A novel fluorometric turn-on assay for lipase activity based on an aggregation-induced emission (AIE) luminogen

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Abstract

Lipase plays critical roles in the industrial catalysis, drug discovery and medical diagnosis of diseases. In this paper, based on the consideration that a tetraphenylethylene (TPE) derivative **P1** with a $-\text{COOC}_6\text{H}_{13}$ group can be hydrolyzed into TPE-COOH which shows low solubility in some solutions and thus aggregation can occur easily, a novel fluorescence turn-on probe bearing aggregation-induced emission (AIE) characteristics to rapidly visualize lipase activities has been constructed with compound **P1**. Lipase of concentrations as low as 0.1 mg mL^{-1} can be detected and the assay range of lipase was located in $0.1\text{--}1.3 \text{ mg mL}^{-1}$. On the other hand, the selectivity of probe **P1**, dynamic monitoring of enzymatic reaction and preliminary commercially enzyme activity screening was also summarized.

Keywords: aggregation-induced emission; lipase activity assay; tetraphenylethylene; hydrolysis reaction; fluorescence “turn-on”

1. Introduction

With the rapid development of green and sustainable chemistry, biocatalysis has been generally employed to synthesize highly stereoselective and enantiomerically pure products.[1-5] As a key component in most extensively used biocatalysts, lipases (triacylglycerol ester hydrolase, E.C. 3.1.1.3) are versatile biocatalysts that can catalyze various types of reactions, such as esterification, interesterification, aminolysis, hydrolysis, and many important classic organic reactions under mild conditions, which produce numerous important chemicals, including pharmaceuticals, agrochemicals, flavouring compounds, biopolymers, and biodiesel.[6-10] Therefore, developing technically convenient yet effective assays for monitoring lipase activity is of critical importance both for the industrial lipases optimization and for the drug discovery and medical diagnosis of diseases. So far, very rare methods have been established for lipase activity assay. Huo and Tian developed a colorimetric probe by using Tween 20 functioned GNPs (gold nanoparticles) as a reporter.[11] J.F. Jette and E. Ziomek described a quantitative lipase assay based on the interaction of Rhodamine B with fatty acids released during the enzymatic hydrolysis of triglycerides.[12]

On the other hand, it is well known that the fluorometric assay is remarkably important owing to its unparalleled sensitivity, simplicity, rapid implementation and applicability to high-throughput screening.[13-17] Fluorophores with aggregation-induced emission (AIE) characteristics have

recently emerged as promising materials for biosensing and imaging applications[18]. Unlike conventional fluorophores suffering from fluorescence quenching in high concentration owing to strong π - π interaction, AIE luminogens such as tetraphenylethene (TPE) and its derivatives enjoy high emission efficiency when their molecules are aggregated or bind to specific analytes.[19-21] To date, a great number of AIE-based “turn-on” biosensors have been reported to target proteins, nucleic acids and glucose with superior sensitivity.[22-37] Recently, some AIE-based probes to detect specific enzymes such as Caspases, MMPs, ACE and SIRTs were reported and opened new opportunities for bioprobe design with optimized performance. [38-43] However, interestingly, there are no reports concerning its utilization in the lipase activity assay. Generally, the lipase can catalyzed hydrolysis of the ester group which was connected to a luminogen then alters the properties itself and thus leads to the variation of fluorescence. In another way, some drawbacks still exist for the reported lipase assays, in these previous work, some specific substrates and indicator assays have been used as the main screening tool, however, all of which possessed relatively complicated production process and were not conveniently synthesized and some of them suffer from needing laborious procedures for preparation. Thus, development of a simple and sensitive biosensor for lipase is essential and significant.

