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A pH responsive AIE probe for enzyme assays†

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By combining leucine (Leu) and tetraphenylethene (TPE), a pH-sensitive aggregation induced emission (AIE) probe **TPE-Leu** was developed. The aliphatic amine in **TPE-Leu** was more easily protonated under acidic conditions, which made **TPE-Leu** more water soluble. Therefore, the protonated AIE probe showed weak fluorescence under acidic conditions. When the pH was changed to basic conditions, it showed strong fluorescence due to the hydrophobic nature of **TPE-Leu**. We demonstrated that the probe showed high selectivity toward pH changes with the coexistence of other potential species such as metal ions, redox agents, and biomolecules. In contrast, **TPE-NH$_2$** did not exhibit obvious pH-sensitive properties. Moreover, **TPE-Leu** was further utilized to develop a sensitive and selective sensing platform for urease and acetylcholinesterase (AChE) detection. The current study not only provides a new strategy for designing pH-sensitive fluorescent probes for bioassays but also broadens the applications of AIE probes.

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Introduction

pH plays a critical role in various biological processes. For instance, the abnormal pH values in living systems are frequently associated with metabolic disturbances such as cancers and Alzheimer's disease.[1–6] On the other hand, many important enzymatic reactions induce pH changes by producing acidic or basic species. For example, urease catalyzes the hydrolysis of urea into ammonia (a basic species) and CO_2 while acetylcholinesterase (AChE) catalyzes the hydrolysis of acetylcholine (ACh) into acetic acid (an acidic species) and choline.[7–10] Therefore, it would be feasible to detect these enzymes' activities by monitoring the catalytic reaction-induced pH changes.

In the past few decades, numerous methods for detecting pH values have been reported, which include acid–base indicator titration, absorbance spectroscopy, potentiometry, *etc*.[11–14] However these traditional measurements either have moderate sensitivity and selectivity, or require frequent calibration. To overcome these drawbacks, recently fluorescence-based techniques for pH sensing have been extensively explored due to their high sensitivity, good selectivity, low cost, and ease-of-operation.[15–21]

It is well known that conventional fluorescent probes usually suffer from aggregation-caused quenching (ACQ), an effect under which these fluorophores show weak or even no fluorescence at high concentrations. The ACQ effect has significantly limited the broad applications of these probes in the development of efficient fluorescence sensing systems, especially in bioassays.[22,23] In contrast, aggregation induced emission (AIE) is an emerging strategy for designing fluorescent probes, which do not suffer from the problems of ACQ. When an analyte induces the aggregation of an AIE fluorophore, its fluorescence sensitively changes from being negligible to strong.[24–29] The unique mechanism endows AIE probes with additional advantages, such as lower background and higher photostability.[26,27] As a result, AIE-based fluorescent probes have attracted much attention and have been widely used in various fields especially in bioanalysis. For example, tetraphenylethene (TPE), a widely used AIE fluorophore, has been utilized to develop biosensing systems since it can be facilely functionalized with varieties of biomolecules.[30,31] However, there were only a few reports that explored AIE fluorophores' pH-sensitive properties for bioassays, especially those that can be used for enzyme detection.[32–36]

Herein, we report a facile pH-sensitive probe **TPE-Leu**, which was synthesized by incorporating a leucine (Leu) moiety into a typical AIE fluorophore TPE. **TPE-Leu** was highly

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insoluble and showed strong fluorescence under alkaline conditions. However, the aliphatic amine in **TPE-Leu** could be easily protonated under acidic conditions, which made the probe more soluble and decreased its AIE fluorescence. Moreover, the emission of **TPE-Leu** showed high specificity to pH changes in the presence of other potential competing species, which was important for its practical applications in enzyme bioassays. We then successfully utilized **TPE-Leu** for selective detection of two biomedically important enzymes (*i.e.*, urease and AChE).

