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PAPER

BSA–tetraphenylethene derivative conjugates with aggregation-induced emission properties: Fluorescent probes for label-free and homogeneous detection of protease and α1-antitrypsin†

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Herein, BSA–tetraphenylethene derivative conjugates with aggregation-induced emission (AIE) properties were constructed and used as fluorescent probes for label-free detection of protease and α1-antitrypsin. Conjugated AIE probes were formed based on the electrostatic induced assembly between an ammonium cation of quaternized tetraphenylethene salt and carboxyl anion groups of BSA. While water soluble quaternized tetraphenylethene salt showed very low fluorescence in its well-dispersed state, obvious enhancement in the fluorescence of the aggregated tetraphenylethene derivative on the BSA templates was achieved due to the abnormal aggregation-induced emission properties of tetraphenylethene. These BSA–tetraphenylethene derivative conjugates enabled label-free detection of protease. In the presence of trypsin, the BSA templates were enzymatically hydrolyzed and the conjugates decomposed. Therefore the quaternized tetraphenylethene molecules became increasingly isolated from each other. Accordingly, the aggregation to dispersing state change of tetraphenylethene derivative resulted in an obvious decrease in the fluorescence of the conjugates probes and enabled the sensitive and selective detection of trypsin. Furthermore, upon addition of α1-antitrypsin, the enzymatic activity of trypsin was inhibited and the fluorescence was consequently preserved. Sensitive detection of α1-antitrypsin was thus realised. The protein–tetraphenylethene derivative conjugates with aggregation-induced emission properties therefore show great promise for the monitoring of biological processes and cancer diagnostics with simplicity, high sensitivity, and rapid response.

1. Introduction

Proteases are involved in the control of a large number of physiological processes.[1,2] These enzymes play their role by catalyzing the hydrolytic cleavage (proteolysis) of peptide bonds at specific sites along the amino acid sequence and breaking down proteins. Many diseases, including cancer, rheumatoid arthritis, cardiovascular and neurodegenerative diseases, are characterized by an altered protease activity.[3–5] For example, trypsin is a very well studied pancreatic serine protease with substrate specificity based upon positively charged lysine and arginine side chains.[3] Inappropriate proteolytic activity can make this enzyme a pluripotent effector in acute and chronic pancreatic diseases. Elevated levels of the trypsin enzyme may be an aggravating factor in pancreatic diseases and have also been found in a variety of tumors.[5] Thus the regulation of physiological proteolytic processes of trypsin needs to be highly controlled. As one of the protease inhibitors, α1-antitrypsin is the major protease inhibitor of human plasma protein, regulating trypsin by the formation of covalently bound complexes.[6] α1-antitrypsin has been shown to be related to emphysema, liver sclerosis and other diseases and has been increasingly proposed as an important biomarker of liver cancer in the very early stages. Consequently, convenient methodologies for the rapid monitoring of the activity of disease-related enzymes (such as trypsin) and the concentration of their inhibitors continue to be in high demand for biomedical research and medical disease diagnostics.[7,8]

Currently, standard techniques for measuring protease activity based on radioisotopes, or chromogenic substrates require considerable sample preparation, thereby making the assays time consuming, costly and error-prone.[9–11] Several strategies to overcome these limitations have been developed, among which the colorimetric- and fluorescence-based homogeneous assays have been widely studied.[12,13] For example, colorimetric assays for protease taking advantage of the aggregation or deaggregation-induced absorption or color change of gold nanoparticles upon analyte hydrolysis were reported by several

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groups.[14,15] Although, these methods are convenient to use, they do not meet the performance parameters necessary for rapid detection of low levels of enzyme activity, making them unsuitable for point-of-care (POC) diagnostic applications. Fluorescence-based methods are the most prevalent techniques and could provide higher detection sensitivity than the colorimetric methods.[16] These methods are based on fluorogenic substrates that allow the monitoring of specific proteases by either turn-on fluorescence or fluorescence resonance energy transfer (FRET).[17] These fluorogenic substrates, usually the doubly labelled molecular beacons or peptides, are expensive and difficult to synthesize.[18–20] It remains a challenge to develop simple, rapid, cost-effective, easy-to-use and sensitive enzyme assays.

