ORGANIC LETTERS

2010
Vol. 12, No. 10
2274−2277

A New Label-Free Continuous Fluorometric Assay for Trypsin and Inhibitor Screening with Tetraphenylethene Compounds

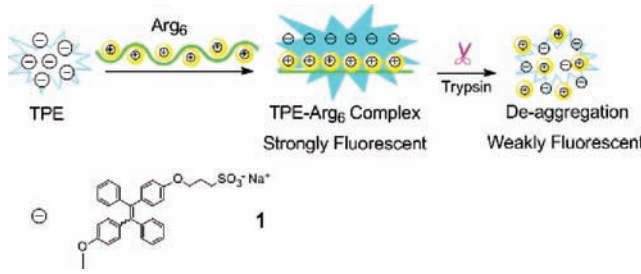
Wangxin Xue, Guanxin Zhang, Deqing Zhang,* and Daoben Zhu

Beijing National Laboratory for Molecular Sciences, Organic Solids Laboratory, Institute of Chemistry, Chinese Academy of Sciences, Beijing 100190, China

dqzhang@iccas.ac.cn

Received March 17, 2010

ABSTRACT



A new label-free continuous assay with the ensemble of compound 1 and Arg_6 for trypsin and inhibitor screening was successfully developed by taking advantage of the aggregation-induced emission feature of tetraphenylethene compounds. Both CLSM and DLS studies confirm the formation of the aggregation complex of compound 1 and Arg_6 and disassembly after further addition of trypsin.

As one class of protease, trypsin is the most important digestive enzyme produced by the pancreas.[1] It is involved in the digestive enzyme activation cascade, which induces the transformation of other pancreatic proenzymes into their active forms within the intestine and then initiates autodigestion. Therefore, trypsin plays a key role in controlling pancreatic exocrine function. It is known that trypsin level is increased with some types of pancreatic diseases.[2] Obviously, convenient and continuous assays for trypsin and inhibitor screening may lead to new diagnostic methods and also have therapeutic implications for these pancreatic diseases. Assays based on solid-phase cleavage,[3] turbidity,[4] and radiometry[5] were described for trypsin, but these methods are either time-consuming or require specific instruments. Ionescu et al.[6] reported a sensitive amperometric biosensor for the detection of trypsin. However, most of these biosensors with enzyme-modified electrodes respond slowly. Fluorometric assay methods based on doubly labeled substrate peptides were also reported for trypsin.[7] A label-free fluorescent assay for trypsin was established with a water-

(1) Hirota, M.; Ohmuraya, M.; Baba, H. *J. Gastroenterol.* **2006**, *41*, 832–836.

(2) (a) Rinderknecht, H. *Dig. Dis. Sci.* **1986**, *31*, 314–321. (b) Lankisch, P. G.; Burchard-Reckert, S.; Lehnick, D. *Gut* **1999**, *44*, 5425–5444. (c) Byrne, M. F.; Mitchell, R. M.; Stiffler, H.; Jowell, P. S.; Branch, M. S.; Pappas, T. N.; Tyler, D.; Baillie, J. *Can. J. Gastroenterol.* **2002**, *16*, 849–854.

(3) Koritsas, V. M.; Atkinson, H. J. *Anal. Biochem.* **1995**, *227*, 22–26.

(4) (a) Trivedi, V. D.; Raman, B.; Ramakrishna, T.; Rao, C. M. *J. Biochem. Biophys. Methods* **1999**, *40*, 49–55. (b) Walker, M.; Retzinger, A.; Retzinger, R. *Anal. Biochem.* **2006**, *351*, 114–121.

(5) Artigas, J. M.; Garcia, M. E.; Faure, M. R.; Gimeno, A. M. *Postgrad. Med. J.* **1981**, *57*, 219–222.

(6) Ionescu, R. E.; Cosnier, S.; Marks, R. S. *Anal. Chem.* **2006**, *78*, 6327–6331.

