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Colorimetric and fluorometric assays for acetylcholinesterase and its inhibitors screening based on a fluorescein derivate

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ABSTRACT

A fluorescein-based sensor was developed for the AChE activity assay and the inhibitor screening. The sensor provided the dual assay methods for the screening of AChE activity in the presence or absence of inhibitor. The colorimetric and fluorometric assays were based on the following processes: (1) owing to the hydrolysis of acetylthiocholine in the presence of AChE, the fluorescein-based probe can rapidly induce 1,4-addition of the hydrolysis product thiocholine to α,β-unsaturated ketone in the compound **1**, resulting in strong fluorescence and absorption changes; (2) in the presence of the corresponding inhibitor, the fluorescence enhancement or the absorption change would be inhibited in that the formation of thiocholine was hindered.

Acetylcholine, a central neurotransmitter, is associated with fatal neurodegenerative disease.[1] One of hypotheses is that a low level of acetylcholine in the hippocampus and cortex is the cause of Alzheimer's disease. The level of acetylcholine could be regulated by acetylcholinesterase (AChE) through hydrolysis reaction.[1] Currently, the clinical treatment of Alzheimer's disease mostly relies on the AChE inhibitor.[2,3] Therefore, developing a highly effective detection method for screening of AChE and its inhibitor should be of great importance.[4] On the other hand, nerve gas and pesticides containing organic phosphorus and carbamate can also serve as AChE inhibitors.[5,6] So, the efficient detection of AChE activity could also be applied to detect nerve gas and pesticides.[7,8] The traditional method for detecting AChE and its inhibitors is based on colorimetric response by employing Ellman's reagent.[9,10] Comparing with colorimetric method, fluorometric method is more desirable because it is sensitive and efficient.[11–14] Therefore, it is very necessary to develop fluorescent methods to carry out the continuous and efficient detection of AChE activity and its inhibitor screening.[15–22]

The methods for monitoring AChE activity based on thiol-trapping turn on probes is highly sensitive.[15,17,23,24] Usually, the concentration of AChE as low as 10^{-2} U/mL is enough to carry out the AChE activity assays, which is lowered by 1–2 order of magnitude than that in the methods using non thiol-trapping turn on probes.[16,21,22] Recently, we reported a fluorescein-based compound **1** for thiols in aqueous solution with high selectivity and sensitivity.[25] The speed of reaction between compound **1** and thiols (Cys or GSH) is so rapid that the reaction is complete in a short time.[25] Based on the previous report, we provided here dual assays by employing fluorometric and colorimetric methods for AChE and its inhibitors screening based on the ensemble of compound **1** and acetylthiocholine (Scheme 1). Owing to the hydrolysis of

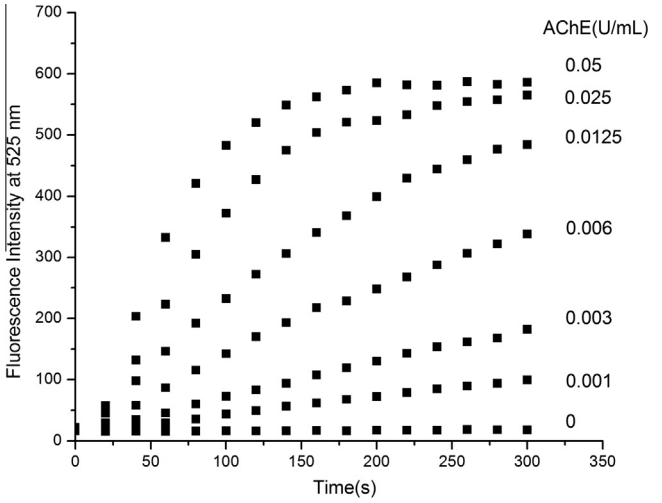

Figure 1. Variation of the fluorescence intensity at 525 nm dependent on the reaction time for the HEPES buffer (10 mM, pH = 7.4) containing compound **1** (10 μM), acetylthiocholine (10 μM), and in the presence of different concentrations of AChE (0, 0.001, 0.003, 0.006, 0.0125, 0.025 and 0.05 U/mL) (λ_{ex} = 485 nm).