Scheme1 is here

Herein, we report a new fluorometric turn-on assay for lipase activity by manipulating the aggregation and deaggregation of probe **P1**. The whole design rationale is illustrated in Scheme 1 and explained as follows: (1) probe **P1** is derived from tetraphenylethylene (TPE) with an ester group. It is well known that TPE and its derivatives show AIE (aggregation induced-emission) behavior; they are almost non-fluorescent when dissolved in organic solvent, but they become strongly emissive after aggregation. In fact, various chemo/biosensors have been disclosed based on the AIE feature; (2) because of the presence of the long alkoxy chain $-\text{OC}_6\text{H}_{13}$ group it is expected that probe **P1** is soluble in a certain solvent, hence probe **P1** is weakly emissive in solvent according to previous studies; (3) lipase-catalyzed cleavage of the ester group in probe **P1** will induce the transformation of **P1** into TPE-COOH, the $-\text{OC}_6\text{H}_{13}$ unit will be cut off, which may release an insoluble TPE residue (TPE-COOH) into the solvent. Accordingly, aggregation of TPE-COOH in solvent will occur and resulting in strong emission, that is to say, turn on the fluorescence. In this way, a simple fluorescence “turn-on” assay for lipase activity can be established with probe **P1**,

through this pathway, the detection of lipase activity with high sensitivity and selectivity is achieved. To our knowledge, this is the first use of AIE luminogen as fluorescence indicators to evaluate lipase activity. We believe that the research of screening lipase activity would not only broaden the spectrum of AIE luminogens-based enzyme assays but also stimulate the development of AIE-based assays for new targets.

2. Experimental section

2.1. Materials

All chemicals and reagents were commercially available and used as received without further purification. Tetrahydrofuran (THF) were dried over and distilled from K-Na alloy under an atmosphere of dry nitrogen. 4-Dimethylaminopyridine (DMAP), 4-bromobenzophenone, n-butyllithium were purchased from Aldrich. Dicyclohexylcarbodiimide (DCC), p-Toluene sulfonic acid (PTSA) monohydrate were purchased from Acros Organics. Lipases from *Pseudomonas cepacia* (PCL), *Candida Antarctica B* (CALB), *Porcine pancreas* (PPL), *Candida rugosa* (CRL), *Pseudomonas fluorescens* (PFL), *Aspergillus niger* (ANL) were all ordered from Novozymes. Caspase-3, lysozyme, pepsin, albumin from human serum (HSA), acylase, glucose oxidase, α -amylase, trypsin and laccase were all purchased from Sigma-Aldrich. 1-(4-Bromophenyl)-1,2,2-triphenylethene (TPE-Br) was synthesized according to a previous literature.[44]

2.2. Instrumentation

^1H and ^{13}C NMR spectroscopy study was conducted with a Varian Mercury 300 spectrometer using tetramethylsilane (TMS; $\delta = 0$ ppm) as internal standard. Fluorescence spectra were performed on a Hitachi F-7000 fluorescence spectrophotometer with a Xe lamp as the excitation source. Elemental analyses were performed by a 73 CARLOERBA-1106 micro-elemental analyzer. EI-MS spectra were recorded with a Finnigan PRACE mass spectrometer. Dynamic laser scattering (DLS) measurements were performed on a commercial laser light scattering instrument (Malvern ZEN3690, Malvern Instruments) at 25 °C.

2.3. Synthesis

2.3.1. Synthesis of 4-(1,2,2-Triphenylethenyl)benzoic acid (TPE-COOH).

1-(4-Bromophenyl)-1,2,2-triphenylethene (TPE-Br) was synthesized according to a reported literature. To a solution of TPE-Br (6.0 mmol, 2.466 g) in distilled THF (80 mL), 5.0 mL (12.0 mmol) of n-butyllithium solution (2.4 M in hexane) was added dropwise under N_2 at 78 °C. The reaction mixture was stirred and kept at 78 °C for two hours. Excess dry ice was added in one portion followed by stirring over six hours at room temperature. The resulting solution was acidified with an

excess amount of 1 M hydrochloric acid and extracted with DCM three times. The organic layer was combined and dried using NaSO₄. After filtration, removal of the solvent gave a crude product, which was purified by silica-gel column chromatography to obtain the desired product (1.93 g, 5.13 mmol) as a white solid. Yield: 85%. ¹H NMR (300 MHz, CDCl₃): δ (TMS, ppm) 7.91-7.86 (d, 2H), 7.19-7.09 (m, 11H), 7.06-6.98 (m, 6H). ¹³C NMR (75 MHz, CDCl₃): δ (TMS, ppm) 172.64, 150.11, 143.79, 143.52, 143.39, 142.97, 140.31, 130.97, 130.73, 130.17, 128.43, 127.97, 127.34, 126.41, 126.08, 125.72.

