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PAPER

Discriminative fluorescence detection of cysteine, homocysteine and glutathione via reaction-dependent aggregation of fluorophore-analyte adducts†

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A novel fluorescence probe capable of discriminatively and simultaneously detecting Cys, Hcy and GSH has been developed. This specially designed probe can selectively react with Cys and Hcy to form thiazinane and thiazolidine derivatives in the presence of diverse amino acids, protected Cys and glucose and display the expected aggregation-induced emission (AIE) properties. Relying on the differences in kinetics, Cys can be easily and discriminately detected over Hcy by the observation of FL responses. GSH shows great interference with the detection of Cys and Hcy and it can be quantitatively detected by the FL spectroscopic titration method. The threshold of the FL turn-off concentration for GSH is measured to be 1 mM. This is the first report of using a single fluorescent probe to discriminately detect Cys, Hcy and GSH by FL turn-on and turn-off strategies. The discrimination relies on the reaction-dependent fluorophore aggregation, or the solubility of adducts of the probe molecule and analytes. The present strategy is intrinsically a fluorescent titration, which combines the high sensitivity of FL spectroscopy and the reliability of precipitate titration methodology. The threshold concentration of Cys (375 μ M, at which the FL is turned-on) coincides with the upper margin of the deficient Cys levels in human plasma, and the primary investigation of the FL response to deproteinized human plasma indicates that this FL probe is a promising one for the discriminatory detection of Cys over Hcy and GSH on a clinical level.

Introduction

Discriminative fluorescence detection of biological thiols, such as cysteine (Cys), homocysteine (Hcy) and glutathione (GSH) is of great fundamental and practical significance because Cys,¹ Hcy² and GSH³ possess various important biochemical functions. So far, several strategies have been applied to detect Cys, Hcy and GSH, for example, high performance liquid chromatography,⁴ gas chromatography-mass spectrometry,⁵ capillary

electrophoresis separations coupled with different detection methods,⁶ electro-chemical assays,⁷ optical detection based on the interplasmon coupling of Au nanorods,⁸ nano-particles⁹ and synthetic reporters.¹⁰ Among these strategies, optical assays based on fluorescent probes have attracted extensive interest in the past decades owing to their high sensitivity, simplicity and low cost.^{10j} The fluorescence (FL) detection is the most appealing one due to its low detection limit and potential for *in vivo* imaging of living cells, whereas the visual or colorimetric protocol possesses inherent merits, including visual detection and real-time *in situ* responses, which are suitable for the *in vitro* analysis of biological samples.^{10j–10l}

In principle, most of the reported fluorescent probes sensitive to Cys/Hcy/GSH are based on the active thiol group on the residue, and different mechanisms such as Michael addition¹¹ and cleavage reactions¹² have been proposed. Though these probes show high sensitivity toward thiol-containing compounds, the selective and simultaneous detection of Cys/Hcy/GSH is hampered because of “cross-talking” between different thiols. Since the pioneering work of Strongin and colleagues, fluorophores bearing aldehydes have been used as fluorescent probes for both Cys and Hcy.¹³ The mechanism was confirmed to be the well-known cyclization of Cys/Hcy with aldehydes to form thiazolidines/thiazinanes. Due to the similarity

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† Electronic supplementary information (ESI) available: Characterization data for DMBFDPS and the resultant thiazolidine derivatives (Fig. S1 and S2), AIE properties of this probe in DMSO/10 mM HEPES (6/4 in volume, pH 7.4) (Fig. S3), absorption spectra, emission spectra, fluorescence photographs taken under ambient and UV light (Fig. S4–S9 and Fig. S12–14), proposed reaction mechanisms for the interference of Hcy and GSH in the detection of Cys (Schemes S1–S4, Fig. S10 and S11). See DOI: 10.1039/c2jm32892e