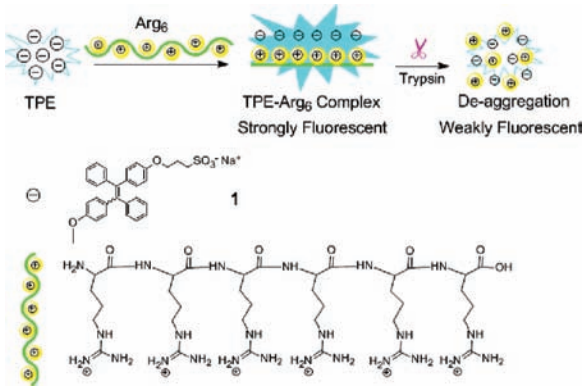
(7) (a) Farmer, W. H.; Yuan, Z. *Anal. Biochem.* **1991**, *197*, 347–352. (b) Kircher, M. F.; Weissleder, R.; Josephson, L. *Bioconjugate Chem.* **2004**, *15*, 242–248. (c) Dean, K. E. S.; Klein, G.; Renaudet, O. R.; Reymond, J. L. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1653–1656.

10.1021/ol100626x © 2010 American Chemical Society
Published on Web 04/20/2010

soluble conjugated polymer.[8] Nevertheless, convenient label-free fluorometric assays for trypsin and inhibitor screening still remain limited. In this paper, we report a new label-free continuous assay for trypsin and inhibitor screening by taking advantage of the abnormal fluorescent behavior of tetraphenylethene (TPE) compounds which are nonfluorescent in solution but become strongly fluorescent after aggregation.[9]

The design rationale for this label-free fluorometric assay is schematically illustrated in Scheme 1 and is explained as follows: (1) Compound **1** is a tetraphenylethene derivative with one sulfonate ($-SO_3^-$) unit which will enable it to be dissolved in aqueous solution. It is expected that compound **1** shows an aggregation-induced emission (AIE) feature; namely, it is weakly fluorescent in solution and its fluorescence is turned on after aggregation.[9] (2) Arg_6, a positively charged peptide, is selected as the substrate for trypsin. We assume that the presence of Arg_6 in the solution would trigger the aggregation of compound **1** because of the electrostatic interaction among the sulfonate ($-SO_3^-$) unit in **1** and the positive arginine residues. The corresponding hydrophobic interaction among molecules of **1** and Arg_6 may also facilitate the aggregation. As anticipated, the fluorescence will increase after the coaggregation of compound **1** and Arg_6. (3) Arg_6 will be hydrolyzed into small fragments in the presence of trypsin and as a result the aggregation complex of **1** and Arg_6 would be disassembled. Accordingly, the fluorescence of **1** will be turned off. Therefore, it is possible to establish a new label-free continuous fluorometric assay for trypsin with the ensemble of compound **1** and Arg_6. (4) The hydrolysis of Arg_6 catalyzed by trypsin will be retarded in the presence of the respective inhibitors. As a result, a smaller amount of the aggregation complex of **1** and Arg_6 will be disassociated; the fluorescence decrease due to the presence of trypsin will become small. In this way, the ensemble of **1** and Arg_6 can be employed for screening the inhibitors of trypsin.

Scheme 1. Illustration of the Formation of the Heteroaggregate between Arg_6 Peptide and Compound **1** (TPE Derivative) and the Disassembly of the Aggregate in the Presence of Trypsin