2.3.2. Synthesis of compound **P1**

To a DCM solution, TPE-COOH (1.5 mmol, 0.565 g), DCC (2.25 mmol, 0.464 g), DMAP (0.3 mmol, 0.036 g), PTSA monohydrate (0.3 mmol, 0.057 g) and 1-Hexanol (6.0 mmol, 0.445 g) were added. The reaction mixture was stirred at room temperature for 24 hours. After solvent evaporation, the crude product was purified by silica gel chromatography to afford the target product (0.32 g, 0.74 mmol) as a gel-like solid. Yield: 49%. ¹H NMR (300 MHz, CDCl₃): δ (TMS, ppm) 7.78 (d, 2H), 7.11 (br, 11H), 7.03-7.01 (m, 6H), 4.26 (t, 2H), 1.72 (t, 2H), 1.41-1.26 (m, 6H), 0.89 (br, 3H). ¹³C NMR (75 MHz, CDCl₃): δ (TMS, ppm) 166.53, 148.62, 143.18, 143.09, 142.28, 139.91, 131.21, 128.89, 128.17, 127.78, 127.64, 126.77, 126.65, 64.98, 31.40, 28.61, 25.64, 22.48, 13.97. MS (EI), m/z [M]: 460.24, (calcd: 459.98). Anal. Calcd for: C₃₃H₃₂O₂: C, 86.05; H, 7.00. Found: C, 86.29; H, 6.89.

2.4. Preparation of the solution of **P1** and lipase

A stock solution of **P1** (1×10^{-4} M) was prepared by dissolving the **P1** solid (0.46 mg) in hexane (10 mL). The solution of **P1** (1 μM) in hexane were prepared by placing 0.1 mL of **P1** stock solution, then diluting the solution to 10 mL with hexane. For a series of commercial purchased lipase (CALB) samples solutions with concentration gradients (0.1 to 1.3 mg mL⁻¹) and other commercial lipases for preliminary screening (1.0 mg mL⁻¹) were dissolved in 0.1 M potassium phosphate buffer (pH=7.0) which were prepared by K₂HPO₄ and KH₂PO₄.

2.5. Lipase activity assay and dynamic monitoring of enzymatic hydrolysis

Different concentrations of lipase stock solutions (0.1 to 1.3 mg mL⁻¹) 2 mL were mixed with 1 μM **P1** solution 2 mL in a 10 mL flask and stirred at room temperature for 20 min. Then the organic layer was taken to proceed fluorescence measurements with an excitation wavelength of 350 nm. The enzymatic hydrolysis process was monitored by the fluorescence spectral measurements which scanned at intervals of 120 s.

Scheme 2 is here.

3. Results and Discussion

3.1. Design, synthesis and structural characterization of **P1**

The design rationale is illustrated in Scheme 1. As is known to all, the hydrolysis of ester group can be carried out by lipase, hence this mode of stimuli can activate the fluorescent response through a simple reaction. The hydrolytic cleavage of the ester bond in **P1** catalyzed by lipase and TPE-COOH was produced, then, the rapid aggregation of TPE-COOH in the same solution will be triggered because of its lower solubility which caused by the loose of -OC₆H₁₃ group.

The biosensor **P1** was synthesized using the synthetic protocol described in Scheme 2 and starting from 4-bromobenzophenone and diphenyl methane leading to compound TPE-Br, which was esterified with 1-hexanol to yield probe **P1**. The structure and purity of **P1** were confirmed by ¹H NMR, ¹³C NMR, EI-MS and elemental analysis.

Fig. 1 is here.

