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A Simple Fluorescent Probe Based on a Pyrene Derivative for Rapid Detection of Protamine and Monitoring of Trypsin Activity

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We report the synthesis of a simple pyrene derivative and its application in protamine detection and monitoring of trypsin activity. This assay can be conducted in aqueous solution and features rapid response, visual detection, high sensitivity and selectivity. The limit of detection of protamine was 0.5 μg mL^{-1}. The IC_{50} value of soybean trypsin inhibitor was estimated to be 0.51 U mL^{-1}.

Introduction

Protamines are a group of strongly basic proteins in which arginine (Arg) accounts for approximately 67% contents with an isoelectric point of 12–13[1]. They are found to play important roles in many biological processes and clinical treatment[2]. Protamine can hardly be detected by the traditional method measuring UV-absorption at 280 nm because it is composed of aliphatic amino acids only. So far, several approaches have been developed for the detection of protamines including HPLC[3], MS[4], electrochemical method[5], colorimetric and fluorometric assay and so forth[6]. However, some limitations such as requirement of sophisticated instruments, tedious sample preparation, and complicated synthesis of the probe still restrict practical application of these techniques. Therefore, developing rapid and simple methods for the selective detection of protamine is still attractive.

Trypsin is one of the most important digestive enzymes which is formed in the pancreatic acinar cells, and responsible for cleaving peptides mainly at the C-terminal side of arginine or lysine (lys) residues[7]. Trypsin not only plays key roles in many biological processes, but also is related with some diseases[8] such as cystic fibrosis[9], pancreatic diseases[10] and certain cancers[11]. This clinical relevance for the diagnosis and treatment of diseases has drawn considerable attention to the rapid detection of trypsin and its activity monitoring. Traditional methods for trypsin detection include enzyme-linked immunosorbent assay[12], gel electrophoresis[13] and radioimmunoassay[14], while many new approaches have been developed in recent years, such as electrochemical assay[15], quartz crystal microbalance (QCM) sensor[16], surface-enhanced Raman scattering (SERS)[17], optical detection based on nanomaterials and organic fluorescent probes[18]. Among them, fluorescent approaches have been widely used because of their simple operation, high sensitivity and ease of observation. Many fluorescent probes have been designed for the detection of cationic peptides or proteins and enzymes in literature[19]. The “fluorescence on-off / off-on” which mainly rely on the changes of fluorescence intensity are the mostly used detection mode of protamine and trypsin assay[20]. Under these circumstances, many external factors such as environmental interference, instrument status and photo intensity of excitation would affect the accuracy of the results. Therefore, a ratiometric sensor based on dual luminescence is preferred because it affords inherent correction and ensures the sensitivity of the probe. Pyrene derivatives are excellent candidates for this purpose owing to their transition between monomer and excimer[18b,21]. However, many of these probes suffer from poor solubility in water and require laborious synthesis[22]. Thus, it is desirable to develop a simple, fast-response and inexpensive probe for monitoring trypsin activity. In this work, we employed an anionic pyrene derivative (sodium 3-(pyren-1-yloxy) propane-1-sulfonate, PyOPS, Scheme 1) for the detection of protamine and trypsin based on the formation and dissociation of pyrene’s excimer. Pyrene derivatives have been widely used for constructing fluorescent sensing systems owing to their chemical stability, high fluorescence quantum yield and long fluorescence lifetime[20]. In our strategy, we envision that PyOPS mainly exists in a monomer form in aqueous media due to the excellent water solubility of sulfonic groups and electrostatic repulsion between them. Accordingly, PyOPS exhibits monomer fluorescence emission. Then, in the presence of protamine, the transformation from the pyrene monomer to the excimer of PyOPS driven by electrostatic interactions between the