Experimental

Reagents and instruments

Commercially available solvents and chemicals were used directly without further purification. Catalase, glucose oxidase (GOx), urease, and AChE were purchased from Sigma. Deuterated solvents for NMR measurements were purchased from J & K Scientific (Shanghai, China). Fluorescence measurements were carried out on a HITACHI F-4600 fluorescence spectrophotometer within a quartz cuvette. ^{1}H NMR spectra were recorded on a Bruker AM400 spectrometer with tetramethylsilane (TMS) as an internal standard.

Fluorescence measurements

1 mL detection solutions were added into a quartz cuvette with a path length of 1 cm. The excitation and emission slit widths were both 5 nm and the PMT voltage was 600 V. Then the spectroscopic experiments were performed at room temperature with an excitation wavelength of 320 nm.

pH titrations and selectivity experiments

Stock solutions of both **TPE-Leu** and **TPE-NH$_2$** (2 mM) were prepared in DMSO. These solutions were further diluted using DMSO and buffers with different pH values to 10 μM in DMSO/buffer (1 : 9, v : v) for further pH titrations. The buffers were 5 mM sodium acetate (pH = 4.03, 4.96, 5.49, 6.12, 7.03, 7.65) and 5 mM PBS (pH = 7.92, 9.07, 9.99, 11.03, 12.00), respectively. For selectivity experiments, competing species were added into 10 μM **TPE-Leu** in DMSO/PBS (1 : 9, v : v); the concentration of PBS was 100 mM and the pH value was 7.

Enzyme detection

For urease detection, different concentrations of urease were added into 10 μM **TPE-Leu** in pH = 5.5 DMSO/buffer (5 mM pH = 5.5 NaOAc) (1 : 9, v : v) with 50 mM urea, which was then incubated at 30 °C for 30 min. (Note: a strongly buffered solution may interfere with the detection of the pH changes of the enzyme reaction.) For AChE detection, different concentrations of AChE were added into 10 μM **TPE-Leu** in pH = 9.5 DMSO/buffer (1 : 9, v : v) with 50 mM acetylcholine (ACh), which was then incubated at 30 °C for 30 min. Then the fluorescence spectra were recorded. The selectivity experiments were carried out by adding other enzymes/proteins instead of urease/AChE.

Synthesis of TPE-NH$_2$ and TPE-Leu

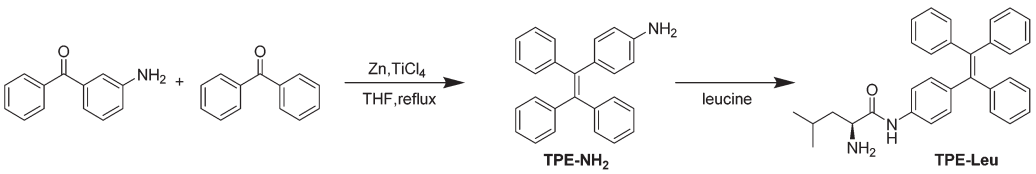
1-(4-Aminophenyl)-1,2,2-triphenylethene (**TPE-NH$_2$**) was synthesized according to a literature method in a final yield of 50.5% (Scheme S1†).[37] ^{1}H NMR (DMSO-d_6, 400 MHz): δ 6.93–7.10 (m, 15H), 6.56 (d, 2H, J = 8.4 Hz), 6.26 (d, 2H, J = 8.4 Hz), and 5.05 (s, 2H).

