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ARTICLE

Discrimination of homocysteine, cysteine and glutathione using an aggregation-induced-emission-active hemicyanine dye

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Elevated levels of homocysteine (Hcy) in blood are indicative of many high risk cardiovascular and neurodegenerative diseases. Thus, development of highly efficient and selective dyes for monitoring Hcy levels has attracted much attention. This paper reports the utilization of TPE-Cy, an aggregation-induced-emission active hemicyanine dye, as a probe for the detection of Hcy. More interestingly, this dye shows high selectivity to the Hcy over cysteine, glutathione and other amino acids in weakly basic buffer solution.

Introduction

Thiol containing small biomolecules, including cysteine (Cys), homocysteine (Hcy) and glutathione (GSH), play a pivotal role in many biological systems.[1] For example, Cys is the only amino acid with thiol functional group that serves as an unique unit in protein construction, enzyme active sites and cofactors.[1a-c] On the other hand, GSH, a Cys containing tripeptide, is the most abundant free thiol in cells and is essential in maintaining the redox homeostasis in the intracellular environment.[1e,f] Recently, much research interest has been paid to Hcy because of its special role as biomarkers in many disease.[2] For instance, an elevated level of Hcy in the blood is a strong indicator for cardiovascular diseases,[2a,b] stroke and arteries or venous thrombosis.[2c] In addition, Hcy has also been reported to be associated with neurodegenerative disorders,[2d] such as neural tube defects, Alzheimer's disease[2g] and other cognitive impairments.[2h] Therefore, tremendous amount of effort has been put into the development of efficient methods for monitoring Hcy levels for the early stage detection of these diseases.

For Hcy detection, one of the most common analytical techniques is liquid chromatography (LC).[3] Although this method offers high accuracy, it typically requires staff with certain skill level, intensive labour, and sophisticated instrument for the analysis, which makes it not suitable for on-site trials and household testing. Fluorescence sensors, on the other hand, possess many advantages, such as superior sensitivity, simple manipulation and low cost,[4] representing an alternative platform for Hcy detection. As a result, much effort has been devoted to the design and fabrication of fluorescent sensors for thiol recognition.[5] Most of the sensing process are designed base on the reaction utilizing of the strongly nucleophilic thiol groups, for example, through covalent bondings with olefins via Michael addition[5f] or with carbonyls via heterocycle formation.[5b-d] Because all Hcy, Cys, and GSH contain thiol moieties, it is quite difficult to differentiate among these molecules using the reaction based fluorescent sensors. In particular, Hcy is a homologue of Cys with only one additional methylene (-CH_2-) group; the differentiation of these two is very challenging. Only a few cases have been reported for the selective detection of Hcy over Cys and GSH.[6] Srongin and coworkers have developed a system for Hcy and Cys discrimination based on the differences in cyclization rate of the two analytes with the probe.[6a] Huang and coworkers have designed a iridium(III) complex which shows relatively high specificity towards Hcy.[6b] Nevertheless, the sensing process of these probes is carried out in a media with large portion of organic solvents, which dramatically limits their application especially in biological systems.

Recently, an aggregation-induced-emission (AIE) active hemicyanine dye, TPE-Cy, has been developed in our group.[7,8] This dye is sensitive toward pH changes: under basic conditions, the nucleophilic OH^- ion can attach to the C1 site (Chart 1) and disrupt the conjugation of the tetraphenylethene (TPE) unit with the cyanine moiety, thus shifting the emission from red to blue. This proposed mechanism is supported by NMR and HRMS analysis. Thiol is a stronger nucleophile than hydroxyl group and could also react with electrophilic TPE-Cy, changing its emission color.[9] In this work, the feasibility of

using TPE-Cy in biothiol detection is investigated, which demonstrates the specificity of the dye for Hcy recognition.