Tetraphenylethene and silole derivatives offer a unique platform for the development of highly sensitive fluorescence-based sensors for chemical and biological targets.[21–23] Tetraphenylethene and silole derivatives are weakly or none fluorescent in well-dispersed state.[24,25] When they aggregate from a well-dispersed state, tetraphenylethene and silole derivatives will change their conformation from a twist state to a coplanar state. The coplanarization could lead to a better molecular conjugation and thus induced enhanced fluorescence emission. This abnormal aggregation-induced emission (AIE) property has been widely used to amplify the fluorescence signal of biomolecules conjugated with AIE-active tetraphenylethene or silole derivative probes for metal ions,[26,27] heparin,[28] nuclease,[29] and phosphatase[30] detection. Similarly, two types of tetraphenylethene derivative-based AIE probes have been developed for nuclease and phosphatase activity studies. One was based on the fluorescence decrease of a quaternized silole salt which has been induced to high fluorescence emission by a negatively charged oligonucleotide. The presence of nuclease catalyzed the hydrolysis of the oligonucleotide backbone, leading to a fluorescence decrease of the silole derivative-based AIE probe.[29] The other AIE probe was constructed based on the electrostatic induced conjugation between adenosine 5′-triphosphate (ATP) and quaternized silole salt.[30] The presence of phosphatase enzymatically hydrolyzed the ATP, and the decrease in AIE probe emission was used to monitor the enzyme activity. Although AIE active fluorescence probe-based methods have emerged as competent strategies for fluorescent bio/chemosensors, it is still highly desired to expand the label-free and homogenous strategy for other proteases activity detection using easily-constructed biomolecule-conjugated AIE probes.

Here, we take advantage of electrostatic attraction between positively charged quaternized tetraphenylethene salt and negatively charged BSA to construct and used as fluorescent AIE-probes. These easily prepared fluorescent probes were induced to high fluorescence emission by BSA templated assembly and conjugation. Importantly, water-soluble bioconjugated complexes were found to be good fluorescent probes for the selective and sensitive detection of the trypsin activity. Subsequently, detection of α1-antitrypsin was realized based on the formation of a covalently bound complex between α1-antitrypsin and trypsin and therefore inhibition of the protease activity. Based on the combination of the protease activity of trypsin to BSA and the AIE phenomenon, a very real time, simple, rapid and sensitive method for sequential detection of trypsin and α1-antitrypsin has been established.

2. Materials and methods

2.1 Materials

The AIE probe (**2**), quaternized tetraphenylethene salt, was synthesized as follows: Into a 250 mL round-bottom flask was added 518 mg (1 mmol) of 1,2-bis[4-(bromomethyl)-phenyl]-1,2-diphenylethene (**1**) and 30 mL triethylamine. The solution was refluxed for 12 h. After being cooled to room temperature, the precipitate was collected by filtration and washed with acetone. A white solid, **2**, was obtained in 76.3% yield (550 mg). ^{1}H NMR (300 MHz, D_2O), δ (TMS, ppm): 1.24 (t, 18H), 3.06 (m, 12H), 4.21 (s, 4H), 7.13 (m, 18H).

Bovine serum albumin, Trypsin and α1-antitrypsin were obtained from Sigma. The water was purified using a Millipore filtration system. All other reagents were of analytical reagent grade and used as received.

2.2 Instrumentation

UV absorption measurements were performed with UV/vis Shimadzu UV-2505 spectrometer. FL spectra were recorded on a Perkin-Elmer LS 55 luminescence spectrometer.

2.3 Construction of fluorescence probe conjugates with BSA and tetraphenylethene derivative

The buffer solution of probes (38 μM) was prepared by mixing 10 μL of AIE probe solution (2.28 mM) and 590 μL of PB buffer solution (10 mM, pH 7.4). The concentration of BSA stock solution was 10 mg mL^{-1}. For the construction of fluorescence probe conjugates, certain small amounts of BSA solution was added intermittently to 600 μL of the buffer solution of probes (38 μM) and carefully mixed. In all the experiments, the FL detections were under the same conditions, the excitation wavelength was set at 303 nm. Both fluorescence and absorption spectral measurements were carried out at room temperature.

