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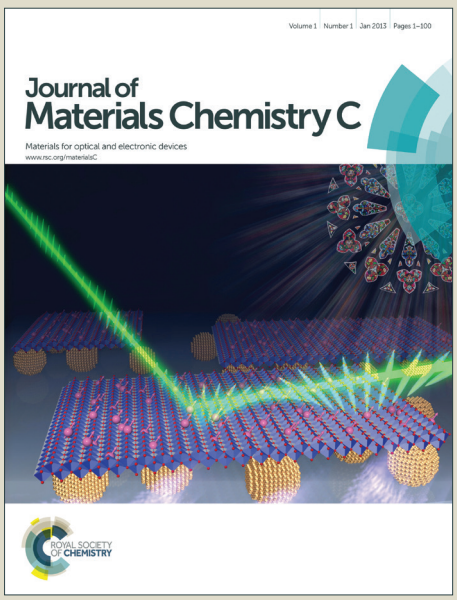

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ARTICLE

A fluorescent probe with aggregation-induced emission characteristic for distinguishing homocysteine over cysteine and glutathione

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Development of fluorescent probe for Homocysteine (Hcy) receives great attention in recent years because abnormal level of Hcy in blood is a risk factor for cardiovascular and neurodegenerative diseases. We herein report a tetraphenyethene derivative, TPE-Py, with aggregation-induced emission (AIE) characteristic. It contains α,β-unsaturated ketone unit and can react with biothiols through 1,4-addition reaction fashion, which results the disruption of molecular conjugation and thus leads to the change of its fluorescence. Due to the intrinsic AIE property of TPE-Py, only Hcy triggers an obvious ratiometric change from yellow emission to blue, whereas the quenching of fluorescence was observed in the case of cysteine (Cys) and glutathione (GSH), respectively. It enables TPE-Py serves as a highly selective and sensitive probe for distinguishing Hcy over Cys and GSH.

Introduction

Biological thiols, including cysteine (Cys), homocysteine (Hcy), and glutathione (GSH), play a vital role in many biological systems.[1] Taken Cys as an example, it is an essential amino acid that is included in protein construction and process of metabolism, and Cys deficiency may lead to many syndromes such as slow growth, hair depigmentation, lethargy, liver damage, skin lesions and weakness.[2] GSH, which consists of a tripeptide of glutamic acid, cysteine and glycine, is the most abundant free thiol in cell. Deficiency of GSH in body can give oxidative stress which is associated with some diseases including cancers and AIDS.[3] Specifically, Hcy, the homologue of Cys with one more methylene group than Cys, has been implicated in vascular and renal diseases. High levels of Hcy in blood is reported as a risk factor for disorders for cardiovascular and Alzheimer's disease, neutral tube defects, complications during pregnancy, inflammatory bowel disease, and osteoporosis.[4] Therefore, detecting the biothiols, particularly Hcy, is quite important for early diagnosis and therapy of some related diseases.

Among various detection methods, optical method has attracted considerable attention in the past decade since it offers many advantages including simplification, high sensitivity and low cost.[5] Significant effort has thus gone into the development of fluorescent probes for biothiols.[6] To date, most of biothiols probes are based on the chemical reactions utilizing strong nucleophilicity of thiol group, and various mechanisms have been employed such as the cyclization of thiols with aldehydes,[7] the Michael addition of thiols with α,β-unsaturated carbonyl moieties,[8] cleavage reaction of sulfonamide and sulfonate esters,[9] conjugate addition cyclization with acrylates,[10] aromatic substitution rearrangement reaction[11] and native chemical ligation[12] and so on. However, it still remains a challenge to discriminate Hcy from Cys through one fluorescent probe due to their similarity in structure and reactivity. Only a few probes with high selectivity for Hcy have been reported. For example, Strongin *et al.* developed a probe based on benzothiazole for Hcy and Cys discrimination.[10] Yoon *et al.* reported a pyrene derivative which shows selective light-up fluorescence response to Hcy.[13] Li *et al.* designed and synthesized an iridium(III) complex that exhibits high selectivity towards Hcy.[14] It's well-known that the α,β-unsaturated ketone unit is a classical building-block for constructing the biothiols probes.[15] As far as we know, nevertheless, there are no fluorescent probes using α,β-unsaturated ketone for specific detecting Hcy.