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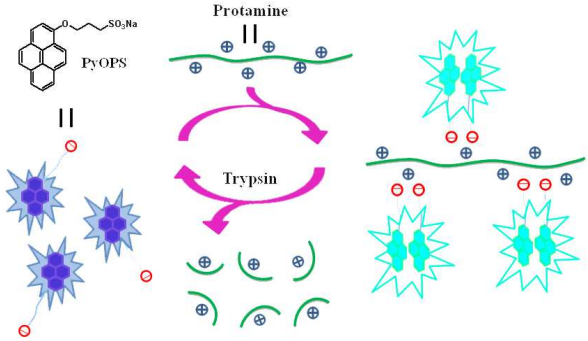
guanidine residues of the protamine and sulfonic groups of PyOPS would occur, thereby leading to much enhanced excimer emission. Next, subsequent addition of trypsin will catalyze the hydrolysis of protamine whose collapse induces the weakening of pyrene excimer emission. These changes provide a two-wavelength fluorescent response which enables PyOPS a ratiometric sensor for the detection of protamine and trypsin (Scheme 1). This assay can be conducted in 100% aqueous solution and features rapid response, visual detection, high sensitivity and selectivity. Details for the preparation of the probe and investigation of the sensing performance are given in the following sections.

Results and discussion

PyOPS was synthesized by a one-step reaction between 1-hydroxypyrene and 1,3-propanesulfonate with a yield of 62.5% (Fig. S1-S4). As expected, the aqueous solution of PyOPS showed typical pyrene monomer emission at 385 nm, 405 nm and 425 nm upon excitation with 365 nm (Fig. 1). It was noted that upon addition of increasing concentrations of protamine, the fluorescence emission of the PyOPS monomer decreased gradually, whereas a new band around 486 nm emerged which is the characteristic of the pyrene excimer. This result was attributed to the formation of PyOPS aggregates induced by protamine through electrostatic attraction and hydrophobic interactions between protamine and PyOPS[23]. In addition, the solution colour of PyOPS upon UV (365 nm) irradiation changed from blue to green in the presence of protamine which could be readily observed with naked eyes (Fig. 1A). From Fig. 1B we can see that a good linear relationship (R = 0.995 from 0.5 to 8 μg mL^{-1}) was obtained by fitting analysis of (I_{486}/I_{385}) and the concentration of protamine, where I_{485} and I_{385} represented the emission intensity at 486 nm and 385 nm of PyOPS, respectively. It is indicated that PyOPS could be used for ratiometric and quantitative detection of protamine and the limit of detection (LOD) is as low as 5×10^{-7} g mL^{-1}. The selectivity of the protamine assay was also examined by comparing the emission spectra of PyOPS responding to various proteins with different pI values under the same conditions including bovine serum albumin (BSA, pI 4.7–5.3), hemoglobin (pI 6.84) and lysozyme (pI 11.0) (Fig. S5 and S6). Also, two small biomolecules (Lys, pI 9.74; Arg, pI 10.76) which have similar levels of cationic charges with protamine were investigated. When 4 μg mL^{-1} of each interferent was added into the PyOPS solutions, little changes in the emission profiles were observed. These results clearly show that this fluorometric assay is highly selective towards protamine due to its specific structure and property.



Scheme 1 Chemical structure of PyOPS and schematic illustration of the process in the detection of protamine and trypsin.

Since trypsin could catalyze the hydrolysis of protamine, which would result in the dissociation of the aggregates of PyOPS templated by protamine, we applied this system in a continuous fluorometric assay for monitoring the activity of trypsin. Fig. 2 shows the emission spectra of PyOPS and its mixtures with protamine, protamine-trypsin and protamine-trypsin-inhibitor. It is observed that in the presence of trypsin, the emission at 486 nm decreased along with the recovery of emission at 385 nm, implying the collapse of protamine/PyOPS complex caused by trypsin. However, with the addition of inhibitor, the emission of PyOPS excimer in the presence of protamine was hardly affected which could be attributed to the retard of protamine hydrolysis by the inhibitor. The time-dependent relative intensity (I_{486}/I_{405}) changes of PyOPS in the presence of different concentrations of trypsin were compared in Fig. 3A. With the increase of trypsin concentration, a faster reaction rate of initial hydrolysis and a reduced intensity change were obtained. The limit of detection of this assay is estimated to be 0.5 μg mL^{-1}. Furthermore, the process of hydrolysis catalyzed by trypsin could also be observed by monitoring the fluorescence colour changes of the PyOPS solutions (Fig. 3B), which makes the visual detection of trypsin possible.