TPE-Leu was further synthesized by incorporating Leu into **TPE-NH$_2$** using Fmoc chemistry by GenScript. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 10.51 (s, 1H), 7.38 (d, 2H, J = 6.8 Hz), 7.15–7.11 (m, 9H), 6.99–6.92 (m, 8H) 4.42–4.37 (m, 2H), 4.15 (s, 1H), 3.89–3.88 (m, 1H), 3.57 (dd, 2H, J = 27.6 and 25.6 Hz), and 0.91 (s, 6H). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 168.2, 143.7, 143.6, 143.5, 140.9, 140.5, 139.5, 136.8, 131.7, 131.1, 131.1, 128.4, 128.3, 127.1, 127.0, 119.3, 71.8, 70.1, 64.3, 52.2, 24.2, 23.0, 22.4. The purity of **TPE-Leu** was found to be 98.76% using an analytical HPLC system on a C_{18} column (Fig. S4†). MS characterization of **TPE-Leu** showed a peak at 461.15 $[M + H]^+$, which matched the calculated value 460.76 very well, indicating that the product was correct (Fig. S5†).

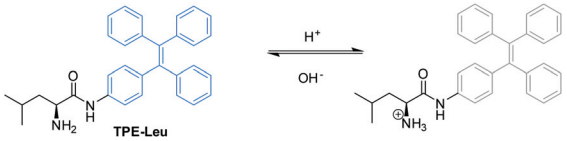
Results and discussion

Design, synthesis and the AIE properties of TPE-Leu

As shown in Scheme 1, **TPE-Leu** was synthesized by incorporating Leu into **TPE-NH$_2$**, which was characterized by nuclear magnetic resonance (NMR), high pressure liquid chromatography (HPLC) and mass spectrometry (MS) (Fig. S1–S5†). To ensure better emission properties of **TPE-Leu**, we monitored its fluorescence intensity in DMSO/PBS mixture solutions with different volume fractions (50%–95%) of PBS buffer (Fig. S6†). The fluorescence intensity of **TPE-Leu** gradually increased with the increasing fraction of PBS buffer, which indicated the aggregation of **TPE-Leu**. Besides, the fluorescence intensity increased dramatically from 70% PBS buffer. Since **TPE-Leu** at 90% PBS buffer showed relatively stable and strong fluorescence, we chose DMSO/PBS (1 : 9, v : v) as the solvent system in the following experiments.



Scheme 1 Synthesis of 1-(4-aminophenyl)-1,2,2-triphenylethene (**TPE-NH$_2$**) and **TPE-Leu**.



Scheme 2 Proposed pH sensing mechanism using **TPE-Leu**.

The **TPE-Leu** probe consists of two parts, both of which play important roles in pH sensing. The TPE moiety is a typical AIE fluorophore, which emits strong fluorescence only after aggregation and acts as the signal reporting group in **TPE-Leu**. The aliphatic amine in **TPE-Leu** is more basic than the aromatic one (the pK_a value for the aliphatic primary amine is about 10.62 and that for the aromatic secondary amine is about 4.4); thus the aliphatic amine is more easily protonated under acidic conditions (Scheme 2). Protonation of the aliphatic amine makes **TPE-Leu** more soluble in acidic solution and decreases its fluorescence intensity while **TPE-Leu** itself is hydrophobic and shows strong fluorescence. Thereby, **TPE-Leu** is sensitive to pH changes and can be applied as a probe for pH sensing. Note: according to the sensing mechanism, a hydrophobic amino acid should be used to construct a pH responsive AIE probe. Therefore, in principle, other hydrophobic amino acids, such as Ile, could also be used for this purpose.

Sensitive and selective fluorescence detection of pH

We evaluated the fluorescence changes of **TPE-Leu** at different pH values (Fig. 1). It showed that the fluorescence intensity of **TPE-Leu** gradually increased with increasing pH values, especially in the range from 5.49 to 9.99 (Fig. 1A). The plots of fluorescence intensity at 455 nm showed clear fluorescence changes *versus* different pH values. As shown in Fig. 1B, **TPE-Leu** was almost non-emissive when the pH value was below ~5.5. As the pH value increased, the protonation of the aliphatic amine gradually reduced and the fluorescence intensity of **TPE-Leu** dramatically increased until the pH value reached ~10. These results indicated that **TPE-Leu** was sensitive to pH and could be used for sensing pH changes. In contrast, **TPE-NH$_2$** only with an aromatic amine was not very sensitive to pH changes. Its fluorescence intensity almost remained unchanged in the pH range from 4 to 12 (Fig. S7†). Therefore, **TPE-Leu** was a promising fluorescent probe for pH sensing.

