ORGANIC LETTERS

2013
Vol. 15, No. 9
2132–2135

Real-Time Fluorometric Assay for Acetylcholinesterase Activity and Inhibitor Screening through the Pyrene Probe Monomer–Excimer Transition

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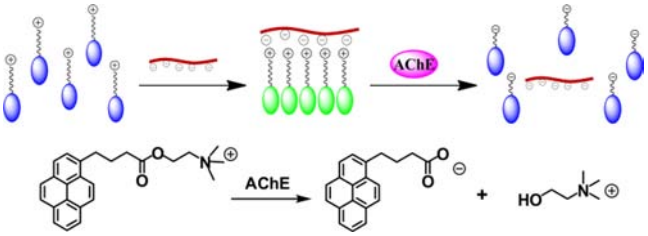
Received March 8, 2013

ABSTRACT



A choline labeled pyrene probe (Py-Ch) was designed and synthesized. Poly(vinylsulfonate) (PVS) could induce Py-Ch aggregation. The aggregation and deaggregation process could be finely controlled by the acetylcholinesterase (AChE) enzymatic hydrolysis of Py-Ch. The resulting excimer–monomer transition provided a facile way for real-time AChE activity fluorometric assay and inhibitor screening.

Acetylcholinesterase (AChE, EC 3.1.1.7) is a hydrolase that can catalyze the hydrolysis of acetylcholine to choline and acetate.[1] It is a key enzyme in the central and peripheral nervous system.[2] AChE inhibitors are currently used for the treatment of a number of neuromuscular disorders and Alzheimer's disease.[3] Detection of AChE activity and the screening for its potential inhibitors are therefore of great importance.

In recent years, AChE has been detected by colorimetric,[4] chemiluminescent,[5] electrochemical,[6] and fluorescent[7−10] methods. Fluorescent methods exhibit higher sensitivity compared with other methods and have drawn more attention. However, certain drawbacks exist. For instance, some methods require a complicated, time-consuming, and expensive synthesis; some fluorescent materials are somewhat toxic; and some may produce false-positive output detection signals. Therefore, the development of a simple, fast, sensitive,

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10.1021/ol400619t
Published on Web 04/16/2013

and inexpensive fluorometric assay for AChE activity and its inhibitors is highly desirable.

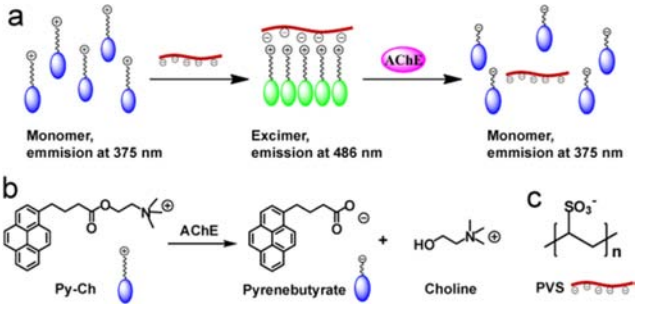
Controlled self-assembly has been widely used by all forms of life for the construction of sophisticated functional units. And in recent years it has also been used for the development of a number of novel biosensing techniques for the selective sensing of enzyme activities, proteins, and other biomolecules.[11]

Pyrene is a planar aromatic compound. The aggregation of pyrene monomer molecules (emission at 370−420 nm) could produce excimer emission. As a result, a red-shifted and broad emission band at 450−550 nm could be observed.[12] Monomer emission could be restored through the deaggregation process.[13] This particular property has been employed for the design of a number of fluorescent sensors.[14] In this work, a choline labeled cationic pyrene probe (**Py-Ch**) was designed and synthesized. It shows considerable water solubility (> 3 mM) and exhibits blue fluorescence in an aqueous solution. Using **Py-Ch** as an AChE substrate, we herein report a simple, fast, inexpensive, and sensitive fluorescent method for real time AChE activity assay.