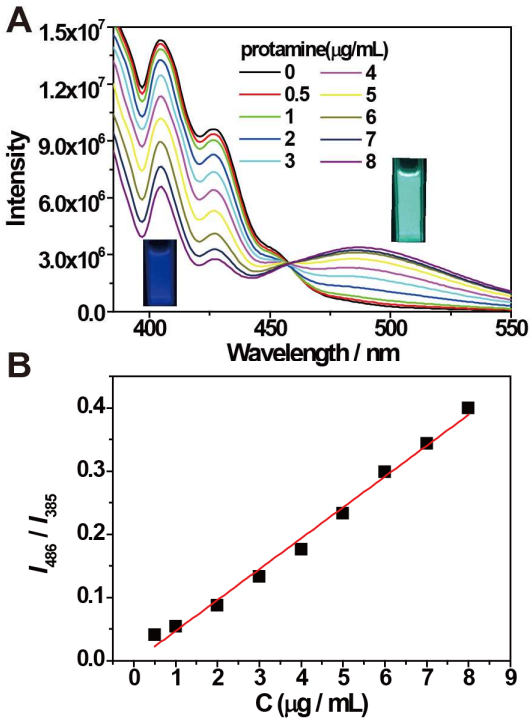


Fig. 1 (A) Emission spectra of PyOPS (3.0 × 10^{-5} M) in the presence of increasing amounts of protamine as indicated in 10 mM HEPES buffer (pH 7.4). (B) Plots of relative intensity (I_{486}/I_{385}) of PyOPS versus protamine concentrations. λ_{ex} = 365 nm.

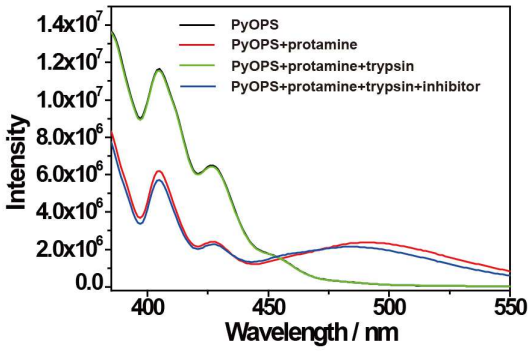


Fig. 2 Emission spectra of PyOPS (3.0×10^{-5} M) in the absence and presence of protamine, protamine-trypsin and protamine-trypsin-inhibitor in 10 mM HEPES buffer (pH 7.4). λ_{ex} = 365 nm; [protamine] = 6.0 μg mL^{-1}; [trypsin] = 8.0 μg mL^{-1}; [inhibitor] = 8.0 U mL^{-1}.

To evaluate the selectivity of the fluorometric trypsin assay, several enzymes such as alkaline phosphatase (ALP), lysozyme, BSA, albumin and pepsin were investigated under the identical conditions. It is shown in Fig. S7 that the presence of these enzymes barely changed the emission spectra of PyOPS–protamine complex except for trypsin. The results were illustrated in Fig. 4 for quantitative appraisal of the specificity of this fluorometric assay. Herein, I_0 and I are the ratios of the intensity at 486 nm to the intensity at 405 nm, I_{486}/I_{405}, in the absence and presence of each enzyme, respectively. It is clearly seen that the value of $(I - I_0)$ in the presence of trypsin is at least 9 times higher than that of other enzymes, indicating the high selectivity of this approach towards trypsin.