2.4 Fluorescence detection of trypsin

The buffer solution of probes (38 μM) was prepared with the addition of 30 μL BSA solution (10 mg mL^{-1}), making the final concentration of BSA 0.5 mg mL^{-1}. For the fluorescence detection of trypsin, a series of trypsin solutions with the same concentration gradients were added to the buffer solution mentioned above, and the final concentrations of trypsin were 0 mU mL^{-1}, 1 mU mL^{-1}, 2.5 mU mL^{-1}, 5 mU mL^{-1}, 7.5 mU mL^{-1}, 10 mU mL^{-1} and 12.5 mU mL^{-1}, respectively. The fluorescence detection was carried out after all the prepared solutions were incubated at 37 °C for 30 min.

In the contrast experiments, the following enzymes were investigated: alkaline phosphatase (ALP), lysozyme, and denatured trypsin with high temperature. The concentrations of these enzymes were all 0.08 mg mL^{-1}, and the fluorescence spectra were measured under the same conditions.

2.5 Dynamic monitoring of enzymatic reaction

The buffer solution of probes were prepared as process 2.4. The enzymatic reaction process was monitored by the fluorescence spectral measurements which scanned at intervals of 90 s.

Fluorescence intensity of the pre-blends were detected with different trypsin concentrations at 0 mU mL^{-1}, 5 mU mL^{-1}, 10 mU mL^{-1}, and 20 mU mL^{-1} at 37 °C.

2.6 Fluorescence detection of α1-antitrypsin

The pretreated solutions were prepared by mixing trypsin solution (15 mU mL^{-1}) and α1-antitrypsin with different concentrations (10 μg mL^{-1}, 100 μg mL^{-1}, 200 μg mL^{-1} and 300 μg mL^{-1}) and each sample was incubated at 37 °C for 20 min. Then the same amount of the pretreated solution was added to the probes solution, and fluorescence spectral measurements were carried out under the same conditions.

3. Results and discussion

3.1 General consideration of the biosensors design

The fluorescence probes were constructed *via* electrostatic attraction between positively charged quaternized tetraphenylethene salt and negatively charged BSA. The design rationale is illustrated in Scheme 1. Quaternized tetraphenylethene salt with excellent solubility in a buffer solution was synthesized to work as the fluorescence probe. Initially, the well-dispersed tetraphenylethene derivative molecules in aqueous solution exhibited rather weak fluorescence. Upon addition of BSA, the aggregation complexes of the tetraphenylethene salt and BSA were expected to be formed due to the electrostatic interactions between ammonium cations of the tetraphenylethene salt and carboxyl anions of BSA. While the pH had a negligible effect on the fluorescence signal of the TPE compound, it really influenced the fluorescence enhancement of the TPE compound and BSA. The fluorescence intensity showed slight increases when pH fell below 3, and then increased further when the pH was raised from 4 to 12 (Fig. S1, ESI†). As the optimal pH for the enzyme activity was 7.4, at this pH, the fluorescence enhancement for the TPE compound and BSA was also obvious, so 7.4 was chosen as the optimal pH. Accordingly, the fluorescence of the ensemble would be enhanced. Moreover, in the presence of trypsin, the BSA could be enzymatically hydrolyzed and the BSA–tetraphenylethene derivative conjugates would be brought to be deaggregated gradually. Therefore the tetraphenylethene salt molecules were brought to be isolated from each other. Accordingly, the aggregation to dispersing state change of the tetraphenylethene derivative resulted in an obvious decrease in the fluorescence of the conjugate probes and enabled the sensitive and selective detection of trypsin. Subsequently, if trypsin activity was inhibited by α1-antitrypsin, the fluorescence was consequently preserved. This process enabled the fluorescence detection of α1-antitrypsin.

3.2 Construction of probing complex

The AIE probe, positively charged quaternized tetraphenylethene salt, was prepared by the reaction of 1,2-bis[4-(bromomethyl)-phenyl]-1,2-diphenylethene with excess amounts of triethylamine. The buffer solution of this probe (38 μM in Tris buffer solution) emitted weakly with a maximum emission wavelength at 467.5 nm. As shown in Fig. 1(a), the fluorescence intensity of the probe increased gradually with the addition of

