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A Fluorescence "Turn-On" Ensemble for Acetylcholinesterase Activity Assay and Inhibitor Screening

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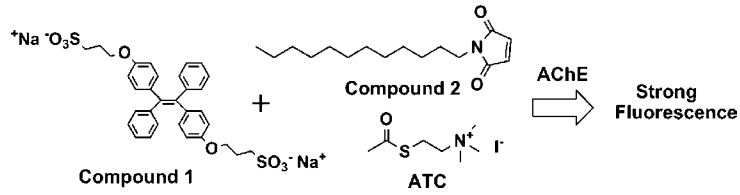
ABSTRACT



By making use of the aggregation-induced emission feature of compound 1 and the cascade reactions among acetylthiocholine iodide (ATC), AChE, and compound 2, a new fluorescence "turn-on" method is developed for AChE assay and inhibitor-screening.

Alzheimer's disease (AD), a neurodegenerative disease characterized by a low concentration of acetylcholine (ACh) in the hippocampus and cortex,[1] is the leading cause of dementia among older people. Hydrolysis of acetylcholine (ACh) by acetylcholinesterase (AChE) is a key process for the regulation of the neutral response system. The "cholinergic hypothesis of AD" has became the neurobiologic incentive for AD treatment. Clinical treatment of AD is mainly based on the AChE inhibitors. Therefore, the development of a reliable assay method for AChE activity and its inhibitor screening is of great importance. Additionally, nerve gases and pesticides of organophosphorus and carbamate types are inhibitors of AChE;[2,3] thus, an efficient assay method for AChE activity will be useful for detection of these nerve gases and pesticides.[4,5]

AChE activity and inhibition are traditionally monitored with absorption spectroscopy (e.g., with the Ellman's reagent), but such assay methods show low sensitivity.[6] Meanwhile, fluorometric approaches with good sensitivity have been reported for screening of AChE inhibitors.[7,8] Chemiluminescent probes have also been reported for AChE activity assay and inhibitor screening.[9] AChE activity assay and the corresponding inhibitor-screening have been successfully achieved by making use of enzyme-involved cascade reactions in which AChE assay can be converted to the detection of H_2O_2.[10,11] However, drawbacks are found for these assay methods. For instance, the use of Ellman's reagent may result in a false-positive effect, and some of these assay methods are discontinuous and time-consuming

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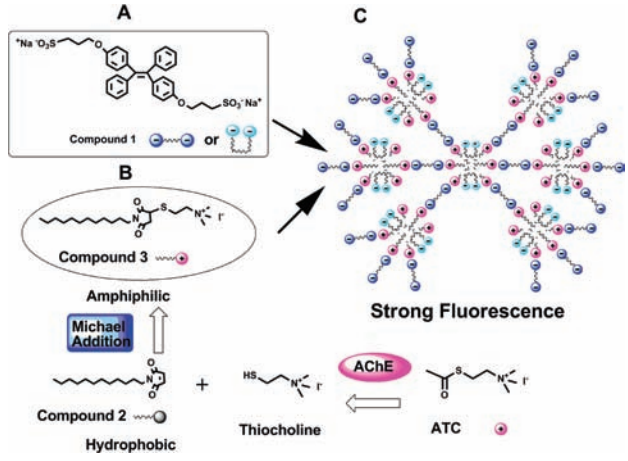
for AChE activity and inhibition studies.[9,12] Therefore, convenient and continuous methods for AChE activity assay and the inhibitor screening are still highly desired.

We have very recently described a convenient and continuous fluorometric assay method for AChE and inhibitor screening based on the ensemble of compound **1** (Scheme 1) with two sulfonate ($-SO_3^-$) units and myristoylcholine by making use of the aggregation-induced emission (AIE) feature of tetraphenyl ethylene (TPE).[13] This assay method is based on the following facts: (1) in the presence of myristoylcholine, an amphiphilic compound with one ammonium group, aggregation of compound **1** occurred leading to strong fluorescence; (2) myristoylcholine can easily be hydrolyzed into myristic acid and choline in the presence of AChE. Accordingly, the aggregation complex of compound **1** and myristoylcholine formed in aqueous solution would be disassembled because of the Coulombic repulsive interaction between **1** and the hydrolysis product of myristoylcholine. As a result, the fluorescence of the ensemble decreases.