The principle of the assay is shown in Scheme 1. (1) In an aqueous solution, **Py-Ch** mainly exists in the monomeric form, because of the positive charge electrostatic repulsive interactions. Strong pyrene probe monomer fluorescence is detected. (2) Poly(vinylsulfonate) (PVS) is a polyanion. When PVS is added to the assay solution, strong attractive electrostatic and hydrophobic interactions between PVS and **Py-Ch** result in the aggregation of **Py-Ch**. Both the increase of the pyrene excimer emission and the decrease of the monomer emission could be observed. (3) AChE can catalyze the hydrolysis of the cationic **Py-Ch** to the anionic 1-pyrenebutyrate and choline. Upon the addition of AChE to the assay solution, the pyrene probe is hydrolyzed and repulsed from PVS becasue of the charge reversal. The pyrene excimer returns to the monomeric state as a result of the deaggregation. An excimer−monomer transition is detected, and a convenient fluorometric assay for AChE activity is therefore established. (4) In the presence of an AChE inhibitor, the activity of AChE is reduced. A reduced degree of excimer−monomer transition is detected, which could be used for the screening for potential AChE inhibitors.

Figure 1 shows that, in the absence of PVS, **Py-Ch** exhibited characteristic monomer emission with peaks at

Scheme 1. (a) Schematic Illustration of the Fluorometric Detection of Ache Activity through the Excimer−Monomer Transition; (b) Hydrolysis of **Py-Ch** by AChE; (c) Structure of PVS



375 and 396 nm. A new and broad emission band with a peak at 486 nm appeared, and the intensity gradually increased upon the gradual increase of the PVS concentration. Meanwhile, a gradual decrease of the monomer emission was also observed. The emission band at 486 nm was attributed to the formation of a pyrene excimer as a result of the PVS-induced **Py-Ch** aggregation. The changes in assay solution emission color from blue to green could be easily observed by the naked eye (inset I, Figure 1). When 300 μM PVS was introduced, the intensity ratio (the I_E/I_M value) of the excimer (at 486 nm) to monomer emission (at 375 nm) reached its maximum (inset II of Figure 1). A further increase of the PVS concentration caused no further increase of the I_E/I_M value, indicating that a saturation point was reached where most of the monomic pyrene probe had been transformed to the aggregated forms.

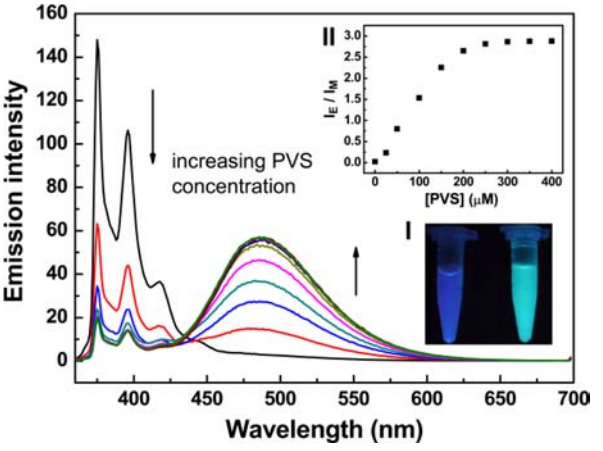


Figure 1. Changes in emission spectra of **Py-Ch** (100 μM) upon the addition of increasing concentrations of PVS. Inset: (**I**) photograph of the **Py-Ch** sample solution in the absence (left) and presence (right) of PVS (300 μM) under UV light illumination at 365 nm; (**II**) Changes in the I_E/I_M value upon the addition of increasing concentrations of PVS.

AChE could catalyze the hydrolysis of the cationic **Py-Ch** to the anionic 1-pyrenebutyrate and choline. 1-Pyrenebutyrate was repulsed from the polyanion (PVS) as a result of the

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negative charge electrostatic repulsive interactions. Deaggregation took place. Therefore, the intensity of the excimer emission decreased, and the intensity of the monomer emission increased. Figures 2 and S1 (Supporting Information) show that, upon the addition of AChE to the sample solution of **Py-Ch** and PVS, with the increase of the enzymatic reaction time, a gradual decrease of the excimer emission and at the same time a gradual increase of the monomer emission were observed. Almost complete recovery of the free **Py-Ch** monomer emission was obtained after 120 min of the enzymatic reaction. The emission color of the sample solution also changed from green back to blue, which could be easily observed by the naked eye (inset of Figure 2). The results clearly show that AChE could catalyze the hydrolysis of **Py-Ch**, which led to the probe excimer-to-monomer emission transition.