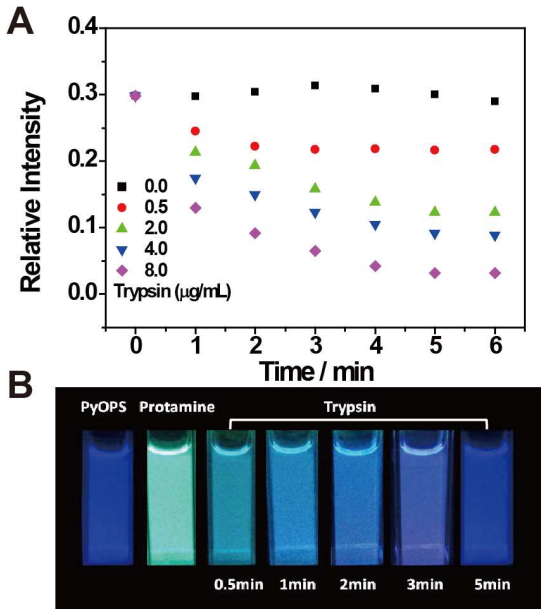


Fig. 3 (A) Relative intensity of the PyOPS–protamine complex versus incubation time for the hydrolysis of protamine with various concentrations of trypsin in 10 mM HEPES buffer (pH 7.4). [PyOPS] = 3.0×10^{-5} M; [protamine] = 6.0 μg mL^{-1}. (B) Changes of PyOPS emission colours induced by the addition of protamine and trypsin with incubation time indicated. [PyOPS] = 3.0×10^{-5} M; [protamine] = 6.0 μg mL^{-1}; [trypsin] = 8.0 μg mL^{-1}; λ_{ex} = 365 nm.

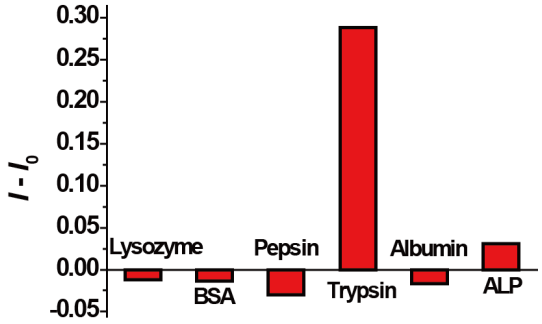


Fig. 4 Relative intensity of the PyOPS–protamine complex in the presence of various proteins in 10 mM HEPES buffer (pH 7.4). [PyOPS] = 3.0×10^{-5} M; [protamine] = 6.0 μg mL^{-1}; [proteins] = 8.0 μg mL^{-1}.

Inhibitor screening for enzymes provided an efficient tool in the discovery of new drugs and the design of sensing systems. Thus, we also employed the present approach to screen soybean trypsin inhibitor (STI). Fig. 5A shows the real-time response of PyOPS to different amounts of STI. It can be seen that the relative intensity of I_{486}/I_{405} increased gradually along with the introduction of more STI. The STI ability for inhibiting trypsin activity is assessed by an IC_{50} value, which represents the concentration of STI required for half inhibition of trypsin activity. This value was calculated to be 0.51 U mL^{-1} according to the plot of inhibition efficiency versus inhibitor concentrations (Fig. 5B). This result suggested that the current strategy could be applied to screening trypsin inhibitors with a good sensitivity and fast response.