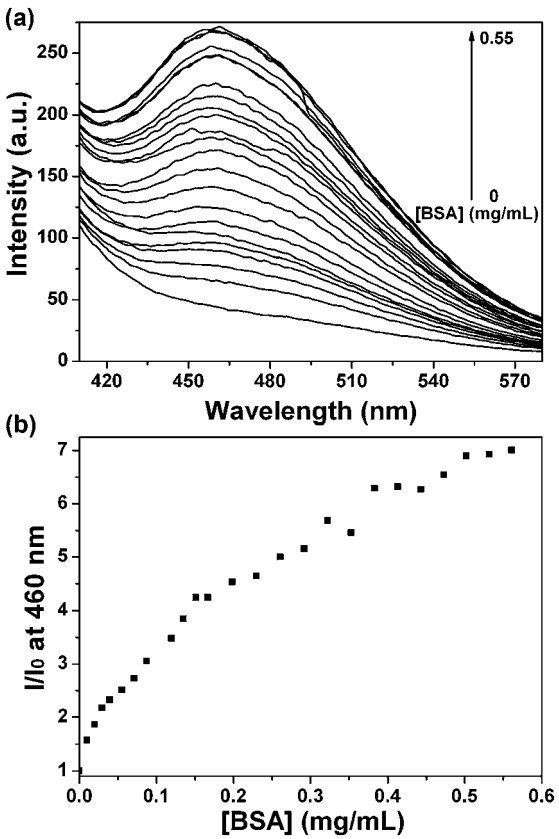
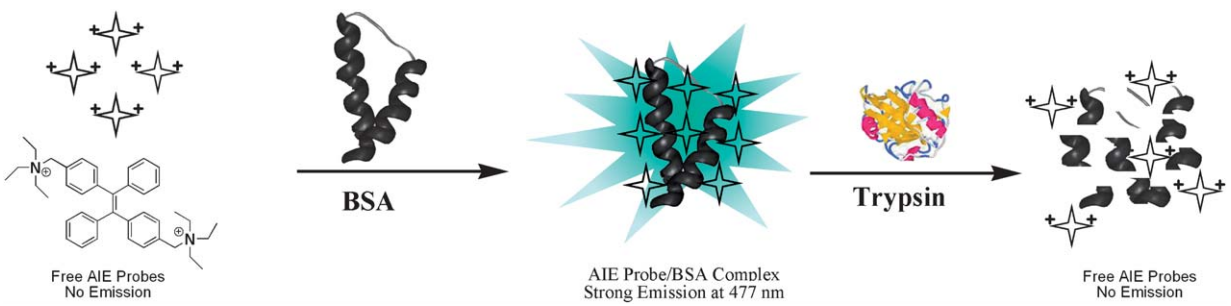


Fig. 1 (a) Fluorescence spectra of the AIE probe (38 μM in 10 mM PB, pH 7.4) in the presence of different concentrations of BSA (from 0 to 0.55 mg mL^{-1}). (b) Plot of the fluorescence intensity (467.5 nm) *vs.* the concentration of BSA.



Scheme 1 Schematic illustration of the sensing mechanism of the probing complex for the detection of trypsin and α1-antitrypsin.

BSA solution. When the final concentration of BSA in the complex reached 0.5 mg mL^{-1}, the fluorescence intensity had increased by 5 times. The increase in the fluorescence of the AIE probe induced by the BSA indicated the induced aggregation effect of BSA towards AIE probes. The fluorescence increase could be ascribed to the restriction of intramolecular rotation of AIE probes due to the conjugation of the cationic AIE probes and the anionic carboxyl groups on the BSA *via* electrostatic interaction. The fluorescence intensity increased along with the concentration of BSA from 0 mg mL^{-1} to 0.5 mg mL^{-1}. The correlation between fluorescence intensity and concentration of BSA was correlated to find out the optimum ratio of BSA to AIE probes in the probing complex and the results are shown in Fig. 1 (b). It was found that the fluorescence reached a plateau when the concentration of BSA exceeded 0.5 mg mL^{-1}. Some negatively charged species that may coexist with BSA, such as nucleic acid and pyrophosphate, didn't influence fluorescence (Fig. S2, ESI†). Therefore the complexes formed from 38 μM of AIE probes and 0.5 mg mL^{-1} of BSA were constructed as the probing complex for the follow-up experiments.