In recently years, one series of propeller-shaped fluorophores with aggregation-induced emission (AIE) feature attracts considerable attention. Unlike the most traditional fluorphores which suffer from concentration caused quenching (ACQ) effect, AIE fluorophores shows non- or very weak emission in its dissolved state, but turns to strong emission when the molecular aggregate is formed based on restriction of intramolecular motion (RIM) mechanism.[16] According to AIE mechanism, the fluorescence of AIE fluorophores can be easily adjusted by analyte through influencing the aggregation behaviour of fluorophores.[17] Based on this interesting phenomenon and principle, a large amount of AIE probes have

been developed for metal ions,[18] anions,[19] pH,[20] biomolecules[21] and so on. It's worth noting that the AIE probes can be successfully exploited for biothiols,[22] and few of them can be even employed for distinguishing Cys or GSH over other biothiols. However, AIE probe for selective detecting Hcy is rare.[23] To expand the utility of the AIE probe for precisely detecting Hcy, we herein design and synthesize two AIE-active tetraphenyethene derivatives, TPE-Py and TPEPh-Py, which contain α,β-unsaturated ketone unit. Both of them can undergo 1,4-addition reaction with biothiols which results the disruption of molecular conjugation and fluorescence change. Interestingly, TPE-Py shows better reaction activity for biothiols. Due to the AIE property of TPE-Py, only Hcy triggers a significant ratiometric fluorescence change from yellow to blue, while the quenching of fluorescence was observed in the presence of Cys or GSH. It makes TPE-Py as a highly selective probe for discriminating Hcy over Cys and GSH.

Experimental section

Material. Tetrahyfrofuran (THF), toluene and ethanol were distilled from sodium, benzophenone ketyl, calcium hydride and magnesium, respectively, under nitrogen immediately prior to use. Homocysteine and cysteine was purchased from TCI. Other materials and amino acids were purchased from 9 Ding Chemistry and GL Biochem (Shanghai) Ltd, and used as received without further.

Instruments. ^{1}H and ^{13}C NMR spectra were measured on a Bruker ARX 400 NMR spectrometer using $CDCl_3$ and tetramethylsilane (TMS) as internal reference. High-resolution mass spectra (HRMS) were obtained on a Bruker Maxis Spectrometer. UV-visible (UV-vis) absorption spectra were measured on a Hitachi U-3900 spectrophotometer. Photoluminescence (PL) spectra were recorded on a Hitachi F-7000 spectrofluorometer.

Preparation of nanoaggregates. Stock solution of TPE-Py and TPEPh-Py in acetonitrile with a concentration of 0.3 mM was prepared. Aliquots of the stock solution were transferred to 5 mL volumetric flasks and appropriate amount of acetonitrile was added. Water was added dropwise under vigorous stirring to furnish 3 μM TPE-Py and TPEPh-Py solution with different water contents (0–90 vol%).

Preparation for fluorescent measurement. Different concentrations of Hcy, Cys and GSH were first dissolved in pH 8 buffer. A solution of TPE-Py (3 μM, 3.0 mL) in acetonitrile/buffer (20 : 80 v/v, 20 mM, pH 8) mixtures was placed in a quartz cuvette. The steady state fluorescent measurement was taken 3 minutes after mixing with appropriate amount of amino acids.