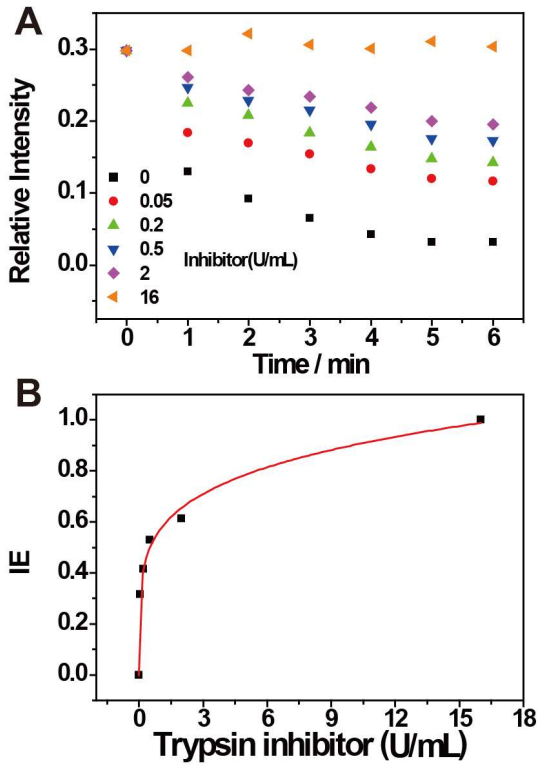


Fig. 5 (A) Relative intensity of the PyOPS–protamine complex versus incubation time in the presence of trypsin at different inhibitor concentrations as indicated in 10 mM HEPES buffer (pH 7.4). (B) Plot of the

inhibition efficiency (IE) versus the concentration of trypsin inhibitor from soybean. [PyOPS] = 3.0×10^{-5} M; [protamine] = 6.0 μg mL^{-1}; [trypsin] = 8.0 μg mL^{-1}.

Conclusions

In conclusion, we have prepared a simple pyrene derivative, PyOPS, and employed it for rapid detection of protamine and monitoring of trypsin activity. The aggregation of PyOPS was induced by protamine through both electrostatic and hydrophobic interactions. A good linear range of 0.5 - 8 μg mL^{-1} and a LOD of 0.5 μg mL^{-1} were obtained. In the presence of trypsin, protamine was hydrolyzed and PyOPS disaggregated gradually along with its excimer transforming to monomer. This transformation could be directly used for trypsin detection with good selectivity and sensitivity. The LOD of this assay is about 0.5 μg mL^{-1}. When the activity of trypsin was inhibited, the dissociation of PyOPS aggregates was retarded. The IC_{50} value of STI was estimated to be 0.51 U mL^{-1}. As a label-free sensing system, the present approach possesses several advantages, including: (1) the preparation of the probe is straightforward because it can be synthesized by a one-step reaction; (2) compared with "turn-on" or "turn-off" sensors, the transition of fluorescence emission of PyOPS between the monomer and excimer provides a ratiometric output and improves the signal to noise ratio by inherent correction; (3) the responses of the probe to analytes could be continuously measured and readily observed with naked eyes.

Experimental

Synthesis of PyOPS 1-hydroxypyrene (**1**) (100 mg,0.454 mmol) was added into 0.5 mL NaOH (2.5M) solution in the flask ,then 0.5 mL dioxane was added to improve the solubility. 1, 3-propanesulfonate (**2**) (52 mg, 0.432 mmol) was dissolved in 2 mL dioxane, and then added into the mixture. The mixture was stirred in nitrogen atmosphere at room temperature for 12 h. The crude product was filtered and then washed with THF and acetone to give PyOPS (103.3 mg; yield 62.5%). ^{1}H NMR (500 MHz, DMSO-*d*6) δ: 8.38 (d, *J* = 9.2 Hz, 1H), 8.24 (d, *J* = 8.5 Hz, 1H), 8.19 (t, *J* = 8.0 Hz, 2H), 8.13 (d, *J* = 9.2 Hz, 1H), 8.07 (d, *J* = 9.0 Hz, 1H), 8.02 (t, *J* = 7.6 Hz, 1H), 7.96 (d, *J* = 9.0 Hz, 1H), 7.75 (d, *J* = 8.5 Hz, 1H), 4.47 (t, *J* = 6.4 Hz, 2H), 2.76 (d, *J* = 7.3 Hz, 2H), 2.20-2.29 (m, 2H). ^{13}C NMR (125 MHz, DMSO-*d*6) δ: 153.16, 131.74, 131.60, 127.79, 126.85, 126.75, 126.49, 125.45, 125.10, 124.96, 124.78, 124.67, 124.58, 121.32, 119.89, 110.38, 68.31, 48.58, 26.06. HRMS (m/z): calculated for $(M-Na)^-$ 339.0691; found 339.0699.

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