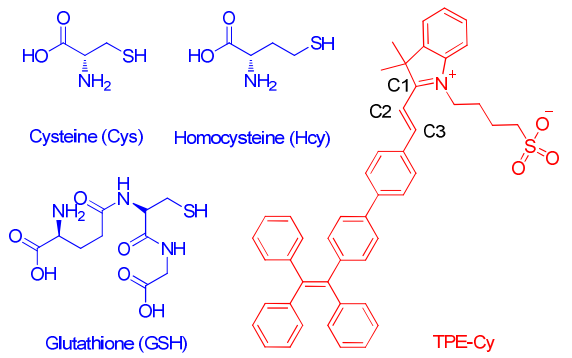


Chart 1. Structure of Cys, Hcy GSH. And TPE-Cy.

Experimental Section

Materials

Buffer solution with pH 8 was purchased from Merck. Homocysteine is purchased from TCI. Cysteine, glutathione, and other amino acids are purchased from Sigma-Aldrich.

Instrumentation

^{1}H and ^{13}C NMR spectra were measured on a Bruker AV 400 NMR spectrometer or a Bruker ARX 300 NMR spectrometer using DMSO-d_6, or D_2O as solvent and tetramethylsilane (TMS) as internal reference. UV absorption spectra were taken on a Milton Ray Spectronic 3000 array spectrophotometer. Photoluminescence (PL) spectra were recorded on a Perkin-Elmer LS 55 spectrofluorometer.

Sample Preparation

TPE-Cy was synthesized according to previous reported procedures.[7] Different concentrations of Hcy, Cys, GSH or other amino acids were first dissolved in pH 8 buffer and prepared by serial dilution. TPE-Cy (1 mM in DMSO) stock solution was then added to the solution containing the analytes. The finial concentration of DMSO is 1%. Measurements were taken 5 minutes after adding TPE-Cy.

Characterization Data

Characterization Data of Cys: ^{1}H NMR (400 MHz, D_2O), δ (TMS, ppm): 3.98 (t, 1H), 3.12–2.99 (m, 2H). ^{13}C NMR (100 MHz, D2O), δ (TMS, ppm): 171.71, 6.10, 24.1.
Characterization Data of Hcy: ^{1}H NMR (400 MHz, DMSO-d_6), δ (TMS, ppm): 3.23 (t, 2H), 2.56 (t, 1H), 2.01–1.91 (1H), 1.85–1.77 (1H). ^{13}C NMR (100 MHz, DMSO-d_6), δ (TMS, ppm): 169.1, 52.9, 35.6, 20.6.
Characterization Data of TPE-Cy+Cys: ^{1}H NMR (400 MHz, DMSO-d_6), δ (TMS, ppm): 7.59–7.52 (m, 4H), 7.45 (d, 2H), 7.16—7.10 (m, 11H), 7.09–6.96 (m, 10H), 6.67 (1H), 6.60 (d, 1H). 5.36 (q, 1H), 4.79 (dd, 1H), 4.04–4.03 (m, 1H), 3.90 (t, 1H), 3.11 (dd, 2H), 3.01–2.867 (m, 3H), 1.67 (s, 4H), 1.58 (d, 3H), 1.29 (d, 3H). ^{13}C NMR (100 MHz, DMSO-d_6), δ (TMS, ppm): 169.6, 169.4, 168.9, 154.0, 153.7, 144.9, 143.2, 142.3, 141.9, 140.81, 140.2, 138.0, 137.5, 131.3, 130.8, 130.7, 128.2, 128.0, 127.9, 127.7, 126.7, 126.7, 126.5, 125.8, 121.4, 118.2, 105.5, 94.1, 93.8, 79.2, 54.7, 54.7, 52.1, 51.4, 51.2, 46.5, 46.2, 44.2, 41.2, 32.0, 28.5, 28.5, 27.9, 25.5, 23.0, 22.9.
Characterization Data of TPE-Cy+Hcy: ^{1}H NMR (400 MHz, DMSO-d_6), δ (TMS, ppm): 7.58–7.44 (m, 7H), 7.15–7.09 (m, 12H), 7.04–6.97 (m, 10H), 6.66 (t, 1H), 6.59 (d, 1H), 5.23 (d, 1H), 4.74 (d, 1H), 2.57 (t, 6H), 2.05–1.96 (m, 5H), 1.93–1.86 (m, 4H), 1.64 (s, 4H), 1.57 (s, 4H), 1.31–1,25 (m, 4H), 1.16 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6), δ (TMS, ppm):170.3, 170.1, 145.0, 143.2,143.2, 142.2, 140.8, 140.2, 137.7, 137.5, 131.3, 130.7, 128.1, 128.0, 127.8, 127.6, 126.7, 126.6, 126.5, 125.8, 118.1, 52.3, 51.3, 45.1, 44.1, 35.3, 28.6, 28.5, 27.7, 22.7, 20.3.