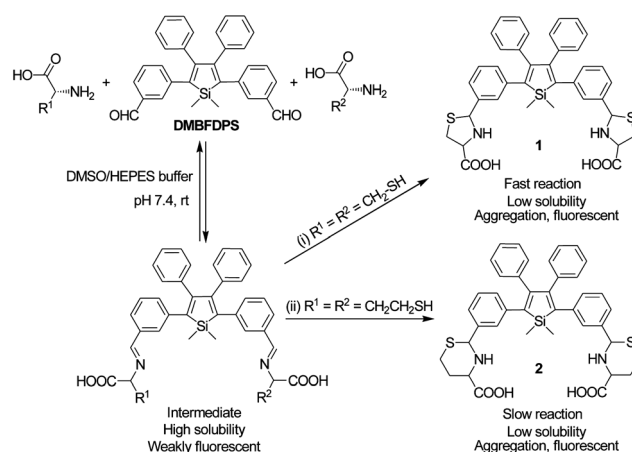
in the reactivity of the amino-thiol moieties of Cys and Hcy towards aldehydes, the methodology of using thiazolidine/thiazinane formation often renders the detection of total content of Cys and Hcy.¹⁴ Recently, selective detection for either Cys or Hcy has been developed by using some aldehyde-containing fluorescent probes. For instance, Strongin *et al.* reported an optical method to discriminate Cys and Hcy from other amino acids and thiols at physiological pH.¹⁵ The discrimination of Cys and Hcy is attributed to different rates of intramolecular cyclizations of their respective thioether adducts derived from 2-(2'-hydroxy-3'-methoxyphenyl)-benzothiazole acrylate.¹⁶ Thus the selective and simultaneous fluorescence detection of Cys and Hcy was achieved. More recently, Yu and colleagues reported a carbazole-based fluorescent probe which is capable of selectively detecting Cys over Hcy, but the exact mechanism has not been clarified yet.^{10m} This progress suggests that a huge space to develop novel fluorescent probes for the selective and simultaneous detection of Cys, Hcy and GSH still exists.

Herein, we demonstrate an unprecedented strategy to selectively and simultaneously detect Cys, Hcy and GSH. In comparison with the reported ones, the most significant modification is that we have taken the advantage of the aggregation of reaction-dependent fluorophore. The fluorophore used in the present work shows distinctive FL behavior, which is termed aggregation-induced emission (AIE). AIE active molecules are non-emissive in dilute solution but become highly emissive upon aggregate formation.¹⁷ It means that the FL turn-on relies on the formation of aggregates. Thus it is rational to conceive that the same aldehyde-functionalized AIE active fluorophore may react with Cys, Hcy and GSH (analyte) in different kinetic rates and produce different adducts of AIE-fluorophore-analyte. These adducts may show different critical concentrations for aggregate formation if they have different solubilities. The differences potentially allow them to be separately and/or stepwise detected by FL turn-on strategy. Such a process is similar to the classical, simple and reliable precipitation titration analysis that is widely used in the detection of different metal cations based on the solubility parameter (K_{sp}) of the compounds.

Results and discussion

Rational design and characterization of probe

Silacyclopentadienes (siloles) are in the list of widely explored AIE active molecules.¹⁸ We used an aldehyde-functionalized silole derivative [DMBFDPS, 1,1-dimethyl-2,5-bis(*meso*-formylphenyl)-3,4-diphenylsilole] (Scheme 1) as an AIE-active fluorescent probe. It is well-known that the cyclization reaction of an aldehyde group with amino and thiol groups results in thiazinane or thiazolidine; this reaction takes place in mild conditions with high efficiency and specialty thus has been widely used for Cys and Hcy detection.^{13,14} We take advantage of the stability of thiazinane or thiazolidine to reduce the side reactions of DMBFDPS molecules with other amino acids. In addition, the transition of aldehyde to thiazinane/thiazolidine by cyclization reaction can change the conjugation mode of the resultant products in comparison with DMBFDPS. It is expected that the detection process can be monitored by the changes in both FL intensity and color. As a result, a ratiometric probing system may



Scheme 1 Mechanistic representation of the discriminative detection of Cys and Hcy.

be fabricated, which could increase the dynamic change of the FL measurement.

DMBFDPS was prepared *via* the coupling reaction of 2-(3-bromophenyl)-1,3-dioxolane and the key intermediate 1,1-dimethyl-2,5-bis(zinc monochloride)-3,4-diphenylsilole, which was derived from bis(phenylethynyl)silanes. The subsequent deprotection reaction of the coupling resultant afforded the final product in a moderate yield. The structures of the final resultant DMBFDPS and the intermediates were fully characterized by multiple techniques including ¹H NMR, ¹³C NMR, FTIR and element analysis (see Experimental section).