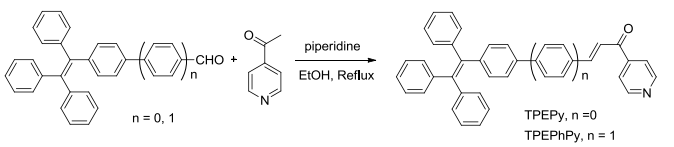
Synthesis (E)-1-(pyridin-4-yl)-3-(4-(1,2,2-triphenylvinyl)-phenyl)prop-2-en-1-one (TPE-Py). The synthesis of TPE-CHO followed the procedure described in literature.[24] TPE-CHO (150 mg, 0.42mmol) were dissolved in ethanol (10 mL), and then 4-acetyl pyridine (92 μL, 0.84 mmol), five drops of piperidine were added. The mixture was heated to reflux for 36h, and then the solvent was removed under reduced pressure. The resulting residue was purified by chromatography on silica gel to give product as a yellow solid (143 mg, 0.31 mmol) in 74 % yield. ^{1}H NMR (400 MHz, DMSO-d_6), δ (TMS, ppm): 8.83(d, J = 4Hz, 2H), 7.96 (d, J = 4Hz, 2H), 7.79 (d, J = 16Hz, 1H), 7.70–7.67 (m, 3H), 7.17–6.97 (m, 17H). ^{13}C NMR (100MHz, $CDCl_3$), δ (TMS, ppm): 191.24, 152.21, 148.70, 148.02, 145.95, 144.74, 144.70, 144.57, 143.85, 141.42, 133.69, 133.49, 132.76, 132.75, 132.69, 129.58, 129.31, 129.29, 129.13, 128.29, 128.18, 122.92, 122.15. LC-MS (ESI-TOF): m/z 464.1993 [$(M+H^+)$, calcd 464.2014].

Synthesis (E)-1-(pyridin-4-yl)-3-(4'-(1,2,2-triphenylvinyl)-[1,1'-biphenyl]-4-yl)prop-2-en-1-one (TPEPh-Py). The synthetic route of TPEPh-Py is similar to that of TPE-Py. ^{1}H NMR (400MHz, $CDCl_3$): δ (TMS, ppm): 8.85–8.84 (m, 2H), 7.85 (d, J = 16Hz, 1H), 7.79–7.78 (m, 2H), 7.69 (d, J = 8Hz, 1H), 7.63 (d, J = 8Hz, 1H), 7.43(d, J = 16Hz, 1H), 7.40 (d, J = 8Hz, 1H), 7.15–7.03 (m, 17H). ^{13}C NMR (100MHz, $CDCl_3$): δ (TMS, ppm): 189.83, 150.85, 146.45, 144.5, 143.79, 143.63, 143.62, 143.58, 143.47, 141.53, 140.27, 137.48, 133.08, 131.99, 131.40, 131.36, 131.33, 129.22, 127.82, 127.77, 127.68, 127.40, 126.61, 126.59, 126.55, 126.18, 121.53, 120.83. LC-MS (ESI-TOF): m/z 540.2312 [$(M+H^+)$, calcd 540.2327].

Results and discussion

Synthesis and optical property

According to the synthetic route shown in Scheme 1, luminogens TPE-Py and TPEPh-Py were prepared in one reaction step and obtained as the yellow solid in a reasonable yield after purification by column chromatography. The structure was characterized using NMR and high resolution mass spectrometry from which satisfactory results corresponding to its structure were obtained (Fig. S1–S7). Both of them are soluble in common organic solvent such as acetonitrile, tetrahydrofuran and dichloromethane, but insoluble in water.



Scheme 1. Synthetic route to luminogens TPE-Py and TPEPh-Py.

The dilute acetonitrile solution of TPE-Py and TPEPh-Py absorbs at ca. 360 nm owed to the intramolecular charge transfer (ICT) transition from the electron-donating tetraphenyethene unit to the electron-accepting pyridine unit (Fig. S8). When the diluted solution is photoexcited, both of them exhibit almost no emission. However, increasing the water fraction (f_w) in the solution of them can lead to the enhancement of their luminescence intensity. As shown in Fig. 1A and Fig. S9A, the emission spectra of TPE-Py and TPEPh-Py are not changed while the f_w is less than 70%, but give

stronger and yellow emission with peak at around 550 nm after the f_w is more than 70%. When the f_w is up to 90%, the fluorescence intensity of TPE-Py and TPEPh-Py boosts sharply and shows 90 and 120 times higher than that in pure acetonitrile solution, respectively (Fig. 1B, Fig. S9B). As both of luminogens are insoluble in water, their molecules must be aggregated in mixture solution with a larger portion of water, which can restrict the intramolecular motion and prohibit the energy dissipation through nonradiative channels. This clearly shows that TPE-Py and TPEPh-Py are typical AIE fluorophors.