3.2. AIE properties of TPE-COOH

As mentioned above, we assumed that the assay of lipase activity was mainly completed by monitoring the fluorescence that attribute to the forming of TPE-COOH. Thence, the AIE properties of TPE-COOH were essential to our study. We firstly investigated the photophysical properties of TPE-COOH in the aggregated state. The fluorescent property of TPE-COOH was investigated using THF and hexane solvent mixtures (Fig. 1). TPE-COOH is almost non emissive in pure THF solution. With increasing the hexane content ranging from 0 to 60%, the fluorescence intensity remains at a low level. Further addition of hexane into the mixture results in a sharp and dramatic enhancement of the fluorescence intensity. At the 95 vol% hexane content, the fluorescence intensity is about 49-fold higher than that in the pure THF solution, suggesting that compound TPE-COOH is AIE-active.

Fig.2 is here.

3.3. Lipase activity assay

Obviously, the difference in solubility and emission behaviors of probe **P1** and its precursor TPE-COOH inspired us to utilize the probe for lipase activity assay. Lipase could catalyze the

hydrolysis reaction of monoester to a hydroxyl group in high efficiency. As the enzymatic product TPE-COOH is insoluble in some poor solvent, it would form aggregates to turn on the fluorescence. Thus, **P1** can work as a lipase activity reporter. To check the feasibility of this design, we investigated the fluorescence change when the probe **P1** was treated with lipase-buffer solution in hexane. Hexane solution of biosensor **P1** (1.0 μM) was prepared for the fluorescent spectral studies and *Candida Antarctica* lipase B (CALB) was selected to evaluate the performance of **P1**. As expected, the fluorescent intensity of probe **P1** (1.0 μM) was much weak in the testing conditions, and nearly no photoluminescence could be seen upon excitation even in a dark room (see Fig. 2). Then, we tried to add some lipase to the diluted solution of probe **P1**, quickly, the blue fluorescence emission turns on after mixing with lipase-buffer solution. As shown in Fig. 2A, after addition of lipase, the fluorescence intensity of the organic phase started to increase; even at the concentration of lipase as low as 0.1 mg mL^{-1} , and the intensity continued to while the concentration of lipase increased. When the concentration of lipase in the probe/lipase complex reaches 1.1 mg mL^{-1} , the fluorescence intensity has increased by 17-fold compared to the blank. Upon addition of 1.3 mg mL^{-1} lipase, the fluorescence increases gradually and the intensity reaches a plateau. This confirmed our idea mentioned above: accompanying with the leakage of the alkoxy chain of the ester group linked to a TPE core in the presence of some lipase, its aggregation behavior changed to give detectable fluorescent signal in response to the analytes. Ideally, the change should be from one extreme case to another, just like in our case, from the “off” state to “on”. Also, Fig. 2B illustrated fluorescence titration curve of compound **P1** (1.0 μM) with lipase in solution, an approximate linear correlation could be observed between the emission intensity and the concentration of lipase within the range from 0 to 1.1 mg mL^{-1} . The photograph was taken from the solution of probe **P1** itself and the probe after incubation with lipase in hexane solution under UV illumination at 365 nm. It is obvious that blue fluorescence is turned on after being treated with lipase, so significant difference in fluorescence emission was observed, and it could be easily distinguished by the naked eye under UV light, directly.

Fig.3 is here.

According to the calibration curve (Fig. 3), the relative fluorescence intensity change ratio (I/I_0) at 420nm also presents a linear increase with the concentration of lipase in the range from 0 to 1.1 mg mL^{-1} ($R^2=0.9915$), which was consistent with the variation of the fluorescence intensity. Furthermore,

to check the reproducibility of the probing behavior, we repeated the above experiments for three times, and the testing results were also shown in Fig. 3. It was easily seen that the error bars were not big and the reproducibility was relatively good.

Fig.4 is here.

To further verify the formation of aggregates of probe **P1** with lipase, particle sizes were carried out by the DLS (Dynamic Laser Scattering) analysis, and the results were displayed in Fig. 4. In the stock solution of probe **P1** (1 μM) with nothing added, the nanoparticles could be observed in the range of 20-100 nm, while the mean diameter of **P1** is approximately 300 nm after treated with lipase and the maximum diameter was even up to 1 μm , which revealed that aggregates are indeed present in the solution. The results suggested that after incubation with lipase, the **P1** was hydrolyzed to the precursor TPE-COOH, due to its insolubility in hexane solution, resulting in the formation of large aggregates, which was consistent with the fluorescence spectra. It suggested that the aggregation size significantly influenced the fluorescence of the solution, because the AIE effect could be triggered by the larger aggregates.