Control experiments were conducted to verify the principle of the assay. **Py-Ch** was incubated with AChE for an ample amount of time, and the emission spectrum was recorded. Figure S2 shows clear pyrene monomer emission, and no excimer emission was observed. The spectrum is quite similar to that of the free **Py-Ch**. The UV−vis absorption spectrum of **Py-Ch** also shows minimal changes upon enzymatic hydrolysis (Figure S3). The results clearly suggest that simple hydrolysis of **Py-Ch** could not produce pyrene excimer emission. Upon the addition of PVS, the absorption bands of **Py-Ch** showed clear broadening, along with a significant decrease in intensity, because of the formation of the probe aggregates. After the enzymatic hydrolysis by AChE, the absorption bands of the pyrene probe changed back to that of the free **Py-Ch** (Figure S3). The results further support the conclusion that the enzymatic hydrolysis of the pyrene probe resulted in deaggregation and restoration of the probe monomer emission. Our results also show that PVS itself shows little effect on the enzymatic activity of AChE (Figure S4). A number of related pyrene and choline derivatives were selected, and their influences on the AChE enzymatic reaction were studied (Figures S5 and S6).

Our method could be used to monitor the AChE activity in real time (Figure 3). The emission spectra of the sample mixture of **Py-Ch** and PVS containing different amounts of AChE were recorded at different reation times. The I_M/I_E value remained mostly unchanged in the absence of AChE, but increased gradually with reaction time in the presence of AChE. The I_M/I_E value increased more quickly at higher AChE concentrations (Figure S7). The results clearly suggest that the increase in AChE concentration led to faster substrate (**Py-Ch**) hydrolysis.

To address the selectivity of our assay, control experiments were conducted. A number of enzymes such as lysozyme, alkaline phosphatase (ALP), collagenase, and exonuclease I (Exo I) were tested. These enzymes can catalyze the hydrolysis of glycoside, phosphate, collagen, and nucleic acid, respectively. Figure S8 shows that none of these enzymes had the ability to induce noticeable I_M/I_E value changes. The results further confirm that the emission changes of **Py-Ch** were due to the AChE-catalyzed **Py-Ch** hydrolysis, and the assay is highly selective for AChE.

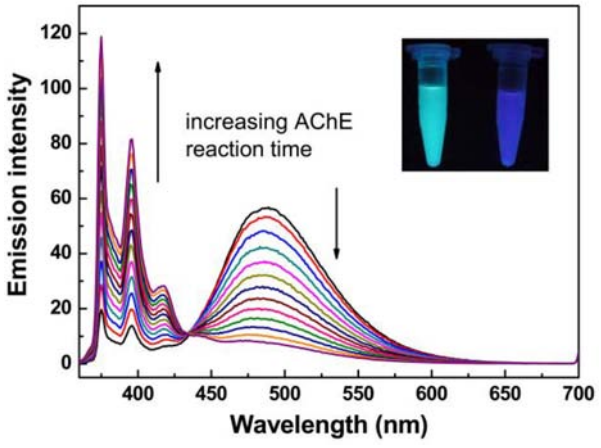

Figure 2. Changes in emission spectrum of **Py-Ch** (100 μM) with the enzymatic reaction time. Emission spectra were recorded at 10 min intervals. The assay solution contained 300 μM PVS and 10 U/mL AChE. Inset: Photograph of the sample solution of **Py-Ch** and PVS in the absence (left) and presence (right) of AChE under UV light illumination at 365 nm.

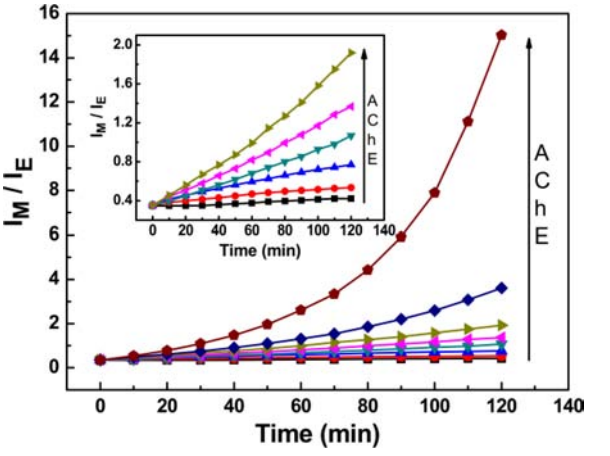

Figure 3. Real-time I_M/I_E value changes of **Py-Ch** (100 μM) at different AChE concentrations. Assay solutions contained 300 μM PVS and different concentrations of AChE (0, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, and 10.0 U/mL, respectively). Inset: Expanded curves with lower AChE concentrations.