3.3 Fluorescence detection of trypsin

To investigate the possibility of detection of trypsin activity with the probing complex, the fluorescence intensity of the BSA–tetraphenylethene derivative probing complex was examined after incubation with trypsin in PB buffer solution. Fig. 2(a) shows the emission of AIE, the AIE–BSA complex before and after incubation with trypsin and the AIE–BSA complex after incubation with trypsin/AAT in PB buffer solution. The AIE probes showed rather low fluorescence intensity owing to the excellent solubility and well-dispersed state in the buffer solution. Upon addition of the BSA templates into the solution, the formation of the AIE probes–BSA complex resulted in an increase in the fluorescence intensity by nearly 5 times (Fig. 2(a)). Control experiments indicated that trypsin at the examined concentration did not show an obvious increasing effect on the fluorescence intensity of the AIE probes. It was important to note that after addition of trypsin and incubation at 37 °C for 30 min, the fluorescence intensity decreased to nearly half of the original value. This phenomenon was not strange if the hydrolyzing activity of trypsin on BSA was taken into consideration. As long as the BSA was enzymatically hydrolyzed, the BSA–tetraphenylethene derivative conjugates were brought to be de-aggregated and consequently the tetraphenylethene salt molecules were brought to be in well-dispersed states. A negligible change in emission intensity was observed when the trypsin was pre-inhibited by AAT by incubating the trypsin and AAT together at 37 °C for 30 min. Fig. 2(b) showed the photograph taken from the solution of the AIE probe itself and the AIE–BSA complex before and after incubation with trypsin in PB buffer solution under UV transilluminator (excitation at 365 nm). It was obvious that the low fluorescence intensity (Fig. 2(b), left) was increased to high fluorescence intensity (Fig. 2(b), middle) with the addition of BSA. After incubation in trypsin solution, the fluorescence intensity decreased again to low levels, (Fig. 2(b), right).

Furthermore, we conducted the experiment of dynamic monitoring of enzymatic reaction. Fig. 3(a) showed the fluorescence intensity changes of the AIE probes–BSA complex as

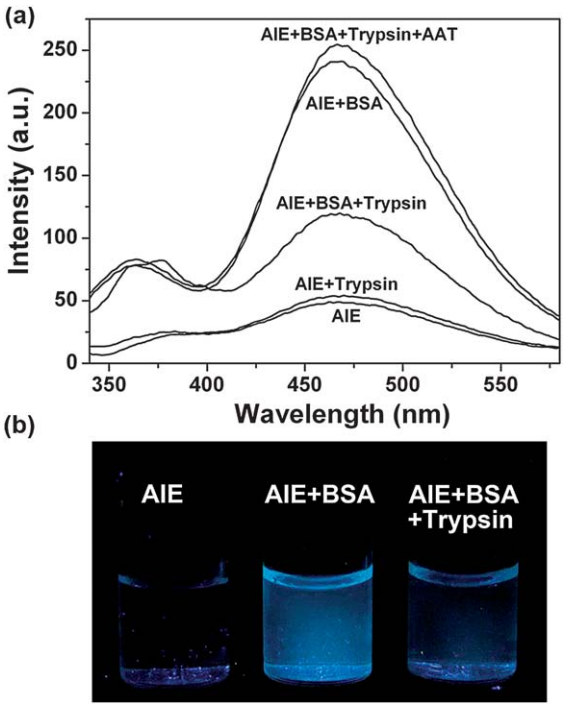


Fig. 2 (a) Fluorescence emission spectra of the AIE probes, the AIE probes–BSA complex before and after incubation with trypsin and the AIE probes–BSA complex after incubation with trypsin/AAT in PB buffer solution (10 mM, pH 7.4). The concentration of the AIE and BSA solution was 38 μM and 0.5 mg mL^{-1} respectively, trypsin's concentration was 20 mU mL^{-1}, and the corresponding concentration of AAT was 0.3 mg mL^{-1}. The excitation wavelength was 303 nm and data was monitored at 467.5 nm. (b) Photograph taken from the solution of the AIE probe itself and the AIE probes–BSA complex before and after incubation with trypsin in PB buffer solution (10 mM, pH 7.4) under UV transilluminator (excitation at 365 nm). [AIE] = 38 mM, [BSA] = 0.5 mg mL^{-1}, [Trypsin] = 20 mU mL^{-1}.

