strongly binding

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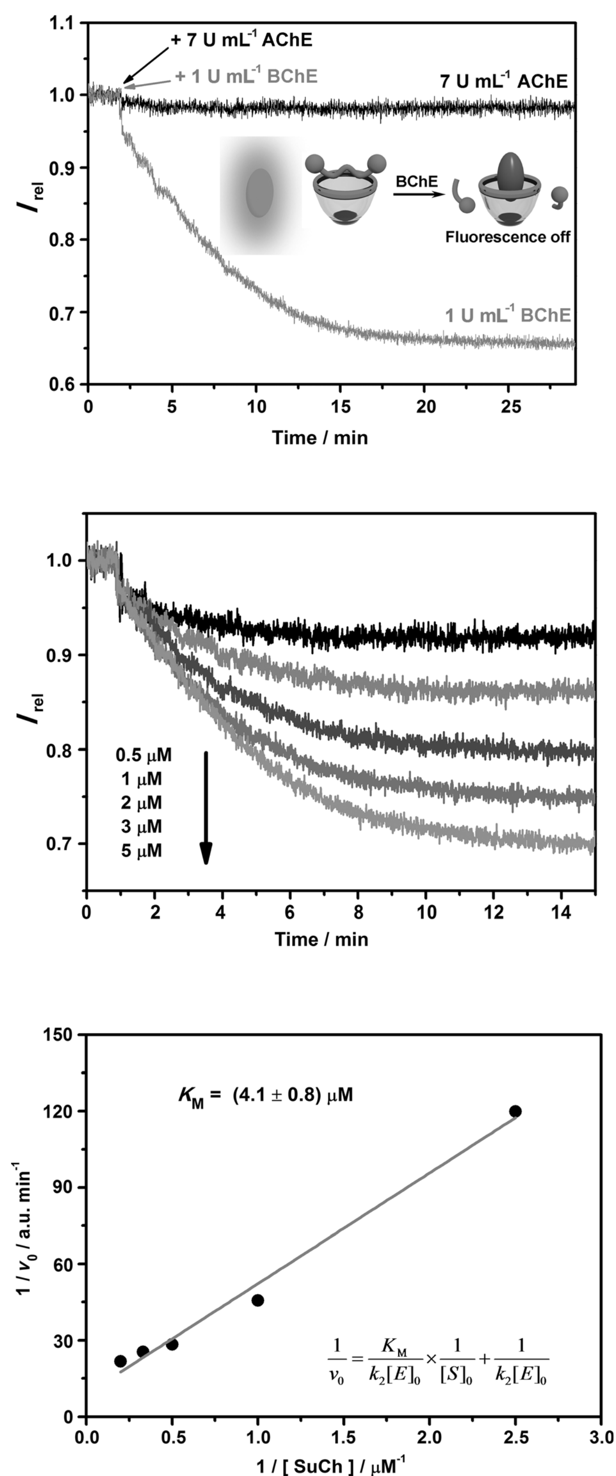

Figure 3. a) Continuous fluorescent enzyme assays for AChE (7 U mL^{-1}) and BChE (1 U mL^{-1}) with the SC4A•LCG reporter pair (10 μM SuCh, 0.5 μM LCG, and 0.5 μM SC4A in 10 mM phosphate buffer at pH 8.0; λ_{ex} = 360 nm, λ_{em} = 504 nm). b) Determination of the enzyme kinetic parameter (K_M) by monitoring BChE activity with various concentrations of SuCh (0.4–5 μM). c) Lineweaver–Burk plot for BChE.

substrate are capable of displacing a larger function of the dye at the outset of the enzymatic reaction. BChE degrades SuCh in two stages: First into succinylmonocholine, then, with a further loss of a Ch molecule, into succinic acid (no competitive binding of SC4A).[3a] Gratifyingly, the largest fluorescence decrease, which originated from the enzymatic reaction, was in good agreement with the fluorescence differentiation between SuCh and Ch, thus indicating that all of the SuCh substrate had been thoroughly conversed into Ch. The complete conversion of SuCh into Ch was further identified by mass spectrometry (see the Supporting Information, Figure S9).