Since the biosystems for bioanalysis are usually complicated, it is important for a pH-sensitive probe to have high selectivity in the presence of other potential competing species. Therefore, a selectivity experiment was carried out in the presence of several competing species in biological systems like metal ions (Ca^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+}, K^+, and Na^+), redox agents (ascorbic acid (AA), NaClO, glutathione (GSH), and H_2O_2), and biomolecules (glucose (Glu), cysteine (Cys), glycine (Gly), histidine (His), and Leu) under neutral conditions. As shown in Fig. 1C, redox agents, biomolecules and most metal ions did not show any obvious interference to the pH sensing property of **TPE-Leu**. All of these results indicated that **TPE-Leu** was highly selective to pH over other potential competing species and thus could be applied in the complicated biological systems for bioanalysis.

Enzymatic assays with TPE-Leu

As **TPE-Leu** was highly sensitive and selective to pH changes, we explored its further applications in detecting enzymes like urease and AChE which can hydrolyze their substrates and induce pH changes. We first explored the capability of **TPE-Leu** for the detection of urease. Urease, an enzyme widely distributed in soil, bacteria, plants, animals and humans, has attracted extensive research interest because of its importance to agriculture, environment, and human health.[8,38] As shown in Fig. 2, **TPE-Leu** was easily protonated and showed low background fluorescence in the absence of urease under acidic conditions. With the addition of urease and subsequent hydrolysis of urea by urease, ammonia was produced and the basicity of the solution was enhanced, resulting in increased AIE fluorescence intensity. We then monitored the fluorescence intensity changes of **TPE-Leu** at 455 nm by adding different concentrations of urease (Fig. 2B). With the increasing concentration of urease, the fluorescence intensity of **TPE-Leu** increased dra-

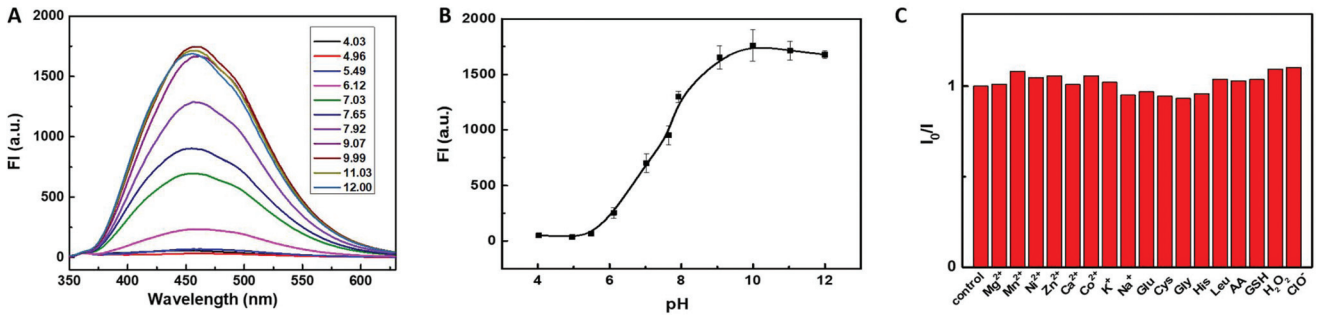


Fig. 1 (A) Fluorescence responses of 10 μM **TPE-Leu** to different pH values. (B) Plots of fluorescence intensity at 455 nm *versus* pH values. Error bars represent standard deviations of three independent measurements. λ_{ex} = 320 nm. (C) Relative fluorescence intensity (I_0/I) of 10 μM **TPE-Leu** in pH = 7 DMSO/buffer (1 : 9, v : v) in the presence of 0.1 mM Mg^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+}, Ca^{2+}, and Co^{2+}; 0.5 mM AA, Glu, Cys, Gly, His, Leu, GSH, H_2O_2, and ClO^-; and 1 mM K^+ and Na^+. I_0 is the fluorescence intensity of **TPE-Leu** at 455 nm (control) and I is the fluorescence intensity of **TPE-Leu** at 455 nm in the presence of competing species. λ_{ex} = 320 nm.