a function of the trypsin digestion time. The fluorescence intensity decreased gradually with the time ranges from 0 to 90 min, and in the later period the decrease speed was far lower than the initial speed. The fluorescence intensity reached the plateau after 90 min. It indicated that BSA was nearly completely disintegrated by trypsin. These results indicated that the AIE probes–BSA complex could be used as a fluorescent probe for the real-time detection of trypsin. Fig. 3(b) indicated the dependence of the intensity ratio of the AIE probes–BSA complex on the concentration of trypsin. In the absence of trypsin, the fluorescence intensity of the AIE probes–BSA complex almost kept unchanged. Then with the addition of 5 mU mL^{-1} of trypsin, the emission intensity changed slowly due to the low enzymolysis speed. The increase of trypsin amounts gave rise to high initial cleavage reaction rates and thus higher level of fluorescence changes.

The dependence of fluorescence changes of the AIE probes–BSA complex upon the concentration of trypsin was used to detect the concentration of trypsin. As shown in Fig. 4(a), the fluorescence of the AIE probes–BSA complex decreased as the amount of added trypsin increased. The correlation between fluorescence intensity and concentration of trypsin was presented as a linear plot which ranges from 1 mU mL^{-1} to 12.5 mU mL^{-1}

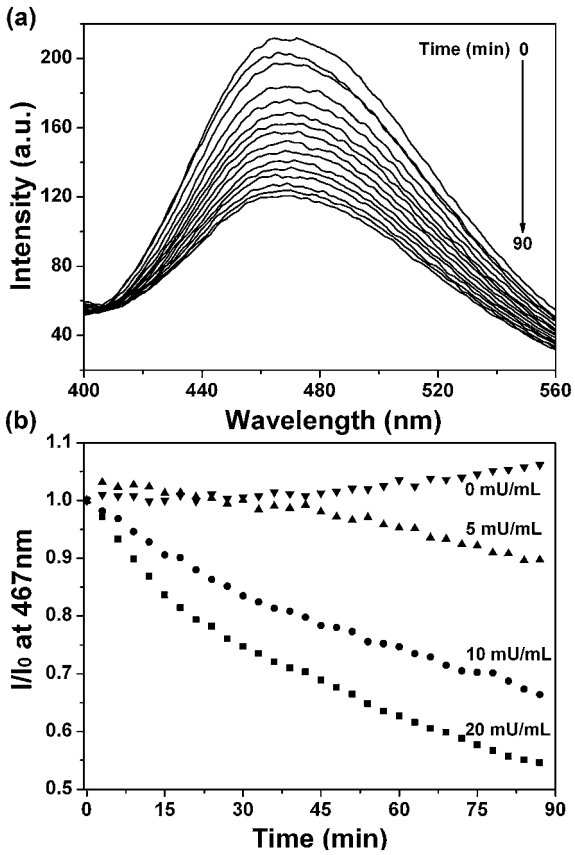


Fig. 3 (a) Fluorescence spectra of the AIE probes–BSA complex after adding 20 mU mL^{-1} trypsin, following time in a PB buffer solution (10 mM, pH 7.4). (b) Plot of the relative fluorescence intensity ratio of the AIE probes–BSA complex emission at 467.5 nm *vs.* the hydrolysis reaction time for different concentration of trypsin (0 mU mL^{-1}, 5 mU mL^{-1}, 10 mU mL^{-1}, 20 mU mL^{-1}). Data recorded at 37 °C.