Results and Discussion

Responses of TPE-Cy to Hcy and Cys

We first examine the reaction between TPE-Cy and Hcy. From the UV-vis spectra, TPE-Cy exhibits two absorption peaks at ~440 (A_{440}) and ~330 nm (A_{330}) in pH 8 buffer that can be attributed to the entire molecule and the TPE unit, respectively (Fig.1). When Hcy is added into the solution, the absorption peak at 440 nm decreased dramatically while the counterpart at 330 nm increased, which is very similar to the phenomenon observed when strong base is added. The change in TPE-Cy absorption indeed indicates the disruption of the conjugation between cyanine and TPE and the formation of new chemical species upon reaction with Hcy.

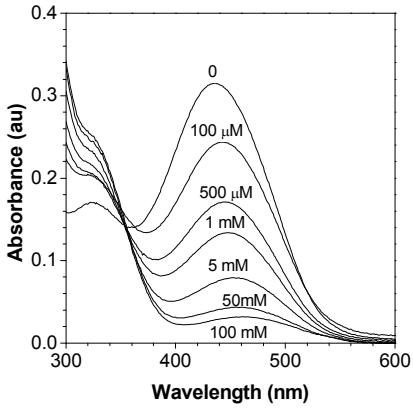


Fig. 1 Absorption spectra of 10 μM TPE-Cy in the presence of different concentration of Hcy in pH 8 buffer.

Similar to absorption spectra, the emission spectra of TPE-Cy changed upon the addition of Hcy (Fig.2A). In pH 8 buffer, a weak red emission peak is predominant. Increasing the concentration of Hcy gradually weakens the intensity of this red emission peak at 615 nm and, on the other hand, promotes the emergence and enhancement of a new blue peak at 465 nm.

Since Cys also carries a thiol group, it can also react with TPE-Cy and induce similar change in its emission spectra when the Cys concentration is above 10 mM (Fig.2B).

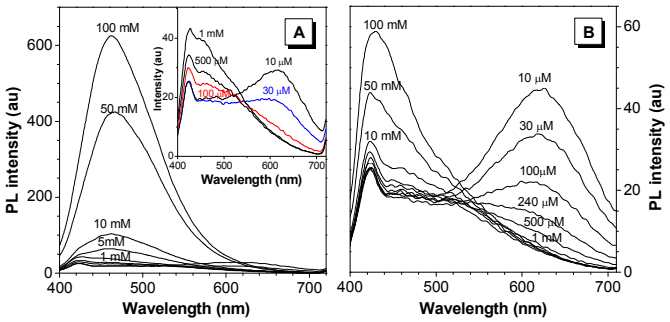


Fig. 2 PL spectra of TPE-Cy in pH 8 buffer with different concentrations of (A) Hcy and (B) Cys. [TPE-Cy] = 10 μM.

The above observations suggest that both Hcy and Cys can react with TPE-Cy, however, the response to Hcy is much more pronounced than Cys. The high sensitivity and selectivity to Hcy can be clearly seen from the ratiometric changes in the absorption or emission of TPE-Cy (Fig.3). The ratio of the absorption peak at 330 and 440 nm (A_{330}/A_{440}) is increased from 0.54 in pure buffer solution to 13.6 in the presence of 100 mM Hcy but only 3.2 with the same concentration of Cys (Fig.3A). When the analyte concentration is down to 100 μM, there is 1.6 times increment of the A_{330}/A_{440} value for Hcy but almost indiscernible for Cys. The detection limit of TPE-Cy for Hcy is calculated to be 12 μM. Note that the physiological level of Hcy in blood is lower than 15 μM, above which is considered to have hyperhomocysteinemia.[2b] In the same condition, the detection limit for Cys is as high as 73 μM.