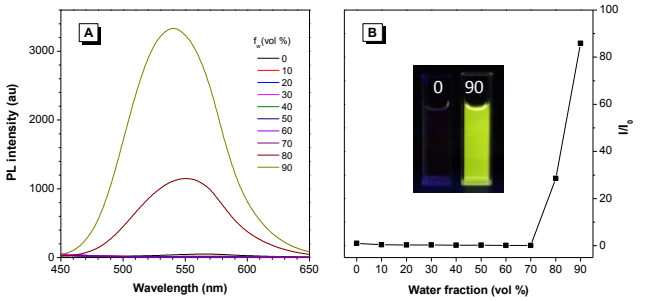


Fig. 1 (A) PL spectra of TPE-Py (3 μM) in acetonitrile and acetonitrile–water mixtures with different water fractions (f_w). (B) Plots of emission intensity *versus* the composition of the aqueous mixtures of TPE-Py. Inset: photographs of TPE-Py in acetonitrile–water mixtures with f_w values of 0 and 90 vol%

Optical response of luminogens to biothiols

As mentioned above, TPE-Py and TPEPh-Py could detect biothiols based on addition reaction of biothiols to α,β-unsaturated ketone unit. To achieve better detecting performance, we examined the optical sensing behaviour of luminogens toward different biothiols in acetonitrile/phosphate buffer (20 : 80 v/v, 20 mM, pH 8). Under such detection media, the molecular aggregate is looser which favour to the process of reaction between luminogens and thiols. On the other hand, the intense yellow emission of luminogens in this media makes the emission color change for sensing biothiols to be possible. Firstly, when the Hcy or Cys is added into the solution of TPE-Py progressively, the absorbance peak at 382 nm decreased gradually and finally total disappeared (Fig. S10), suggesting the reaction indeed proceed between TPE-Py and biothiols. With the addition of Hcy into the solution of TPE-Py, the emission at 550 nm is decreased, meanwhile, a new blue emission peak at 455 nm emerges and the intensity enhances along with increasing of concentration of Hcy (Fig. 2A). In contrast, upon addition of Cys or GSH to the solution of TPE-Py, only drastic decreasing of fluorescence was observed (Fig. 2B, Fig. S11). Above observations demonstrate that TPE-Py exhibits good selectivity for Hcy over Cys and GSH. Compared to TPE-Py, TPEPh-Py exhibits poorer fluorescence response towards Hcy at the same condition (Fig. S12), which mainly attributed to tighter aggregate of TPEPh-Py in detection media.

The high sensitivity and selectivity of TPE-Py for Hcy also can be clearly seen from the ratiometric changes in the emission intensity of TPE-Py. As described in Fig. 3A, the ratio of the emission intensity at 455 and 550 nm (I_{455}/I_{550}) shows a variation from 0.02 to 5.93 in the presence of 60 mM Hcy, but to 1.55 and 2.15 at the same concentration of Cys and GSH, respectively. When the biothiols concentration is down to 100 μM, TPE-Py still shows higher selectivity for Hcy (Fig. 3B). Finally, Hcy detection using TPE-Py was found to have a detection limit of 3.46×10^{-7} M and a linear range between 1.5 and 18.0 μM (Fig. S13).

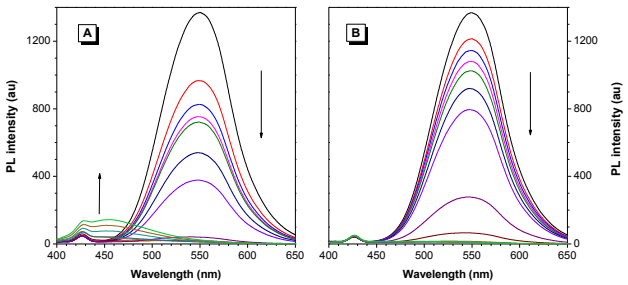


Fig 2. PL spectra of TPE-Py (3 μM) in acetonitrile/phosphate buffer (20 : 80 v/v, 20 mM, pH 8) with different concentration (0–60 mM) of (A) Hcy and (B) Cys.