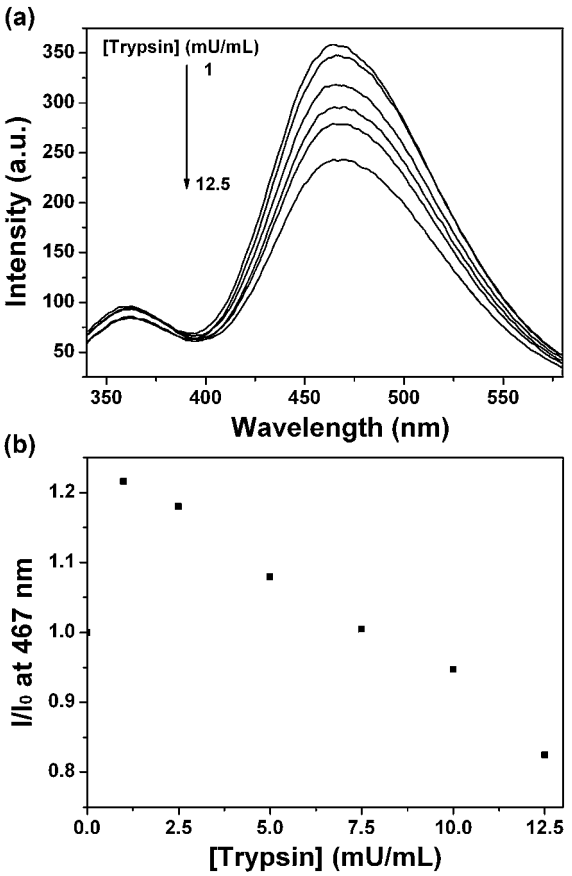


Fig. 4 (a) Fluorescence spectra of the AIE probes–BSA complex (38 μM AIE probe and 0.5 mg mL^{-1} BSA in 10 mM PB, pH 7.4) in the presence of different concentrations of trypsin at 1, 2.5, 5, 7.5, 10 and 12.5 mU mL^{-1}. (b) Plot of the fluorescence intensity ratio of the AIE probes–BSA complex emission at 467.5 nm *vs.* the concentration of trypsin.

(Fig. 4(b). The detection limit for this method, at a signal-to-noise ratio (*S*/*N*) of 3, was measured to be 1.43 mU mL^{-1}, namely 5.7 μg mL^{-1}.

To address the selectivity of the AIE probes–BSA complex toward trypsin, control experiments with other nonspecific enzymes, namely alkaline phosphatase (ALP), lysozyme and denatured trypsin (boiled for 10 min) at the same experimental conditions were further investigated. As shown in Fig. 5(a), while the addition of trypsin into the AIE probes–BSA complex caused the highest decrease in the fluorescence intensity, ALP, lysozyme and denatured trypsin did not induced obvious changes in the fluorescence intensity. The time-dependent fluorescence responses of the AIE probes–BSA complex were also studied in the absence of an enzyme or in the presence of a nonspecific enzyme (Fig. 5(b)). Fluorescence responses of the AIE probes–BSA complex kept stable in the absence of any enzyme. Also, BSA was resistant to ALP, lysozyme and d-trypsin, and no interferential responses were observed for the fluorescence of the AIE probes–BSA complex in the presence of ALP, lysozyme and d-trypsin. These results demonstrated that the enzymolysis of BSA by trypsin specifically caused the fluorescence intensity decrease. Hence, the AIE probes–BSA complex can be used as a fluorescent probe for the sensitive detection of trypsin with minor interference from other nonspecific substrates and proteins.

3.4 Fluorescence detection of α1-antitrypsin

Based on the fact that the fluorescence decrease was strictly related to the trypsin enzymatic cleavage of BSA into small fragments, the AIE probes–BSA complex can be used as a fluorescent probe for the α1-antitrypsin (AAT) concentration detection. It has been well recognized that to diagnose cancer and begin treatment as early as possible directly determines its cure rate and has resulted in decreasing the death rate. α1-antitrypsin is a well-known tumor marker and it is possible to diagnose liver cancer in the very early period by detecting the abnormally high concentrations of α1-antitrypsin. As a well-known inhibitor for trypsin, it is possible to develop a detection method for α1-AT based on the inhibition effect of α1-antitrypsin on the enzymatic cleaving reaction in our probes. As shown in Fig. 6, the inhibition effect increased along with the concentration of AAT. The correlation between fluorescence intensity and concentration of AAT was presented as a linear plot which ranges from 0.01 to 0.3 mg mL^{-1}. In the presence of 0.3 mg mL^{-1} AAT, the activity of trypsin was well inhibited and no significant fluorescence signal decrease, compared to the initial probing solution, was observed.