Fig.5 is here.

To examine the possibility of quantitative analysis of lipase activity, we conducted the experiment of dynamic monitoring of enzymatic reaction. Fig. 5 displays the variation of the fluorescence intensity at 420 nm of probe **P1** (1 μM) after incubation with different amounts of lipase (0-1.3 mg mL^{-1}) for different times at room temperature. Obviously, the fluorescence intensity increased gradually by prolonging the incubation time, with different concentrations of lipase incubated, the intensity of the probe increases gradually with different magnitudes. The fluorescence changes more obviously at the beginning for all lipase concentrations but becomes slower in the later period. The fluorescence intensity reaches the plateau after about 14 min, indicating that probe **P1** is nearly completely hydrolysed by lipase and the test could be completed within 14 minutes, which was much faster than traditional turbidimetric assays. Moreover, the fluorescence intensity was enhanced more rapidly when the concentration of lipase was higher. This is understandable by considering the fact that the hydrolysis of probe **P1** would be facilitated in the presence of a high concentration of lipase. As depicted in Fig. 5, such a fluorescence enhancement was still detectable even when the concentration

of lipase was as low as 0.2 mg mL^{-1} within 5 minutes. These results reveal that the lipase turn-on mode could be applied potentially for the real-time activity assay of lipase. Furthermore, to address the selectivity of probe **P1**, the selectivity of this fluorometric commercial assay for lipase was also tested, a control experiment with other nonspecific enzymes including caspase-3, lysozyme, pepsin, HSA, acylase, glucose oxidase, α -amylase, trypsin and laccase was performed with a fixed concentration (1.0 mg mL^{-1}). As shown in Fig. 6, the results revealed that about 15-fold fluorescence enhancement of the probe was recorded with the effect of lipase, while negligible changes in emission intensity were observed for other enzymes. Thus, interferences from other enzymes can be neglected and the probe **P1** is highly specific to lipase. To further demonstrate that fluorescence enhancement was due to lipase-catalyzed hydrolysis rather than other effects, a control experiment between lipase and inactivated lipase was conducted. Herein, lipase was deactivated in high temperature (100°C) and it is well known that high temperature is one of the traditional protein denaturants. According to our proposed mechanism, inactivated lipase could not hydrolyze the probe **P1** and no fluorescence enhancement is expected. The fluorescence result in Fig.7 shows that inactivated lipase is not able to turn on the fluorescence, which reveals that it is the catalysis effect of lipase that functions on this process, excluding other interference effects such as hydrophobic interaction and electrostatic interaction between proteins and probes.

Fig.6 is here.

Fig.7 is here.

A preliminary screening was carried out using this system to test the activities of six commercial purchased lipase samples (CRL, PPL, RFL, ANL, CALB, and PCL). The fluorescence intensity and the I/I_0 value of probe **P1** solution in the presence of a fixed concentration (1.0 mg mL^{-1}) of lipase samples were monitored, respectively, within a fixed time interval. As shown in Fig. 8, probe **P1** incubated with CALB, PPL, and ANL led to significant fluorescence changes, whereas with RFL, PCL and CRL no obvious fluorescence changes were observed. Moreover, the different fluorescence changes (strong emission in CALB, PPL, and ANL and weak emission in PFL, PCL and CRL) were attributed to the different lipase activities. With fluorescence spectroscopy quantitative detection, we conclude that the lipase activity decreases in the following order: $\text{CALB} > \text{ANL} > \text{PPL} > \text{CRL} > \text{RFL} > \text{PCL}$. This order is consistent with the results detected with a pH-stat method,[11]

demonstrating the possibility that the fluorometric turn-on assay protocol can be used as an alternative to conventional methods for a sensitive assay of lipase activity. The inserted photograph in Fig. 8 was taken from the probe **P1** after incubation with different commercial lipase in hexane solution under UV illumination at 365 nm. It is obvious that different blue fluorescence emission related to the activity of lipases, it could be easily distinguished by the naked eye under UV light.