Compound **1** was synthesized similarly according to the reported procedure,[10] and the synthetic details including characterization are provided in the Supporting Information. The aqueous solution of **1** (60.0 μM) in PBS buffer (2.0 mM, pH = 8.5) was prepared for the following fluorescent spectral investigations. As anticipated, the aqueous solution of **1** was almost nonfluorescent. As shown in Figure 1, the fluorescence intensity of **1** increased gradually after addition of Arg_6 to the solution. For instance, the fluorescence intensity at 475 nm of **1** was enhanced by 47 times when the concentration of Arg_6 reached 10.0 μM in the solution. In fact, the fluorescence quantum yield of the solution of **1** (60.0 μM) increased from 0.005 to 0.156 (by reference to quinine hemisulfate monohydrate) after Arg_6 (10.0 μM) was introduced to the solution. Such fluorescence enhancement observed for **1** after addition of Arg_6 can be distinguished by the naked eye as depicted in the inset of Figure 1, where photos (under UV light irradiation) of two PBS buffer solutions of **1** in the absence and presence of Arg_6 were shown. Interestingly, the fluorescence intensity of the ensemble solution of compounds **1** and Arg_6 increased almost linearly with the concentration of Arg_6 in the range of 0−10 μM as displayed in the inset of Figure 1 ($I_{475\ nm} = 38.4[Arg_6] - 1.28$, $r = 0.99$).

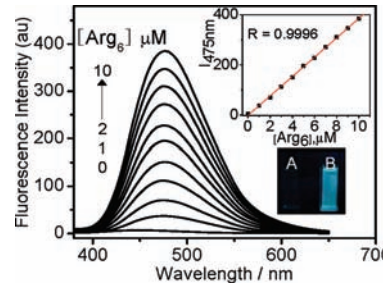


Figure 1. Fluorescence spectra of **1** (60.0 μM) in PBS buffer solution (2.0 mM, pH = 8.5) in the presence of different amounts of Arg_6 peptide (from 0.0 to 10.0 μM); the insets show (1) the photos of the corresponding buffer solutions of **1** (60.0 μM) in the absence (A) and presence(B) of Arg_6 peptide (10.0 μM) under UV light (365 nm) illumination and (2) variation of the fluorescence intensity at 475 nm vs the concentration of Arg_6.

(8) (a) An, L.; Tang, Y.; Feng, F.; He, F.; Wang, S. *J. Mater. Chem.* **2007**, *17*, 4147–4152. (b) Wosnick, J. H.; Mello, C. M.; Swager, T. M. *J. Am. Chem. Soc.* **2005**, *127*, 3400–3405.

(9) (a) Wang, M.; Zhang, G.; Zhang, D.; Zhu, D.; Tang, B. *J. Mater. Chem.* **2010**, *20*, 1858–1867. (b) Hong, Y.; Lam, J.; Tang, B. *Chem. Commun.* **2009**, *29*, 4332–4353. (c) Luo, J.; Xie, Z.; Lam, J.; Cheng, L.; Chen, H.; Qiu, C.; Kwok, H.; Zhan, X.; Liu, Y.; Zhu, D.; Tang, B. *Chem. Commun.* **2001**, 1740–1741. (d) Ning, Z.; Chen, Z.; Zhang, Q.; Yan, Y.; Qian, S.; Cao, Y.; Tian, H. *Adv. Funct. Mater.* **2007**, *17*, 3799–3807. (e) Chung, J.; An, B.; Kim, J.; Park, S. *Chem. Commun.* **2008**, 2998–3000. (f) Wang, M.; Gu, X.; Zhang, G.; Zhang, D.; Zhu, D. *Anal. Chem.* **2009**, *81*, 4444–4449. (g) Peng, L.; Zhang, G.; Zhang, D.; Xiang, J.; Zhao, R.; Wang, Y.; Zhu, D. *Org. Lett.* **2009**, *11*, 4014–4017. (h) Peng, L.; Wang, M.; Zhang, G.; Zhang, D.; Zhu, D. *Org. Lett.* **2009**, *11*, 1943–1946. (i) Liu, L.; Zhang, G.; Xiang, J.; Zhang, D.; Zhu, D. *Org. Lett.* **2008**, *10*, 458–461. (j) Wang, M.; Zhang, D.; Zhang, G.; Wang, Y.; Wang, S.; Zhu, D. *Anal. Chem.* **2008**, *80*, 6443–6448.