It was also necessary to determine whether the homogeneous AChE affected this assay of BChE activity. Thus, we performed control experiments to show that the SC4A•LCG reporter pair gave no fluorescence response upon the addition of a seven-fold excess of AChE in the presence of SuCh (Figure 3a), because AChE did not hydrolyze SuCh.[13] More excitingly, there was almost no effect on the monitoring of BChE, even in the presence of excess AChE (see the Supporting Information, Figure S8). A seven-fold excess of AChE was employed because it was above the normal ratio of AChE/BChE in vivo.[3b] Undoubtedly, we have established an appealing STA principle for the specific detecting of BChE. We also confirmed that the presence of the enzyme itself did not affect the fluorescence of the SC4A•LCG reporter pair and that no fluorescence response was obtained from SuCh in the absence of BChE (a response could occur if hydrolysis occurred otherwise).

To demonstrate the applicability of this method to the screening of BChE inhibitors, which is critical for the evaluation of drug candidates, we performed inhibition measurements with Tacrine (an approved Alzheimer's drug) as a reversible competitive inhibitor. We observed that, as is typical for competitive inhibitors, the addition of increasing amounts of the additives led to an efficient suppression of BChE activity, as reflected in a steep decrease in the initial reaction rates (Figure 4). The IC_{50} value was calculated to be (10 ± 1) nM, which agreed well with the literature value.[20]

In summary, by taking advantage of the reaction specificity of BChE with SuCh and the binding selectivity of calixarene towards the SuCh substrate and the Ch product, we have successfully implemented the STA principle for the real-time, continuous, direct, and label-free monitoring of BChE activity through a fluorescence "switch-off" assay. This assay can facilely discriminate between BChE and AChE and the monitoring of BChE activity remains almost unaffected, even in the presence of excess AChE. Furthermore, we have demonstrated the potential of this assay for the screening of inhibitors. In view of the increasing significance of BChE, the application of tandem assays to selectively monitor BChE activity has feasible implications in disease diagnosis and drug screening, in which BChE is an important disease marker.

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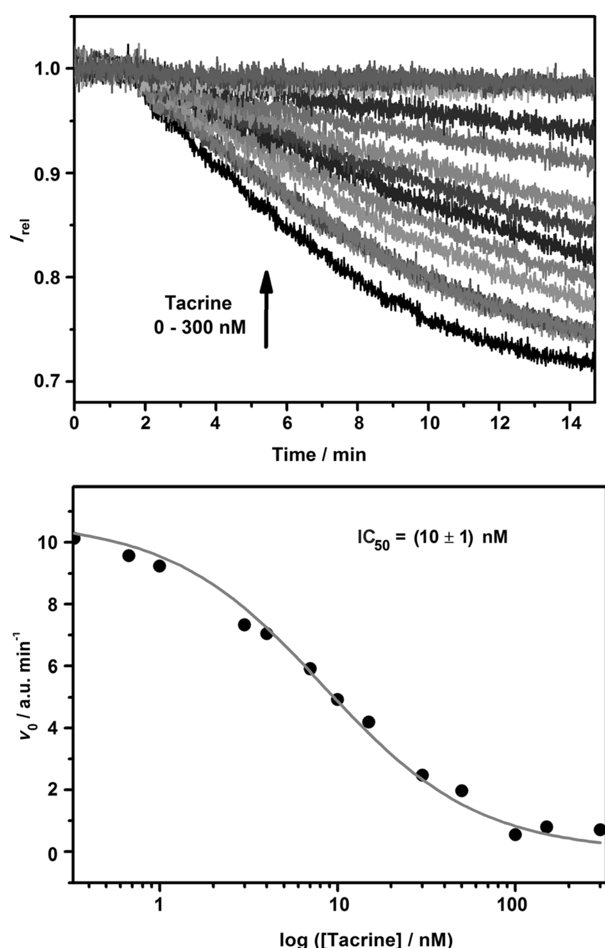

Figure 4. a) Continuous fluorescent enzyme assay for BChE inhibition by Tacrine (0–300 μM) with the SC4A•LCG reporter pair (1 U mL^{-1} enzyme, 10 μM SuCh, 0.5 μM LCG, and 0.5 μM SC4A in 10 mM phosphate buffer at pH 8.0; λ_{ex} = 360 nm, λ_{em} = 504 nm). b) Dose-response curve and associated plot analysis for BChE inhibition by Tacrine.