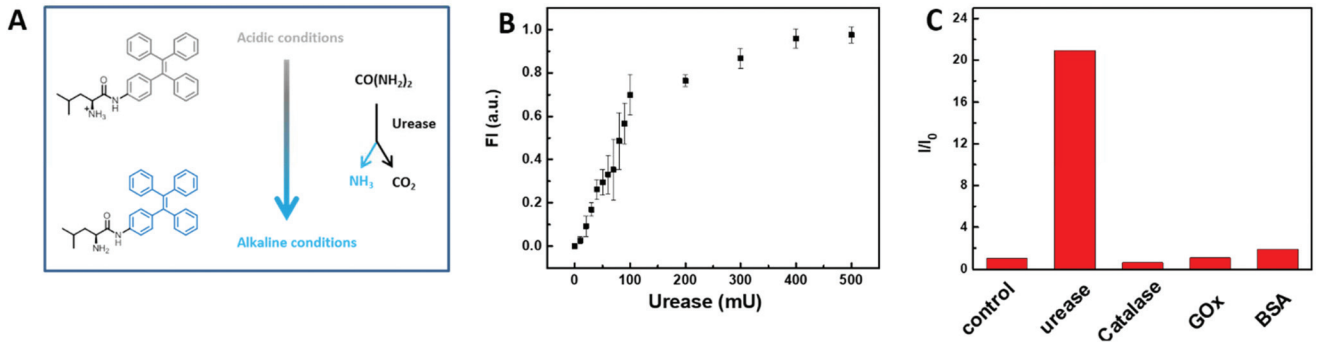


Fig. 2 (A) Schematic diagram of **TPE-Leu** used for urease detection. (B) Plots of normalized fluorescence intensity of 10 μM **TPE-Leu** at 455 nm in the presence of different concentrations of urease in pH = 5.5 DMSO/buffer (5 mM pH = 5.5 NaOAc) (1 : 9, v : v). (C) Selectivity of the **TPE-Leu** based bioassay toward urease detection. I_0 is the fluorescence intensity of 10 μM **TPE-Leu** with 50 mM urea at 455 nm (control) and I is the corresponding fluorescence intensity in the presence of 10 U catalase, 10 U GOx, 1 U urease, and 0.1 mg mL^{-1} BSA, respectively. Error bars represent standard deviations of three independent measurements. λ_{ex} = 320 nm.