(10) Tong, H.; Hong, Y. N.; Dong, Y. Q.; Haussler, M.; Li, Z.; Lam, J. W. Y.; Dong, Y. P.; Sung, H. H. Y.; Williams, I. D.; Tang, B. Z. *J. Phys. Chem. B* **2007**, *111*, 11817.

Both confocal laser scanning microscopic (CLSM) and dynamic light scattering (DLS) studies confirm the formation of the aggregation complex of **1** and Arg_6, which induces the fluorescence enhancement of **1**. As displayed in Figure S1 (Supporting Information), no detectable fluorescence aggregates were observed for the solution of **1** (0.1 mM) with CLSM. However, fluorescence aggregates with sizes ca. 1.0 μm were observed for the buffer solution of **1** (0.1 mM) and Arg_6 (20.0 μM) (see Figure S1, Supporting Information). DLS data indicated that aggregates of around 1000 nm were formed in the solution of **1** (60.0 μM) and Arg_6 (10.0 μM) (see Figure S2, Supporting Information).[11]

Next, we want to demonstrate that the ensemble of **1** and Arg_6 can be employed to establish a label-free continuous fluorometric assay for trypsin. As mentioned above, Arg_6 is hydrolyzed in the presence of trypsin. It is expected that hydrolysis of Arg_6 will destabilize the aggregation complex of **1** and Arg_6; as a result, the fluorescence of the ensemble will decrease. As discussed above, the initial PBS buffer solution of **1** (60.0 μM) and Arg_6 (10.0 μM) showed strong fluorescence. But, the fluorescence of the ensemble started to decrease after introduction of trypsin into the solution, and more fluorescence reduction was observed by prolonging the incubation time as shown in Figure 2. The fluorescence

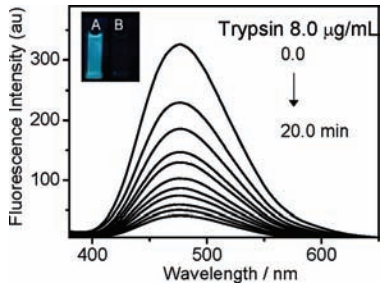


Figure 2. Fluorescence spectra of the ensemble of **1** (60.0 μM) in PBS buffer solution (2.0 mM, pH = 8.5, containing $CaCl_2$ (10.0 μM)) and Arg_6 peptide (10.0 μM) in the presence of trypsin (8.0 μg/mL) incubated at room temprature for different times; the inset shows the photos of the corresponding solutions of **1** and Arg_6 peptide without (A) and with (B) trypsin (8.0 μg/mL) after incubation at room temperature for 20.0 min under UV light (365 nm) illumination.

reduction observed for the ensemble of **1** and Arg_6 upon addition of trypsin could also be detected with the naked eye as indicated in the inset of Figure 2, where the photos of the solution of **1** and Arg_6 in the absence and presence of trypsin were displayed. It should be noted that the fluorescence variation of **1** upon addition of only trypsin can be neglected under the same conditions.

The hydrolysis of Arg_6 should be dependent on the concentration of trypsin used. Five concentrations of trypsin (0.2, 0.8, 1.6, 3.2, 8.0 μg/mL) were prepared to react separately with Arg_6 (10.0 μM). The hydrolysis reaction was followed by measuring the fluorescence spectrum of **1** in the reaction mixture. Figure 3 shows the variation of the

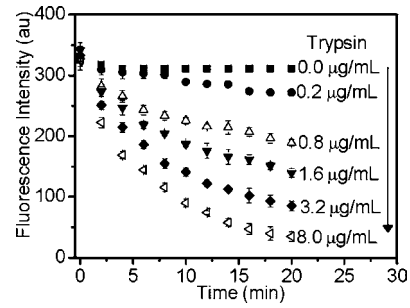


Figure 3. Variation of the fluorescence intensity at 475 nm vs the reaction time for the ensemble of **1** (60.0 μM) in PBS buffer solution (2.0 mM, pH = 8.5, containing $CaCl_2$ (10.0 μM)) and Arg_6 peptide (10.0 μM) in the presence of different concentrations of trypsin (0.0, 0.2, 0.8, 1.6, 3.2, 8.0 μg/mL); the excitation wavelength was 340 nm.