Subsequently, the selectivity of TPE-Py toward Hcy over other amino acids was investigated by emission spectra. Upon addition of same amount amino acids (60 mM), only Hcy switched the fluorescence from 550 nm to 455 nm, while Cys and GSH lead to decreasing in emission intensity at 550 nm. In contrast, the emission spectrum shows negligible alteration for other amino acids compared with biothiols (Fig. S14). Furthermore, the increment of I_{455}/I_{550} for Hcy is much larger than Cys, GSH and other amino acids as shown in Fig. 4. Above sensing process can also be seen visually from the photos taken under 365 nm UV illuminations. TPE-Py exhibits bright yellow emission color in detection media, and this color almost faded away entirely after addition of Cys or GSH. But in the presence of Hcy, the yellow color was changed to blue, which can be easily differentiated from Cys or GSH treated sample. We also tested the fluorescence response of TPE-Py to typical thiol compounds and non-thiol nucleophiles. As described in Fig. S15, the increment of I_{455}/I_{550} for them is negligible compared with that of Hcy. The result shows that TPE-Py exhibits highly specificity for Hcy, and the sensing process can be easily distinguished by naked eyes owing to its clear change of emission color.

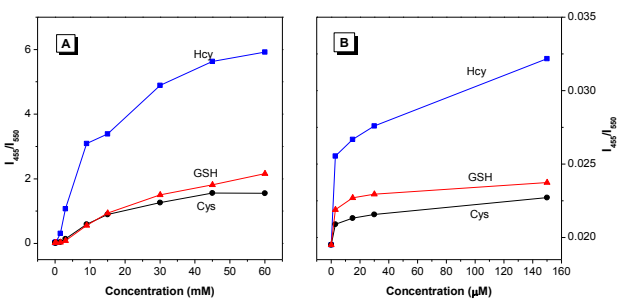


Fig. 3 (A) Plots of the I_{455}/I_{550} *versus* the concentration of Hcy, Cys and GSH, respectively. (B) The magnified range from 0 to 150 μM of panel A.

Mechanistic study

To verify our hypothesis that the fluorescence change comes from addition reaction between TPE-Py and biothiols and give a better understand of above detecting mechanism, NMR spectrum are used. In the ^{1}H NMR spectrum of TPE-Py, a distinct resonance signal at around 7.79 ppm was observed, assigned to the H_a of the ethylenic bond. Conversely, upon addition of 5 equiv Hcy to solution of TPE-Py, the ethylenic proton signal disappeared, but a new resonance signal at 4.26 ppm distinctly appeared which attributed to the thioether methine proton H_b (Fig. 5C), demonstrating unambiguously 1,4-addition reaction of Hcy to the α,β-unsaturated ketone sub-unit in TPE-Py. Mass spectrum analysis also reveals the occurrence of 1,4-addition reaction between TPE-Py and Hcy. As described in Fig. S3, the molecular ion peak of TPE-Py appears at m/z = 540.2312 ($M+H^+$). Upon addition of Hcy, a new fragment peak at m/z = 599.2356 is observed, which is consistent to the molecular ion fragment of the adduct product TPE-Py-Hcy ($M+H^+$) (Fig. S4).