Experimental Section

Materials: The four host compounds, that is, *para*-sulfonatocalix[4]arene tetrasodium (SC4A),[21] *para*-sulfonatocalix[5]arene pentasodium (SC5A),[22] *para*-sulfonatomethylcalix[4]arene tetrasodium (SMC4A), and *para*-sulfonatomethylcalix[5]arene pentasodium (SMC5A),[23] were synthesized and purified according to literature procedures. Lucigenin nitrate (LCG) and succinylcholine chloride (SuCh) were purchased from TCI and choline chloride (Ch) was purchased from Acros. For the enzymatic assays, acetylcholinesterase (AChE, from human erythrocytes, 2712.50 U mg^{-1}), butyrylcholinesterase (BChE, from equine serum, 246 U mg^{-1}) and Tacrine (9-amino-1,2,3,4-tetra-hydroacridine hydrochloride hydrate) were purchased from Sigma–Aldrich. All of these compounds were used without further purification. The phosphate buffer solution (pH 8.0) was prepared by dissolving sodium dihydrogen phosphate in distilled deionized water to make a 10 mM stock solution, which was then adjusted to pH 8.0 by the addition of NaOH. The reagent solutions were freshly prepared daily. The pH values of the buffer solutions were verified by using a pH meter that was calibrated with two standard buffer solutions.

NMR spectroscopy: ^{1}H NMR spectra were recorded on a Brucker AV400 spectrometer in D_2O at 298.15 K by using 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as an external standard. For the NMR titrations, the concentration of the guest species was kept constant while the concentration of the host species was varied. The SC4A host and the SuCh guest were mixed in molar ratios of about 0.5–4:1, with a concentration of SuCh of 2.0 mM. Solutions for the ^{1}H NMR titrations were prepared by mixing 4.0 mM solutions of the guests in D_2O (250 μL) with appropriate quantities of 16.0 mM solutions of SC4A in D_2O in the NMR tubes and then diluted with D_2O to 500 μL.

Optical spectroscopy: The steady-state fluorescence spectra were recorded on a Varian Cary Eclipse spectrometer that was equipped with a Varian Cary single-cell Peltier accessory. Fluorescence titrations between the calixarenes and LCG were performed at ambient temperature (25 °C). The 1:1 fitting equations that were used to analyze the binding titrations are discussed below. The competitive fluorescence titrations were performed by the successive addition of known amounts of a competitor to solutions that contained the calixarene and LCG and following the induced fluorescence intensity within the spectroscopic area of the largest variation. Care was taken to keep the concentrations of the calixarene and LCG constant during the course of the titrations. Enzyme assays were performed in 4.0 mL quartz cuvettes and the fluorescence was followed in the time-scan mode. The BChE (or AChE) assays with the SC4A•LCG reporter pair were performed in solutions that contained 0.5 μM LCG and 0.5 μM SC4A, 0.4–5 μM SuCh, and 1 U mL^{-1} BChE in 10 mM sodium phosphate buffer, pH 8.0, 37 °C (λ_{ex} = 360 nm, λ_{em} = 504 nm).

Acknowledgements

We thank the 973 Program (2011CB932502) and the NSFC (20932004 and 21172119) for financial support. We also thank Prof. Werner M. Nau and Dr. Garima Ghale (Jacobs University, Bremen) for their help in fitting the competitive titrations.

Keywords: calixarenes • cholinesterases • enzymes • inhibitors • tandem assay

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Received: March 14, 2013
Published online: May 27, 2013

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