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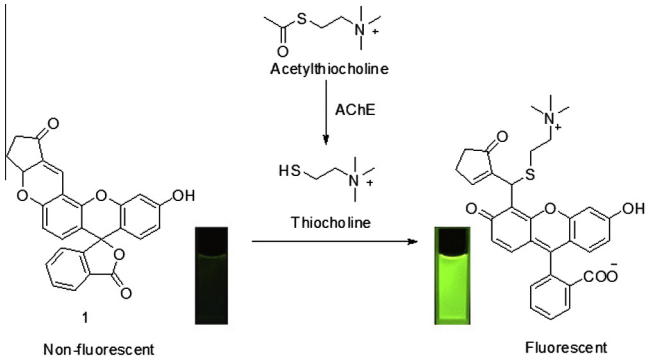

Scheme 1. The mechanism of acetylcholinesterase activity screening based on compound **1**.

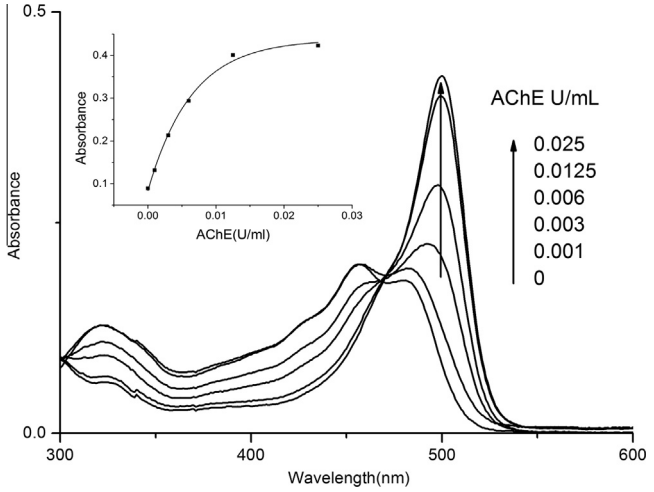

Figure 2. UV/vis absorption spectra of the HEPES buffer (10 mM, pH = 7.4) containing compound **1** (10 μM), acetylthiocholine (10 μM), and in the presence of different concentrations of AChE (0, 0.001, 0.003, 0.006, 0.0125, 0.025 U/mL) after incubation for 5 min. Inset: the plot of the absorption changes at 499 nm with various concentration of AChE.

acetylthiocholine in the presence of AChE, the fluorescein-based probe can rapidly induce 1,4-addition of the hydrolysis product thiocholine to α,β-unsaturated ketone in the compound **1** to form thioether, resulting in strong fluorescence enhancement and absorption change. However, in the presence of the corresponding inhibitor, the fluorescence enhancement or the absorption change would be inhibited in that the formation of thiocholine was hindered. Based on this strategy, this compound was successfully applied to the detection of the AChE activity and its inhibitors screening.

Compound **1** was synthesized according to our previous report.[25] In order to demonstrate that the compound is efficient for the activity assay and the inhibitor screening, we choose acetylthiocholine as substrate of AChE in that acetylthiocholine could be hydrolyzed into thiocholine by AChE. Initially, compound **1** is non-fluorescent in HEPES buffer (10 mM, pH 7.4). Upon addition of AChE (0.05 U/mL) to aqueous solution containing compound **1** (10 μM) and acetylthiocholine (10 μM), the fluorescence intensity at 525 nm increased. Time-dependent fluorescence intensity assays show the reactions including the hydrolysis induced by AChE and addition reaction between compound **1** and thiocholine were almost complete within 5 min (Fig. S1).