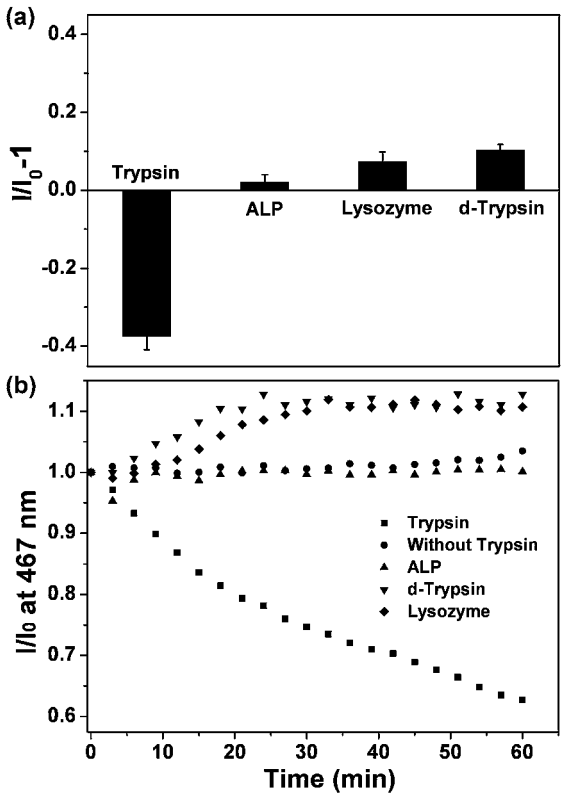


Fig. 5 (a) Fluorescence intensity changes at 467.5 nm, $[(I - I_0)/I_0]$ of the AIE probes–BSA complex itself in the presence of a different protein/enzyme at 0.08 mg mL^{-1}. (b) Variation of the intensity ratio of the AIE probes–BSA complex emission on different enzymes at 0.08 mg mL^{-1}. The excitation wavelength is 303 nm. Data recorded at 37 °C.

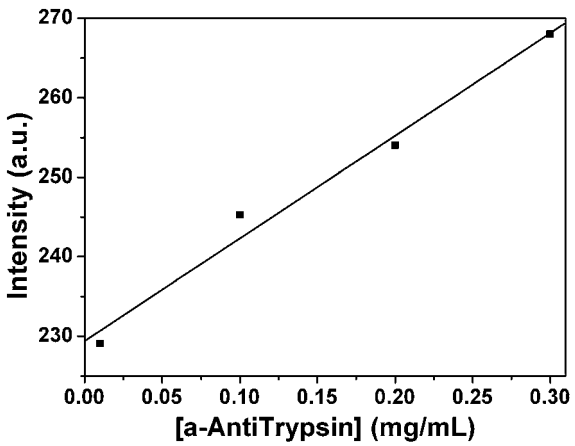


Fig. 6 Plot of the fluorescence intensity of the AIE probes–BSA complex emission at 467.5 nm *vs.* the concentration of AAT. Trypsin and AAT were incubated together at 37 °C for 30 min, [trypsin] = 0.08 mg mL^{-1}, [AAT] = 0.01, 0.1, 0.2 and 0.3 mg mL^{-1}.

These results illustrated that the decrease in fluorescence of the complex was indeed induced by trypsin, exactly speaking, the enzymatic cleaving reaction catalyzed by trypsin. Therefore, tumor marker α1-antitrypsin was likely to be detected by the AIE probes–BSA complex.

4. Conclusions

In summary, we have described the construction of BSA–tetraphenylethene derivative conjugates and used them as fluorescent AIE probes for label-free and homogenous detection of protease and α1-antitrypsin. The probe in this manuscript could generate fluorescence change through aggregation or disaggregation, thus making our method easy for sample preparation and possible for rapid detection. Most importantly, our method didn't need complex modification and label processes compared to traditional organic dyes. The disadvantage of our method, if any, was that the probe combined with the aptamer through electrostatic force which was not analyte-specific, so other charged materials may bring interference.

Conjugated AIE probes were easily formed based on the electrostatic induced assembly between positively charged quaternized tetraphenylethene salt and negatively charged BSA in a buffer solution. Water soluble conjugated AIE probes showed enhanced fluorescence due to the abnormal aggregation-induced emission properties of tetraphenylethene. This BSA–tetraphenylethene derivative conjugates enabled label-free detection of protease. The presence of trypsin enzymatically hydrolyzed the BSA, and the decrease in AIE probe emission was used to monitor the enzyme activity. Sensitive detection of α1-antitrypsin was realised based on the inhibition effect on the activity of trypsin. The protein–tetraphenylethene derivative conjugates with simplicity, high sensitivity, and rapid response show great promise for the monitoring of biological processes and cancer diagnostics.

Acknowledgements

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