AIE property of probe and detection medium

We firstly measured the FL spectrum of DMBFDPS in dimethyl-sulfone (DMSO), considering that DMSO is widely used in bioassay systems as a biocompatible organic solvent. The results shown in Fig. S1† indicate that DMBFDPS is very weakly emissive in its 10 mM DMSO solution. To examine the possibility of using DMBFDPS as a fluorescent probe in the biological medium, we then checked its FL behavior in the mixture of HEPES buffer/DMSO (8/2 in v/v). As shown in Fig. S3,† the emission is considerably intense because DMBFDPS is insoluble in the buffer and suspension rather than the solution which was formed [$F_b/F_0 = 65$, $F_b = 946$; where F_0 and F_b are the FL intensity recorded in DMSO and the mixture of HEPES buffer/DMSO (8/2 in v/v), respectively]. Fig. S1† also demonstrates the variation of FL intensity (F) with the volume fraction of HEPES buffer (f_b) respect to DMSO. When f_b is lower than 50%, F is small and remains unchanged; once f_b is up to 50%, F raises abruptly and is boosted continuously with the increase of f_b . These data indicate that DMBFDPS is a typical AIE active fluorophore with a threshold of f_b at which aggregates generate and FL is turned on. To ensure the biocompatibility, f_b should be as high as possible; however, to assure strong “turn-on” effect, f_b should be as low as possible. To achieve a balance between the two aspects, the mixture of 10 mM HEPES buffer and DMSO with $f_b = 40\%$ and pH = 7.4 was chosen as the action ratio and was subsequently used as the detection medium without specific notation in the following works.

The recognition of Cys and Hcy in different kinetics

DMBFDPS has moderate solubility in such medium and the solution with a concentration of 25 μM DMBFDPS displays an FL band peaked at 479 nm at a very low intensity (Fig. 1a). When Cys was added to the solution, it readily reacts with DMBFDPS at room temperature (rt), leading to thiazolidine derivative [1, Scheme 1, route (i)]. Molecule 1 should have higher solubility in the medium than DMBFDPS, because of the carboxylic and thiazolidine groups. But in fact, the intermolecular hydrogen-bonding between these groups may help to form aggregates thereby results in lower solubility. Since the reaction solution turned from transparent to turbid, this process could be observed by naked eyes. Monitoring the process with FL revealed that the FL response started immediately after the addition of Cys to the reaction medium and leveled off in about 100 minutes (Fig. 1b). The FL band peaked at 479 nm enhanced gradually. In comparison with Fig. S1,[†] this band is assigned to the emission of DMBFDPS and its aggregates. When Cys was introduced to the reaction system, the solubility of DMBFDPS was decreased and its aggregates were formed, thereby the emission was enhanced. Meanwhile, a new band peaked at 424 nm appeared and its intensity grew quickly with the increasing amount of the added Cys (Fig. 1a). This band is assigned to the resultant 1 and its aggregates. The blue-shift of the emission feature can be associated with the transformation of the aldehyde to the thiazolidine auxochrome, which eliminates the conjugation between the aldehyde and the silole core. The precipitation of molecule 1 may also take some DMBFDPS molecules along due to their intrinsic hydrophobicity. According to the restricted intramolecular rotation mechanism of AIE,^{17c,18a} this process can

induce the emission of DMBFDPS. As a result, an increase in intensity of the FL band peaked at 479 nm has been observed. When more and more resultant 1 was produced, the new emission band (424 nm) became predominant.

A quantitative evaluation of the changes in emission intensity and peak wavelength is displayed in Fig. 1b. The maximum intensity shows a drastic elevation from ~ 7 in the absence of Cys to ~ 102 in the presence of Cys (100 equiv.), or about 13-fold enhancement (for short, the emission enhancement is defined as $F/F_0 - 1$). Concurrently, the emission peak shows a significant blue-shift (~ 55 nm), and the emission color changes from greenish-blue to purple-blue. The emission enhancement can be ascribed to the poor solubility (precipitate) of the resultant thiazolidine in the buffer mixture. The precipitate is resulted from molecular aggregation, which induced the observed emission enhancement. This observation suggests that the probe could be employed for Cys detection by simple visual inspection.