Our AChE fluorometric assay was further tested in complex sample mixtures. The emission spectra of the sample mixtures containing **Py-Ch**, PVS, and AChE of various concentrations in 2% fetal calf serum (or 2% cell lysate) were measured, and the I_M/I_E values were obtained. Figures S9 and S10 show that the more AChE is spiked, the larger the I_M/I_E value increase is obtained. The activity of 0.1 U/mL of AChE could be easily detected. The results clearly show that our assay could be used in complex sample mixtures.

The assay could also be used for the screening of potential AChE inhibitors. To demonstrate this, donepezil

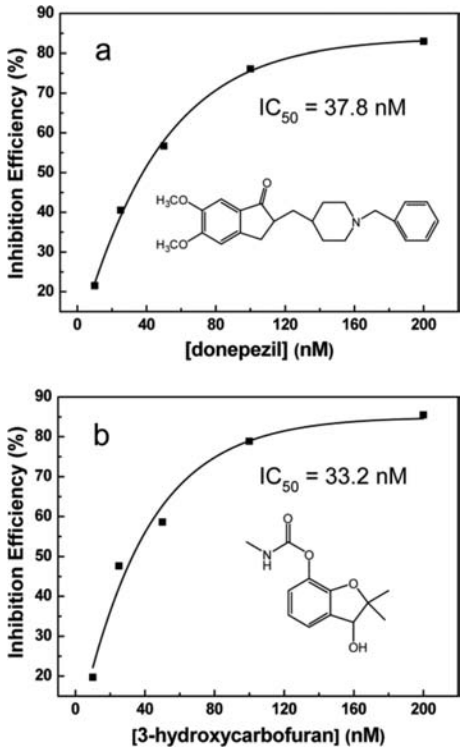

Figure 4. Plots of the inhibition efficiency of donepezil (a) and 3-hydroxycarbofuran (b) versus the inhibitor concentration. Assay solutions contained 100 μM **Py-Ch**, 300 μM PVS, and 10 U/mL AChE.

and 3-hydroxycarbofuran, two known AChE inhibitors,[7b,8a] were tested. The emission spectra of the sample mixtures of **Py-Ch**, PVS, AChE, and the inhibitors of different concentrations were recorded. Figures S11 and S12 show that both the excimer emission and the monomer emission changed significantly upon the addition of the inhibitors. And the I_M/I_E value decreased with the increase of the inhibitor concentration. The results indicate that the inhibition was more effective at higher inhibitor concentrations. The IC_{50} values for donepezil and 3-hydroxycarbofuran were estimated to be 37.8 and 33.2 nM, respectively (Figure 4).[7b,8a] The results clearly demonstrate that our assay could be used not only for the real-time monitoring of AChE activity but also for the screening of potential AChE inhibitors.

In summary, a choline labeled pyrene probe (**Py-Ch**) was designed and synthesized. **Py-Ch** was used as an AChE substrate. Based on the finely controlled aggregation and deaggregation process, and the resulting excimer−monomer transition, a facile real-time fluorometric assay for AChE activity has been developed. Our assay has several important features. First, it is based on the "excimer−monomer transition" mode, which could considerably reduce the likelihood of false-positive signals associated with other fluorometric assays. Second, it offers a convenient "mix-and-detect" approach for the rapid and sensitive detection of AChE activity and inhibition. Third, the emission spectral changes could be monitored in real time using a common spectrophotometer. Fourth, **Py-Ch** could be easily prepared, and the polyanion (PVS) is commercially available. All materials used are fairly inexpensive. The assay is thus fairly cost-effective. Fifth, the principle of our assay may also be used for the detection of other enzymes (such as protease and phosphatase) and the corresponding inhibitors with the properly designed substrates.

Acknowledgment. This work was supported by the "100 Talents" program of the Chinese Academy of Sciences, the National Natural Science Foundation of China (Grants 21075119, 91027036, and 21275139), the National Basic Research Program of China (973 Program, Grant No. 2011CB911002), and the Pillar Program of Changchun Municipal Bureau of Science and Technology (Grant No. 2011225).

Supporting Information Available. Experimental details, synthesis and characterization of **Py-Ch**, UV−vis and emission studies. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.