Scheme 1. (A) Chemical Structures of Compounds **1**−**3**. (B) Cascade Reactions among ATC, AChE, and Compound **2**. (C) Illustration of the Aggregation of Compound **1** in the Presence of Compound **3**



In this paper, we want to report an alternative fluorescence turn-on assay method for AChE activity and the inhibitor-screening based on the ensemble of compound **1** and compound **2** (Scheme 1). The design rationale is illustrated in Scheme 1 and explained as follows: (1) compound **2** is a hydrophobic compound with a maleimide group; (2) acetylthiocholine iodide (ATC) is a good substrate of AChE; i.e., ATC can be hydrolyzed into thiocholine in the presence of AChE. Michael reaction of thiocholine with the maleimide group in **2** would lead to the formation of an amphiphilic compound **3**; (3) such amphiphilic compound would induce the aggregation of compound **1**, and accordingly the fluorescence of the ensemble would increase significantly; (4) in the presence of the corresponding inhibitor the hydrolysis of acetylthiocholine would be retarded, and as a result, a smaller amount of the amphiphilic compound would be generated. Consequently, the fluorescence enhancement for the ensemble would be reduced. Therefore, the ensemble of compounds **1** and **2** can be used for the AChE activity assay and the inhibitor-screening by making use of the AIE feature of TPE compounds.

Compound **1** was prepared according to a previous report.[14] Compound **2** was obtained simply by the reaction of maleic anhydride and dodectylamine, and compound **3** was obtained by further reaction of compound **2** with thiocholine (see the Supporting Information). First, we discuss the fluorescence enhancement of compound **1** in the presence of compound **3** (Scheme 1), the Michael adduct of compound **2** with thiocholine. As anticipated, compound **1** [20 μM in HEPES (10 mM) buffer solution, pH = 7.35] showed rather weak fluorescence. After addition of compound **3**, the fluorescence of compound **1** increased greatly. For instance, the fluorescence intensity at 490 nm for the aqueous solution of compound **1** (20 μM) was enhanced by 45.5 times when the concentration of compound **3** in the ensemble reached 24 μM. In fact, the fluorescence quantum yield of the solution of **1** (20 μM) increased from 0.0090 to 0.064 (by reference to quinine hemisulfate monohydrate) after introduction of compound **3** (24 μM) to the solution. Interestingly, the fluorescence intensity of the ensemble solution of compounds **1** and **3** increased almost linearly with the concentration of compound **3** in the range of 0−20 μM as displayed in the inset of Figure 1.

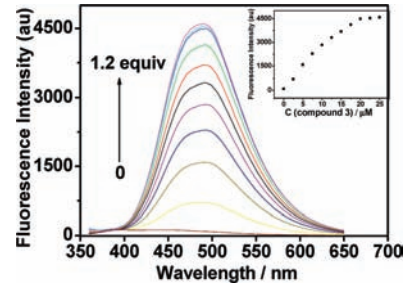


Figure 1. Fluorescence spectra of the ensemble of compound **1** [20 μM in HEPES (10 mM) buffer solution, pH = 7.35] in the presence of different concentrations of compound **3** (from 0 to 1.2 equiv); inset shows the plot of fluorescence intensity of compound **1** at 490 nm vs the concentration of compound **3**.

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According to previous studies, such fluorescence enhancement observed for compound **1** should be ascribed to the aggregation of compound **1** in the presence of compound **3**. The dynamic light scattering (DLS) and surface tension experimental results support this conclusion. The DLS signal shown in Figure S1 (Supporting Information) indicates the formation of aggregates with sizes around 460 nm in the ensemble solution of compounds **1** (20 μM) and **3** (30 μM).[15] The variation of the surface tension vs the concentrations of compounds **1** and **3** (see Figure S2, Supporting Information) implies that there is a phase-transition when the concentrations of **1** and **3** are 15 and 22 μM, respectively. These results clearly indicate the aggregation of compound **1** in the presence of compound **3**. The aggregation of compound **1** in the presence of compound **3** may occur via the following mechanism: molecules of **1** and **3** may form comicelle, in which the hydrophobic moieties of **1** and **3** (TPE framework and alkyl chain) may stack to generate the core and their hydrophilic parts (sulfonate and ammonium) may form the corona. These comicelles may be further interconnected by molecules of compound **1** due to electrostatic interaction to form large aggregates as illustrated in Scheme 1.