In contrast, when Hcy was added to the DMSO/HEPES buffer mixture under the same conditions, little change in emission features was recorded for hours. In a long interval of 160 minutes, $F/F_0 - 1$ just increased from 0 to 1.2 and the emission peak showed a red-shift of about 15 nm (Fig. 1d). Three days later, white solids came out of the transparent solution (inset of Fig. 1c). Simultaneously, the FL peak blue-shifted from 472 to 424.5 nm and $F/F_0 - 1$ was measured to be 31.4. These observations imply that the reaction of Hcy with the probe molecule to produce thiazolidine is quite slow in comparison with Cys [Scheme 1, route (ii)]. According to literature, upon reaction with aldehyde, Cys forms the more favored 5-membered ring heterocycle, which is beneficial to the reaction proceeding, as compared to Hcy (6-membered ring formation).^{13,14} ^1H NMR and KBr pellet FTIR measurements were carried out to confirm the mechanism (Fig. S2 and S3[†]). It is worth noting that the discriminative detection of Cys and Hcy can be reflected qualitatively and concurrently by three parameters: precipitate formation, FL intensity enhancement and spectral shift.

The sensitivity of the probe to Cys

The sensitivity of DMBFDPS to Cys was investigated by FL spectroscopic titration experiments. The changes in FL features with Cys concentration in DMSO/HEPES buffer mixture are displayed in Fig. S4a.[†] From 0 to 2.5 mM, FL intensity increases slowly. The plot of $F/F_0 - 1$ versus Cys concentration is nearly a flat line parallel to the abscissa before the concentration of Cys increased to 375 μM . Once the Cys concentration is above 375 μM , the plot takes off with a sharp inflexion, the FL peak starts to blue-shift (Fig. S5[†]) and the clear mixture turned turbid at this point as well. At 2.5 mM of Cys, $F/F_0 - 1$ grows to over 8 in 1 h and white solid is clearly visible (inset in Fig. S4b[†]).

Since AIE behavior depends on the formation of aggregates or the solubility of the fluorescent species, it is expected that the FL intensity of DMBFDPS-Cys adduct will show temperature dependence because solubility normally decreases with the lowering of temperature. This was confirmed by monitoring the changes of $F/F_0 - 1$ versus Cys concentration at different temperatures. As shown in Fig. S6,[†] the FL enhancement at 18 $^\circ\text{C}$ (lower temperature) is more pronounced than that at 36 $^\circ\text{C}$ (higher temperature).

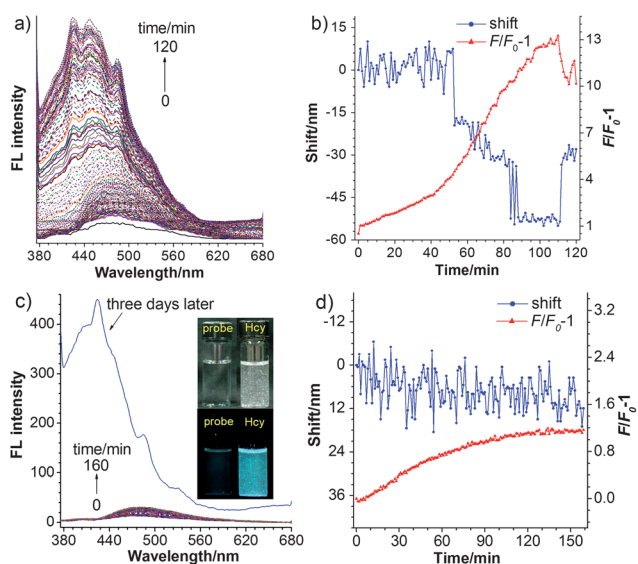


Fig. 1 Time dependent FL spectra when (a) Cys or (c) Hcy was added to the mixture of DMSO and 10 mM HEPES buffer (6/4 in volume, pH 7.4) containing DMBFDPS; (b) and (d) are corresponding plots of the changes in FL intensity and FL peak for (a) and (c), respectively. [DMBFDPS] = 25 μM , [analyte] = 2.5 mM, λ_{ex} = 356 nm. F for FL intensity in (a) and (c); $\Delta\lambda$ for wavelength shift, F for peak FL intensity and F_0 for the peak FL intensity of DMBFDPS at $t = 0$ min in (b) and (d). Inset of (c): FL photographs of DMBFDPS and Hcy in the above mixture taken 3 days later under ambient (top row) and UV-light (bottom row, λ_{ex} = 365 nm).