Fig.8 is here.

Conclusions

In conclusion, a fluorescence “turn-on” probe **P1** for lipase activity assay was established by making use of the AIE technology. The obtained results demonstrated that: 1) the high sensitivity of probe **P1**, coupled with its simple structure and easy preparation, and not require specific instrumentation or complicated experimental steps, would surely contribute to its practical applications in different fields. 2) Lipase of concentrations as low as 0.1 mg mL^{-1} can be detected and the assay range of lipase was located in $0.1\text{--}1.3 \text{ mg mL}^{-1}$. DLS studies confirmed the formation of aggregates and accordingly lipase-catalyzed hydrolysis of the fluorescent probe led to self-aggregation of insoluble products, resulting in strong fluorescence. The fluorescence “turn-on” method provided a platform for the research on lipase activity assay and other probes, including those of “on-off”, are still under way in our laboratory.

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Legend of Figures

Scheme 1. Schematic illustration of sensing mechanisms of probe **P1** in lipase activity assay.

Scheme 2. Synthesis route to probe **P1**.

Fig. 1 (A) fluorescence spectra of TPE-COOH (1 μM) in THF-hexane mixtures with different fractions of hexane (f_H). (B) Plot of I/I_0 at 425 nm versus f_H , where I_0 is the fluorescence intensity in pure THF solution. Excitation wavelength: 350 nm.

Fig. 2 The fluorescence spectra of probe **P1** (1.0 μM) after incubation with different concentrations of lipase-buffer (0.1 M potassium phosphate) solution at 25 °C; inset shows the photo of the corresponding solutions of probe **P1** (1.0 μM) without (left) and with (right) lipase (1.3 mg mL⁻¹) after incubation at 25 °C for 20 min under UV light (365 nm) illumination. Excitation wavelength: 350 nm.

Fig. 3 Plot of I/I_0 and expanded linear region versus the different concentration (0.1-1.1 mg mL⁻¹) of lipase, where I and I_0 are the fluorescence intensities in the presence or absence of analytes. Excitation wavelength: 350 nm.

Fig. 4 Dynamic light scattering (DLS) results for the solution of probe **P1** in the absence and presence of lipase: (a) probe **P1** (1.0 μM) in hexane solution; (b) probe **P1** (1.0 μM) in hexane solution after incubated with lipase (1.0 mg mL^{-1}) for 20 min.

Fig. 5 Time-dependent fluorescence intensity of probe **P1** (1.0 μM) in hexane solution at 420 nm versus the hydrolysis reaction time in the presence of different concentrations of lipase at 25 $^{\circ}\text{C}$. Excitation wavelength: 350 nm.

Fig. 6 Variation of the fluorescence intensity of probe **P1** (1.0 μM) in hexane solution at 420 nm after treated by 1) caspase-3, 2) lysozyme, 3) pepsin, 4) HSA, 5) acylase, 6) glucose oxidase, 7) α -amylase, 8) trypsin, 9) laccase, 10) lipase was performed with a fixed concentration (1.0 mg mL^{-1}).

Excitation wavelength: 350 nm.

Fig. 7 Fluorescence spectra of the probe **P1**, probe **P1**/activited lipase and probe **P1**/inactivited lipase. Concentration: probe **P1** ($1.0 \mu\text{M}$); activited lipase (1.0 mg mL^{-1}); inactivited lipase (1.0 mg mL^{-1}); Excitation wavelength: 350 nm. Inset: photographs of corresponding solutions (a) probe **P1** only, (b) probe **P1**/activited lipase and (c) probe **P1**/inactivited lipase under UV light illumination at 365 nm.

Fig. 8 Plot of I/I_0 intensity of probe **P1** upon the addition of a fixed concentration (1 mg mL^{-1}) of commercial lipase samples, respectively, and within a interval of 20 min. (Inset) Corresponding visual fluorescence under UV light illumination at 365 nm.