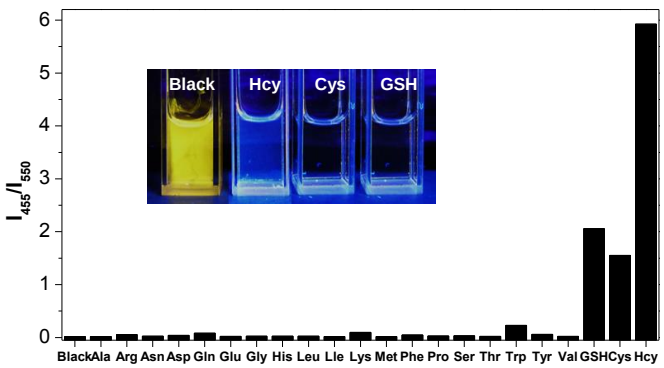


Fig.4 Emission ratio I_{465}/I_{550} of TPE-Py in the absence or presence of 60 mM different amion acids in solution. Inset: photo of TPE-Py in blank or 60 mM Hcy, Cys and GSH under 365 nm UV lamp illumination.

Combining the results of NMR and mass spectra analysis, the biothiols can react with TPE-Py, which break the conjugation of molecular backbone and should theoretically switch its fluorescence. The fluorescence of TPE-Py, however, is selectively changed to blue region by Hcy rather than Cys and GSH in the detection media. We are curious about the origin of such appearance. Taken into consideration the AIE property of TPE-Py, the difference in fluorescence response may be ascribed to the varied solubility of adduct products between TPE-Py and biothiols. Hcy is more hydrophobic than Cys and GSH, the adduct product of TPE-Py and Hcy thus possess poor solubility and form aggregate in detection media. On the basis of AIE mechanism, the aggregate formation restricts the intramolecular motion leads to the intense blue emission for adduct product of TPE-Py and Hcy. On the contrary, the better hydrophilic feature of Cys and GSH can prompt the dissolution of their adduct product in detection media, and thus generate the very faint fluorescence. To the best of our knowledge, this is the first example of fluorescent probe based on α,β-unsaturated ketone unit for distinguishing the Hcy over Cys and GSH.

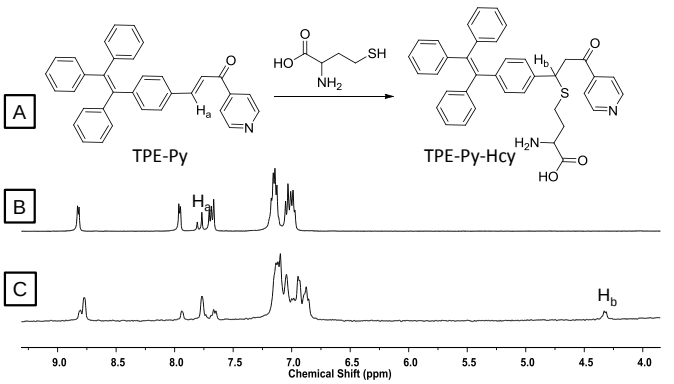


Fig.5 (A) Reaction of TPE-Py with Hcy. ^{1}H NMR spectra of TPE-Py (B) and TPEPy-Hcy adduct (C) formed by addition of 5 equiv Hcy in d_6-DMSO solutions.

Conclusions

In conclusion, an AIE probe, TPE-Py, containing a α,β-unsaturated group was developed for detecting biothiols. TPE-Py displays strong yellow emission in acetonitrile/phosphate buffer (20 : 80 v/v, 20 mM, pH 8). Upon addition of Hcy, the yellow emission of TPE-Py is switched to blue. However, in the presence of Cys or GSH, the yellow emission of TPE-Py is quenched without appearance of blue emission. NMR and mass spectra analysis suggests that the biothiols can react with TPE-Py based on 1,4-addition reaction. Combining the AIE property of TPE-Py, the difference in sensing behaviour of TPE-Py for biothiols can be attributed to the solubility of their adduct products in detection media. The synergistic effect of the reaction and AIE property enables TPE-Py possesses high selectivity toward Hcy over other biothiols and other amino acids.

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Electronic Supplementary Information (ESI) available: NMR and high resolution mass spectra; supplementary figures. See DOI: 10.1039/b000000x/

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TOC: In this work, an novel AIE fluorescent probe, TPE-Py, containing an α,β-unsaturated ketone unit was developed for discriminating homocysteine over cysteine and glutathione.