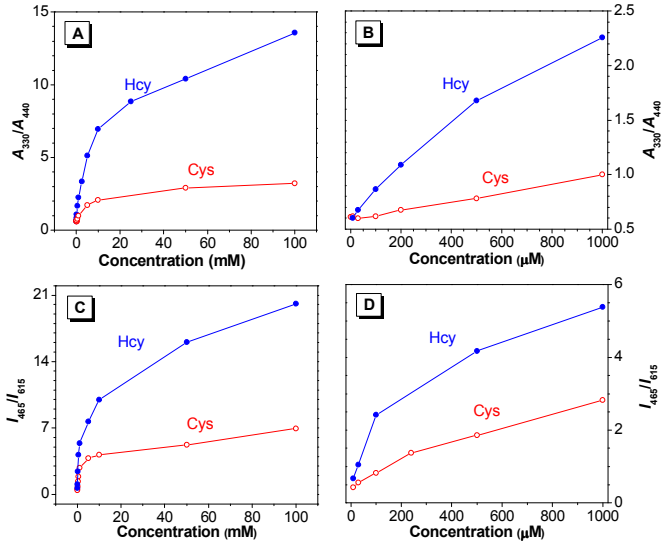


Fig. 3. (A) Plots of A_{330}/A_{440} versus the concentration of Hcy or Cys, where A_{330} and A_{440} are the absorption values at wavelengths of 330 and 440 nm, respectively. (B) The magnified range from 0 to 1 mM of panel A. (C) Plots of I_{465}/I_{615} versus the concentration of Hcy or Cys. I_{465} and I_{615} are the emission intensities at wavelengths of 465 nm and 615 nm, respectively. (D) The magnified range from 0 to 1 mM of panel C. [TPE-Cy] = 10 μM; λ_{ex} = 380 nm. Tests are taken in pH 8 buffer.

In terms of fluorescence, the ratio of the blue and red emission (I_{465}/I_{615}) also demonstrates the sensitivity and selectivity of TPE-Cy towards Hcy (Fig.3B). With 100 μM Hcy, the I_{465}/I_{615} value reaches 2.4, which is 3 times higher than the value with the same concentration of Cys. This ratio is further increased to 20.4 when the Hcy concentration is 100 mM, while for Cys, the highest I_{465}/I_{615} value is only 6.9. These results indicate that TPE-Cy shows higher response in both absorption and emission change toward Hcy over Cys.

Selectivity Test

The selectivity of TPE-Cy to Hcy over other amino acids and GSH is then investigated. As shown in Figure 4, the increment of the blue to red emission ratio induced by Hcy is much larger than that triggered by Cys and GSH, as well as other amino acids. The selectivity of TPE-Cy to Hcy can be clearly seen from the photos taken under UV irradiation. TPE-Cy shows red emission in buffer solution (blk) without any amino acid And the intensity of this red emission is slightly weakened when 100 mM GSH is added. On the toher hand, when the same amount of Cys is introduced to the buffer solution, the emission color is changed from red to weak blue and intense blue emission is observed when Cys was replaced with Hcy. All these changes in emission color and intensity can be easily distinguished by naked eyes. With all other amino acids, the emission remains almost unchanged as the blank TPE-Cy solution.

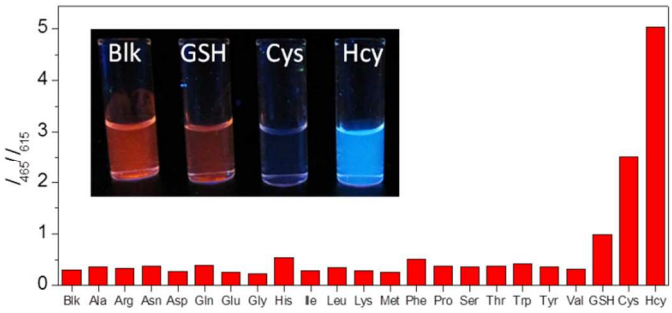
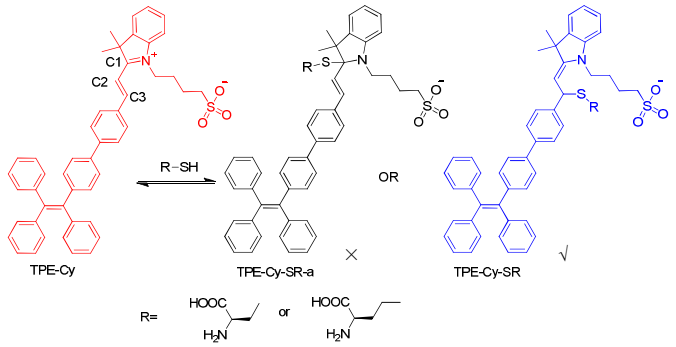