fluorescence intensity at 475 nm with hydrolysis time for the five concentrations of trypsin. It is obvious that the fluorescence intensity of the solution of **1** and Arg_6 decreased rapidly with high concentration of trypsin. This fluorescence reduction for ensemble of **1** and Arg_6 after incubation with trypsin is ascribed to the disassembly of the aggregation complex of **1** and Arg_6, which is supported by both CLSM and DLS studies. The initial fluorescence images detected for the solution of **1** (0.1 mM) and Arg_6 (20.0 μM) disappeared after the solution was incubated with trypsin (8.0 μg/mL) for 30 min (see Figure S1, Supporting Information). The number of large aggregates around 1000 nm was significantly reduced for the solution of **1** (60.0 μM) and Arg_6 (10.0 μM) after addition of trypsin (8.0 μg/mL) as shown in Figure S2 (Supporting Information).

The hydrolysis of Arg_6 catalyzed by trypsin was also studied with different concentrations of Arg_6. Four buffer solutions of Arg_6 with concentrations of 3.4, 4.7, 6.8, and 10.8 μM were prepared separately, and each solution containing compound **1** (60.0 μM) and trypsin (3.2 μg/mL) was incubated for different times at room temperature. The fluorescence spectrum of each solution after certain incubation time was measured. In this way, the hydrolysis reaction was followed by measuring the fluorescence intensity of **1** at 475 nm, and the concentration of Arg_6 remained at certain hydrolysis reaction time was deduced accordingly. The corresponding initial reaction rate (V_0, in $\mu M \cdot min^{-1}$) was calculated for the hydrolysis reaction at 4.0 min with different concentrations of Arg_6. Figure S3 (Supporting Information) shows the plot of $1/V_0$ vs $1/[Arg_6]$, the corresponding Lineweaver−Burk plot. The kinetic parameters for trypsin, K_m and V_{max}, were estimated to be 2.17 μM and 13.8 $\mu M \cdot min^{-1}$, respectively, by fitting the plot with Michalis−Menten equation. The K_m value is close to those reported for trypsin with other assay method.[7a] The above results

(11) Aggregates of ca. 100 nm were detected with DLS for the solution of **1** before addition of Arg_6. Compound **1** is an amphiphilic compound, and thus, it is understandable that molecules of **1** tend to assemble in aqueous solution.

clearly indicate that a label-free fluorometric assay for trypsin can be established with the ensemble of compound **1** and Arg_6. With the ensemble of **1** and Arg_6, trypsin with a concentration as low as 0.2 μg/mL can be analyzed.

The hydrolysis of Arg_6 catalyzed by trypsin will be retarded when the corresponding inhibitors of trypsin were present in the solution. Accordingly, less fluorescence reduction for the ensemble of **1** and Arg_6 containing trypsin is expected after further addition of the inhibitors. Therefore, the ensemble of **1**, Arg_6 and trypsin can be used to screen the trypsin inhibitors. A Bowman−Birk inhibitor (BBI) from soybean[12] was selected to demonstrate the usefulness of the ensemble to screen trypsin inhibitors. Similarly, the fluorescence spectra of the ensemble of **1** (60.0 μM), Arg_6 (10.0 μM), and trypsin (3.2 μg/mL) in the presence of different amounts of BBI (0.0, 1.0, 2.0, 5.0 μg/mL) were recorded after incubation for different times at room temperature. Figure 4 shows the variation of the fluorescence intensity of