We now demonstrate the application of the ensemble of compounds **1**, **2**, and acetylthiocholine (ATC) for AChE activity assay and the inhibitor screening. A clear solution of **1** (20 μM), **2** (30 μM), and ATC (30 μM) in mixture of HEPES buffer (10 mM) and THF (v/v, 1000/3) was prepared for the following spectral studies. Initially, the ensemble solution of compounds **1**, **2**, and acetylthiocholine (ATC) exhibited rather weak fluorescence as shown in Figure 2. After addition of AChE (0.1 U/mL), the fluorescence intensity of the ensemble started to increase; moreover, the fluorescence intensity increased gradually by prolonging the incubation time (see Figure 2). For instance, the fluorescence intensity at 490 nm of the ensemble was enhanced 43 times after AChE (0.1 U/mL) was introduced and the solution was incubated for 15 min. Actually, the fluorescence enhancement, observed for the ensemble solution after addition of AChE, can be distinguished by naked eye when the buffer solution is illuminated with UV light (365 nm) as indicated in the inset of Figure 2.

The control experiments confirm that the fluorescence enhancement observed for compound **1** requires the presence of compound **2**, ATC, and AChE. Neither the ensemble of compound **1**/AChE or that of compound **1**/compound **2**/AChE or that of compound **1**/ATC/AChE exhibits obvious fluorescence enhancement as displayed in Figure S3 (Supporting Information). The fact that the fluorescence of the ensemble of **1**, **2**, and ATC increases after addition of AChE can be understood as follows: ATC is hydrolyzed to generate thiocholine, which in turn reacts with compound **2** to form the amphiphilic compound **3**. As discussed above, aggregation of compound **1** occurs in the presence of compound **3**

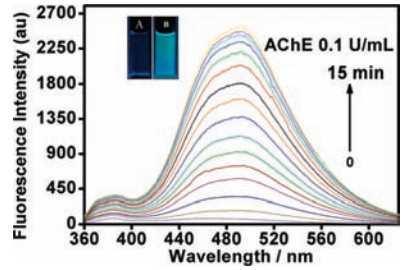

Figure 2. Fluorescence spectra of the ensemble of compound **1** [20 μM in HEPES (10 mM) buffer solution, pH = 7.35], compound **2** (30 μM), and ATC (30 μM) in the presence of AChE (0.1 U/mL) incubated at room temperature for different periods; inset shows the photos of the corresponding mixed buffer solution in the absence (A) and presence (B) of AChE (0.1 U/mL) after incubation for 15.0 min under UV light (365 nm) illumination.

(see Scheme 1); accordingly, the fluorescence of the ensemble is enhanced. In fact, the formation of compound **3** within the ensemble of compounds **1** and **2**, ATC, and AChE was confirmed by HPLC analysis as shown in Figure S4 (Supporting Information).

The fluorescence spectra of the ensemble of **1** (20 μM), **2** (30 μM), and ATC (30 μM) containing different concentrations of AChE (0, 0.005, 0.01, 0.02, 0.05, 0.1U/mL) were measured after incubation for different times. As shown in Figure 3, the fluorescence intensity at 490 nm remained almost unchanged in the absence of AChE, but the fluorescence intensity increased gradually in the presence of AChE by prolonging the reaction time at room temperature. Obviously, a large degree of fluorescence enhancement was detected for the ensemble containing a high concentration of AChE. AChE with concentrations as low as 0.005 U/mL can be analyzed. This is understandable by considering the fact that the presence of more AChE would induce the formation of more amount of compound **3**, which would trigger the aggregation of compound **1** leading to fluorescence enhancement.

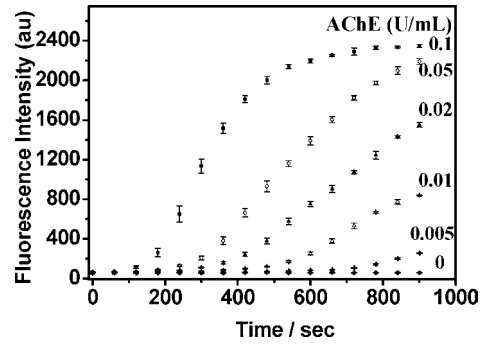

Figure 3. Variation of the fluorescence intensity at 490 nm vs the reaction time for the ensemble of compounds **1** [20 μM in HEPES (10 mM) buffer solution, pH = 7.35], **2** (30 μM), and ATC (30 μM) in the presence of different concentrations of AChE (0, 0.005, 0.01, 0.02, 0.05, and 0.1 U/mL); the excitation wavelength was 340 nm.

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(15) Because of the amphiphilic feature, compound **3** can also self-assemble into aggregates with sizes around 44 nm in aqueous solution as indicated by the DLS result (see Figure S1, Supporting Information).