Fig. 4 Emission ratio I_{465}/I_{615} of TPE-Cy in the absence or presence of 1 mM different amino acids or GSH in pH buffer. [TPE-Cy] =10 μM; λex=380 nm. Inset: photo of 10 μM TPE-Cy in blank buffer or 100 mM GSH, Cys and Hcy (from left to right) under a handhold UV lamp illumination.

Mechanistic Study

The phenomenon of biothiol triggered emission changes of TPE-Cy prompts us to investigate the underlying working mechanism. The above absorption and emission behaviours all suggest a chemical reaction between TPE-Cy and Hcy and the reaction product is thus fully analysed by NMR spectroscopy.

Retrostructural analysis of TPE-Cy suggests that the potential reactive site could be the carbon atoms labeled with C1, C2, and C3 in the chemical structure shown in Scheme 1. The thiol group can act as a nucleophile to attack the electron-deficient C1, C2, or C3, resulting in the breakage of the conjugation and switch the emission of TPE-Cy from red to blue. Considering the α,β-unsaturated iminium moiety in in TPE-Cy, C1 and C3 are the possible sites for the addition reaction to occure. The 1,2-addition mode at C1 gives TPE-Cy-SR-a while the 1,4-addtion mode at C3 generates TPE-Cy-SR (Scheme 1). In our previous work using TPE-Cy as a pH sensor, we have proposed that the nucleophilic OH^- prefers to attack the C1 position and result in pH-switched red/blue emission.[7] Based on the hard soft acid base theory,[10] we believe the hard nucleophile like OH^- favours to react at C1 position while soft nucleophile like RSH would prefer to react at the C3 site.



Scheme 1. Proposed Mechanism of the Reaction Between TPE-Cy and Biothiols.

To verify our hypothesis, NMR analyses are used. Upon addition of Cys or Hcy, most of the proton signals in aromatic region are shifted upfield, presumably due to the absence of electron-withdrawing iminium functional group, except for protons on phenyl groups that forms the TPE core which remains unshifted (Fig.5). Closer examination of the resulting spectra reveals the absence of the olefin protons for α,β-unsaturated iminium molecule (7.83 and 8.48) and a new set of signals corresponding the enamine olefin (5.0–5.5) and benzylic allylic proton (4.5–5.0) appeared, indicating the reaction of Cys or Hcy with TPE-Cy proceeds in a 1,4-addition fashion. It is clear that the excessive Hcy/Cys can attack the C3 position of the TPE-Cy molecule, which breaks the conjugation, and hence switch the red emission into blue. Compared with Cys, Hcy has one additional methylene ($-CH_2-$) group, the longer spacer provides less steric effect, which could facilitate the addition reaction. On the other hand, presumably due to the greater steric hindrance, the tripeptide biothiol GSH has the lowest reactivity towards TPE-Cy compared to Cys or Hcy. The ^{13}C NMR spectra of these reactions are provided in the ESI (Fig S1).

In addition to the reactivity differences, we believe the AIE property of TPE-Cy also plays an important role in its different responses to Hcy, Cys and GSH. Upon the addition reaction with biothiol, the positively charged nitrogen on TPE-Cy is neutralized which will increase the hydrophobicity of the resulting product. Because Hcy is the most hydrophobic molecule among the three biothiol examined, the product from the TPE-Cy and Hcy reaction would have higher tendency to aggregate in the aqueous buffer solution. Because of its AIE property, the aggregate formation restricts the intramoleuclar rotations, upon which, the radiative decay will be populated and the fluorescence will be enhanced. The collective effect makes the TPE-Cy-Hcy adduct the most emissive than TPE-Cy-Cys and TPE-Cy-GSH.