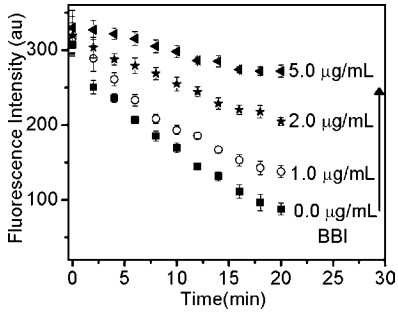


Figure 4. Plot of the fluorescence intensity at 475 nm vs the reaction time for the ensemble of **1** (60.0 μM in PBS buffer solution (2.0 mM, pH = 8.5, containing $CaCl_2$ (10.0 μM))), Arg_6 peptide (10.0 μM), and trypsin (3.2 μg/mL) in the presence of different concentrations of BBI (0, 1.0, 2.0, 5.0 μg/mL); the excitation wavelenth was 340 nm.

the ensemble at 475 nm with the hydrolysis time in the presence of four different concentrations of BBI. As expected, in the absence of BBI, the fluorescence intensity decreases by prolonging the hydrolysis time, but in the presence of BBI, the degree of the fluorescence reduction for the ensemble is reduced gradually. Based on the plot of the inhibition efficiency[13] vs the concentration of BBI (Figure S4, Supporting Information), the corresponding IC_{50} value of BBI from soybean toward trypsin was estimated to be 1.53 μg/mL. This IC_{50} value is different from those determined with other assay methods.[14] But, it is understandable because the reported IC_{50} values are affected by several parameters including the concentrations of trypsin and the substrate. All these results obviously indicate that the ensemble of **1** and Arg_6 can be utilized not only for trypsin activity assay but also for the corresponding inhibitor screening.

In summary, we successfully developed a new label-free continuous assay with the ensemble of **1** and Arg_6 for trypsin and inhibitor screening by taking advantage of the aggregation-induced emission feature of tetraphenylethene compounds. Both CLSM and DLS studies confirm the formation of the aggregation complex of **1** and Arg_6, which is driven by the electrostatic and hydrophobic interactions, and disassembly after further addition of trypsin. Compared to the reported assay methods for trypsin, this new fluorometric assay has the following advantages besides being label-free and having continuous features: (1) the assay can be carried out in pure aqueous solution, (2) the response time is acceptable, and (3) compound **1** is easily prepared and Arg_6 is commercially available; thus, this new assay should be cost-effective. Therefore, this fluorometric assay is potentially useful for high-throughput screening of trypsin inhibitors that may have implications for diagnostic methods for pancreatic diseases and anticarcinogenic drug discovery.[15]

Acknowledgment. The present research was financially supported by the NSFC, Chinese Academy of Science, and State Key Basic Research Program.

Supporting Information Available: Synthesis and characterization of compound **1**; CLSM and DLS data; the Lineweaver−Burk plot of the hydrolysis of Arg_6 peptide for trypsin; plot of the inhibition efficiency of BBI toward trypsin. This material is available free of charge via the Internet at http://pubs.acs.org.

OL100626X

(12) BBI is a protein of a molecular weight of 8 KDa with a well-characterized ability to inhibit trypsin and chymotrypsin and has been studied extensively as an anticarcinogenic protease inhibitor: (a) Kennedy, A. R. *Protease Inhibitors As Cancer Chemopreventive Agents*; Plenum Press: New York, 1993. (b) Birk, Y. *Int. J. Peptide Protein Res.* **1985**, *25*, 113–131. (c) Rawlings, N. D.; Tolle, D. P.; Barrett, A. J. *Biochem. J.* **2004**, *378*, 705–716. (d) Ye, X. Y.; Ng, T. B.; Rao, P. F. *Biochem. Biophys. Res. Commun.* **2001**, *289*, 91–96.

(13) Ho, V. M.; Ng, T. B. *J. Pept. Sci.* **2008**, *141*, 278–282.

(14) Wang, H. X.; Ng, T. B. *Biochem. Biophys. Res. Commun.* **2006**, *342*, 349–353.

(15) Kennedy, A. R. *Am. J. Clin. Nutr.* **1998**, *68*, 1406S–1412S.