It is known that the hydrolysis of ATC catalyzed by AChE will be retarded in the presence of corresponding AChE inhibitors. Thus, it is anticipated that the degree of fluorescence enhancement for the ensemble of compounds **1**, **2**, ATC, and AChE will become small after addition of the inhibitors of AChE. Accordingly, the ensemble of compounds **1**, **2**, ATC, and AChE can be employed for screening the inhibitors of AChE. Neostigmine (see Figure 4),[16] a typical inhibitor of AChE, was selected to demonstrate the application of the ensemble to screen the AChE inhibitors. The fluorescence spectra of the ensemble of compounds **1** (20 μM), **2** (30 μM), ATC (30 μM), and AChE (0.1U/mL) containing different concentrations of neostigmine (0, 10, 50, 100, 500 nM) were measured after incubation for different times at room temperature. As expected, in the absence of neostigmine the fluorescence intensity increases by prolonging the reaction time, but in the presence of neostigmine the degree of the fluorescence enhancement for the ensemble is reduced gradually (see Figure S5, Supporting Information). On the basis of the plot of the inhibition efficiency[4] vs the concentration of neostigmine (Figure 4), the corresponding IC_{50} value was estimated to be 50.3 nM.

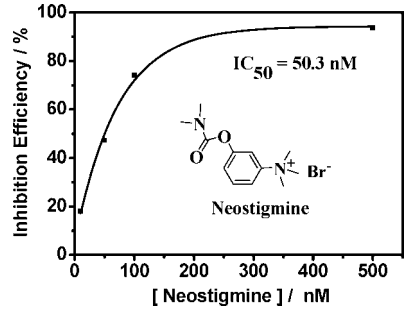

Figure 4. Plot of the inhibition efficiency of neostigmine to AChE vs the concentration of neostigmine; the data were obtained with the ensemble of compounds **1** [20 μM in HEPES (10 mM) buffer solution, pH = 7.35], **2** (30 μM), and ATC (30 μM) and AChE (0.1 U/mL) in the presence of different concentrations of neostigmine (10, 50, 100, 500 nM).

This value of IC_{50} is close to that obtained with other AChE assay methods (36 nM).[17] The fluorescence spectra of the ensemble were recorded in the presence of tacrine and donepezil, which are also the inhibitors of AChE. Figures S6 and S8 (Supporting Information) show the plots of the fluorescence intensity at 490 nm of the ensemble vs the reaction time in the presence of different amounts of tacrine and donepezil, respectively. Similarly, the IC_{50} values were estimated to be 22.5 and 7.3 nM for tacrine and donepezil, respectively, based on the plots of the inhibition efficiency vs the respective concentrations of tacrine and donepezil (see Figures S7 and S9, Supporting Information). These IC_{50} values are close to those obtained with other AChE assay methods (11.8 nM, 6.4 nM) .[11,7]

Additionally, cysteine and reduced glutathione (GSH) have negligible interference toward this fluorometric assay method for AChE activity and the inhibitor-screening. As shown in Figure S10 (Supporting Information), the fluorescence intensity of the ensemble of compounds **1** (20 μM) and **2** (30 μM) remained almost unchanged after addition of either cysteine (up to 10 equiv) or GSH (up to 10 equiv). It is probable that the corresponding Michael adducts formed between compound **2** and cysteine/GSH do not possess net positive charges and as a result aggregation of compound **1** may not take place efficiently. It should be noted that thiol-containing compounds such as reduced glutathione (GSH) have interference on the AChE assay with Ellmans's reagent.

In summary, we successfully demonstrate a new switch-on fluorometric assay method for AChE and its inhibitor-screening based on the ensemble of compounds **1**, **2**, and ATC. This fluorometric assay is designed by making use of the following features of the ensemble: (1) cascade reactions from ATC hydrolysis to the Michael reaction with compound **2** to generate the amphiphilic compound **3**; (2) aggregation of compound **1** in the presence of the amphiphilic compound **3**; (3) the AIE (aggregation-induced emission) feature of tetraphenyl ethylene compounds. Fluorescent spectral investigations show that this fluorometric assay can be performed continuously and AChE with concentrations as low as 0.005 U/mL can be analyzed. Moreover, the ensemble of compounds **1**, **2**, ATC, and AChE can be conveniently used to screen the inhibitors of AChE. Given its speed, simplicity, and ease of operation, this fluorescence turn-on assay may extend to high-throughput screening of AChE inhibitors and relevant drug discovery.

Supporting Information Available: Synthesis and characterization data of compounds **2** and **3**; DLS and HPLC data; fluorescence spectra and relevant data for the ensemble. This material is available free of charge via the Internet at http://pubs.acs.org.

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