After incubation for different time, the fluorescence enhancement of compound **1** in the presence of acetylthiocholine (10 μM) and various concentrations of AChE were shown in Figure 1. The fluorescence intensity at 525 nm induced little change in the absence of AChE. However, upon the addition of as low as 0.001 U/mL of AChE, the fluorescence intensity displayed an obvious enhancement by extending the reaction time. Incubation for a same time, increase of the concentration of AChE led to the enhancement of the fluorescence intensity at 525 nm since the hydrolyzing rate of acetylthiocholine into thiocholine is faster. As shown in Figure S2, after the addition of AChE (0.025 U/mL), the fluorescence intensity at 525 nm increased with the reaction time. Similarly, upon the addition of AChE (0.025 U/mL), the absorption intensity at 499 nm of the assemble solution increased while the absorption peak at 460 nm decreased (Fig. 2). Using fluorometric or colorimetric method, the activity of AChE upon a concentration as low as 0.001 U/mL could be detected. It further proved that AChE is highly active for the formation of thiocholine. Through monitoring the reaction kinetics of compound **1** (10 μM) and thiocholine (10 μM), the reaction was completed within 20 s. In contrast, it will take more than 300 s for getting to the end of the reaction of **1** (10 μM), acetylthiocholine (10 μM) and AChE (0.0125 U/mL) (Fig. S3), which showed the thiol trapping reaction is much faster than the enzyme hydrolysis and the fluorescence generated is a real time reflection of the enzyme action.

To further investigate the corresponding AChE inhibitor, neostigmine, the typical inhibitor of AChE, was used to carry out the inhibitive activity assay. The ensemble solution containing compound **1** (10 μM), acetylthiocholine (10 μM) and AChE (0.0125 U/mL) was treated with various concentrations of neostigmine (0.5, 1, 2.5, 5 nM) after incubation for 5 min. After neostigmine was added to the ensemble solution, the fluorescence intensity at 525 nm decreased comparing to that in the absence of inhibitor after incubation for the same time (Fig. 3a inset). Similarly, when incubated with neostigmine for 5 min, the absorption intensity at 460 nm of the ensemble solution decreased while the absorption intensity at 499 nm increased. When more AChE inhibitor was added to the ensemble solution, the hydrolysis of acetylthiocholine would be discouraged and less thiocholine would be generated, resulting in the less changes both in fluorescence and absorption. On the basis of the plot of the inhibition efficiency dependent on the concentration of neostigmine, the IC_{50} value was examined to be 1.4 nM using the fluorometric method (method A) while 3.2 nM using the colorimetric method (method B) (Fig. 3). Furthermore, tacrine was the first centrally-acting cholinesterase inhibitor approved for the treatment of Alzheimer's disease and also used to carry out the inhibiting experiment. Similarly, on the basis of the plot of the inhibition efficiency dependent on the concentration of tacrine, the IC_{50} value was examined to be 11 nM and 12.8 nM using the fluorometric and colorimetric methods, respectively (Fig. 4). Thus, using the fluorescein-based sensor, the colorimetric and fluorometric assays for acetylcholinesterase and inhibitor screening were carried out successfully.

In contrast with fluorescein-based sensor, Ellman's reagent (DTNB) was used to substitute compound **1** to carry out the inhibitive activity assay, and other condition remain unchanged. As shown in Figure 5, after the addition of AChE, with the increasing of the concentration of AChE (0, 0.003, 0.006, 0.0125 and 0.025 U/mL), the absorption peak at 416 nm of the assemble solution increased while the absorption intensity at 328 nm decreased. When treated the ensemble of Ellman's reagent (DTNB) [10 μM in HEPES (10 mM) buffer solution, pH 7.4], acetylthiocholine (10 μM) and AChE (0.0125 U/mL) with different concentrations of neostigmine (1, 2.5, 5, 10 nM) and tacrine (5, 10, 20, 50 nM) after incubation for 5 min, the inhibition efficiencies were obtained. On the basis of the plot of the inhibition efficiency dependent on the con-