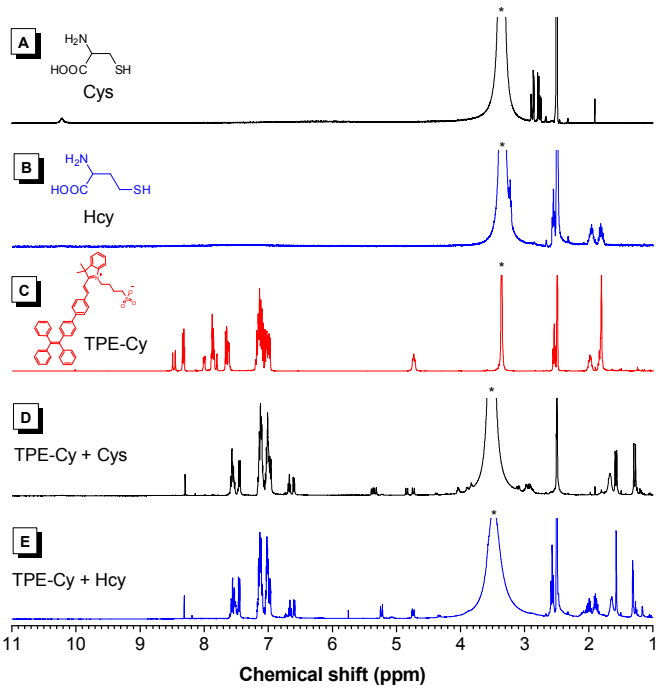


Fig. 5 ^1H NMR spectra of (A) Cys, (B) Hcy, (C)TPE-Cy, (D) TPE-Cy with Cys and (E) TPE-Cy with Hcy in DMSO-d_6. The water peaks are marked with asterisks.

In summary, a red-emission hemicyanine AIE-active dye, TPE-Cy, has been utilized for the discrimination of Hcy, Cys and GSH. TPE-Cy possesses high selectivity toward Hcy over other amino acids, including the thiol containing Cys and the tripeptide, GSH. Both the absorption and fluorescent emission of TPE-Cy are sensitive to Hcy. In the presence of Hcy, the red emission of TPE-Cy in weakly alkalized buffer is suppressed while a blue emission appears. Cys, although can also decrease the red emission of TPE-Cy, cannot promote the blue emission as much as Hcy and results in a weak blue emission. NMR analysis suggests that the thiol group can attack the double bond between the TPE unit and the cyanine unit of TPE-Cy through a 1,4-addition fashion which is facilitated by the less sterically hindered Hcy. Because of the steric hindrance, GSH is the least reactive and no obvious difference in terms of fluorescence can be observed of TPE-Cy with GSH. The collective effect of the reaction and the AIE property of the dye

enables its sensitivity and selectivity to Hcy over other biothiols.

Acknowledgements

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Notes and references

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† Electronic supplementary information (ESI) available: ^{13}C NMR spectra.

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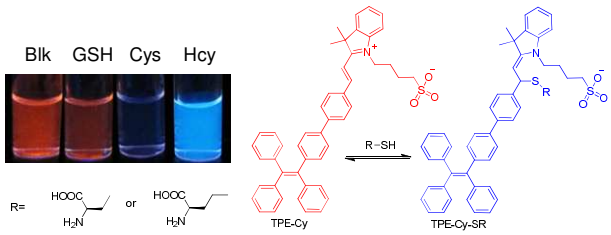
Discrimination of homocysteine, cysteine and glutathione using an aggregation-induced-emission-active hemicyanine dye

Sijie Chen, Yuning Hong, Jianzhao Liu, Nai-Wen Tseng, Yang Liu, Jacky Wing Yip Lam, and Ben Zhong Tang*



An aggregation-induced-emission active hemicyanine dye, TPE-Cy, shows high selectivity to the homocysteine over glutathione and other amino acids.

Graphical abstract