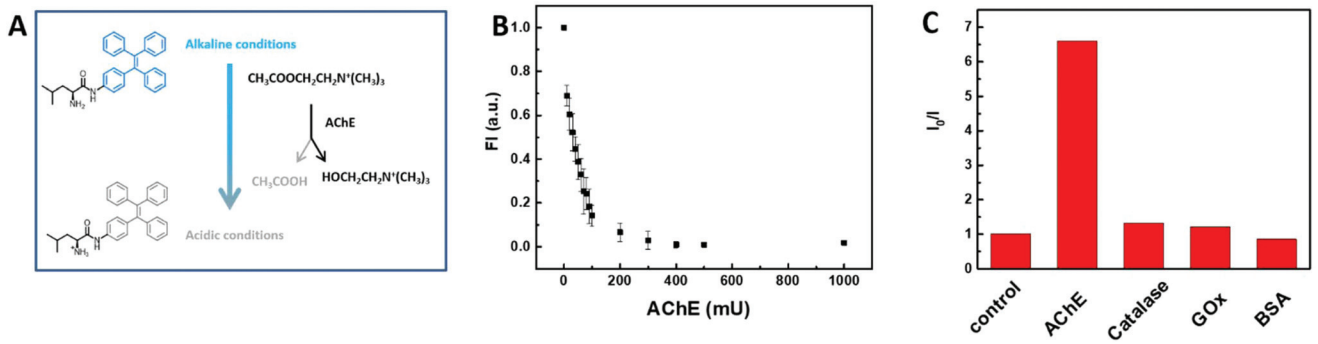


Fig. 3 (A) Schematic diagram of **TPE-Leu** used for AChE detection. (B) Plots of normalized fluorescence intensity of 10 μM **TPE-Leu** at 455 nm in the presence of different concentrations of AChE in pH = 9.5 DMSO/buffer (5 mM pH = 9.5 PBS) (1 : 9, v : v). (C) Selectivity of the **TPE-Leu** based bioassay toward AChE detection. I_0 is the fluorescence intensity of 10 μM **TPE-Leu** with 50 mM ACh at 455 nm (control) and I is the corresponding fluorescence intensity in the presence of 10 U catalase, 10 U GOx, 1 U AChE, and 1 mg mL^{-1} BSA, respectively. Error bars represent standard deviations of three independent measurements. λ_{ex} = 320 nm.

matically, especially in the initial range from 0 to 100 mU. And the fluorescence intensity gradually reached a plateau with further addition of urease. This result indicated a good sensitivity of **TPE-Leu** to urease. Moreover, the sensing system with **TPE-Leu** was highly selective to urease (Fig. 2C). Other enzymes (such as catalase and GOx (glucose oxidase)) or proteins (such as BSA (bovine serum albumin)) could not hydrolyze urea to cause pH changes, and thus did not influence the fluorescence intensity of **TPE-Leu**. These results suggested that the pH-sensitive probe **TPE-Leu** can be successfully used for sensitive and selective detection of urease.

To demonstrate the generality of our sensing strategy, the detection of another enzyme AChE was then investigated. AChE is an important enzyme in the nervous system as it can hydrolyze the neurotransmitter acetylcholine (ACh). Dysregulation of AChE may affect nervous system functions like learning and cognition and even cause Alzheimer's disease. Therefore, it is necessary to develop effective methods for AChE detection.[7,9,39,40] AChE could hydrolyze its substrate ACh into acetic acid and choline, and the produced acetic acid increased the acidity of the solution, thus promoting the protonation of **TPE-Leu** and decreasing its fluorescence intensity (Fig. 3). The bioassay of AChE was carried out by monitoring the fluorescence intensity of **TPE-Leu** at 455 nm (Fig. 3B). The fluorescence intensity of **TPE-Leu** dropped rapidly with the increasing concentration of AChE and finally achieved a plateau at about 400 mU. Besides, **TPE-Leu** showed a high selectivity toward AChE over other enzymes (such as catalase and GOx) and proteins (such as BSA) (Fig. 3C). Therefore, the sensing system with **TPE-Leu** was demonstrated to be an excellent probe for the detection of AChE.

Conclusions

In summary, we developed a pH-sensitive AIE probe **TPE-Leu**, which has an aliphatic amine moiety that is easily protonated under acidic conditions. The protonation reaction at the aliphatic amine moiety changed the water solubility of **TPE-Leu**, which was accompanied by significant emission intensity changes. Moreover, **TPE-Leu** can be used to detect pH changes in

complicated environments such as that with the coexistence of various metal ions, ensuring its further applications in bioanalysis. More encouragingly, we have successfully applied this AIE probe for sensitive and selective detection of enzymes like urease and AChE. The current study will not only provide a new strategy for designing pH-sensitive fluorescent probes for bioassays but also broaden the promising applications of AIE probes.

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Conflicts of interest

There are no conflicts to declare.

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