The specificity of probe to Cys

In addition to sensitivity, the specificity of a fluorescent probe is a crucial parameter for potential applications. To examine the specificity of DMBFDPS to Cys, the responses of the probe to other amino acids, glucose, GSH and protected Cys (Cya and Cyt) were investigated. As shown in Fig. 2 and S7,† among all the tested analytes, Cys gave the highest FL response. FL enhancement is more than 11-fold and the blue-shift of the FL peak is 45 nm for Cys in 60 min (Fig. 2a and b). About 4, 1.7 and 1-fold enhancement was recorded for Cyt, Hcy and Lys, respectively. And below 0.67 was observed for the rest analytes. The order of the sensitivity is Cys > Cyt > Hcy > Lys > other amino acids, glucose and GSH. Although both Cys and Hcy present a similar blue-shifted emission peak, it is easy to recognize one from the other by checking the emission intensity and formation of white precipitate (Fig. 2).

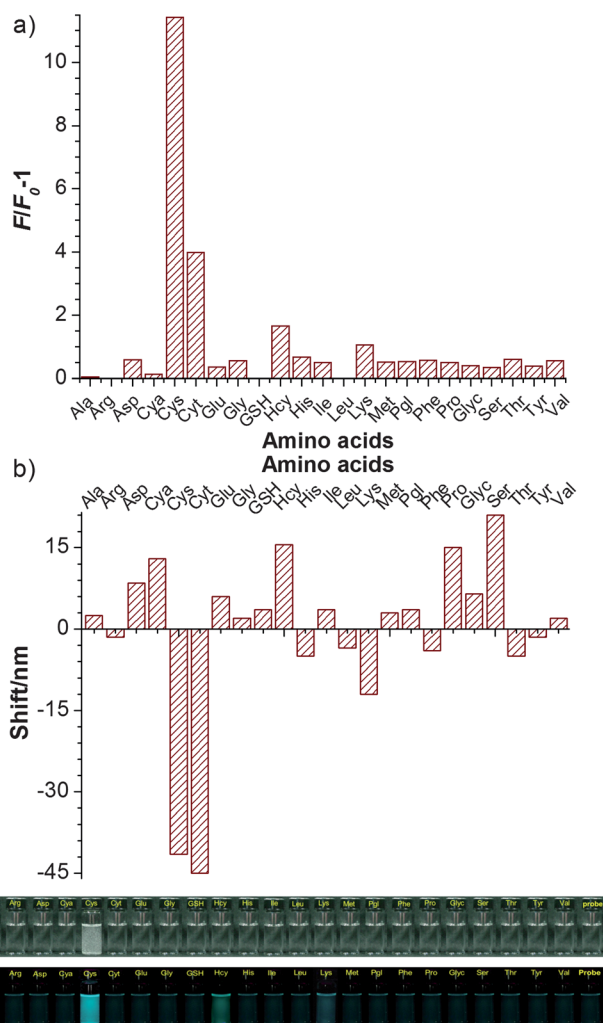


Fig. 2 Ratiometric and specific response of DMBFDPS to diverse amino acids (Ala, Arg, Asp, Cys, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Ser, Thr, Tyr, Val, Pro, Hcy, Pgl and GSH), glucose or Glyc and protected Cys [Fmoc-Cys(Trt)-OH, or Cyt and *N*-acetyl-L-Cys, or Cya] in the mixture of DMSO and 10 mM HEPES buffer (6/4 by volume, pH 7.4), [probe] = 25 μ M, [analyte] = 2.5 mM, λ_{ex} = 356 nm. All measurements were conducted at rt 60 min later. The probe is DMBFDPS.

The selectivity of the probe to Cys

In practical detection, Cys may co-exist with other amino acids, peptides and polypeptides containing free thiol group (*e.g.* GSH). Examination on the selective detection of Cys in the presence of other analytes is an essential step. When only 2.5 mM of Cys exists in the buffer solution (control sample), $F/F_0 - 1$ value is 11.4, and the shift of emission peak ($\Delta\lambda$) is about -40 nm. By measuring the FL spectra of mixtures containing Cys (2.5 mM) and other analytes (2.5 mM), it was found that most of the analytes, except GSH, Hcy and Cya, have exerted little impact on the detection of Cys in FL spectroscopic titration method (Fig. 3a and b, S8 and S9†). While, in the presence of Hcy, Cya and GSH (2.5 mM), the $F/F_0 - 1$ values are 2.4, 2.2 and 0.4-folds, respectively, which are evidently lower than that of the control one. Meanwhile, the shifts of emission peak ($\Delta\lambda$) are about -38, -1 and 25.5 nm for Cya, GSH and Hcy, respectively. The interference responses can be associated with the formation of different Schiff-base intermediates and the successive competing reaction of the

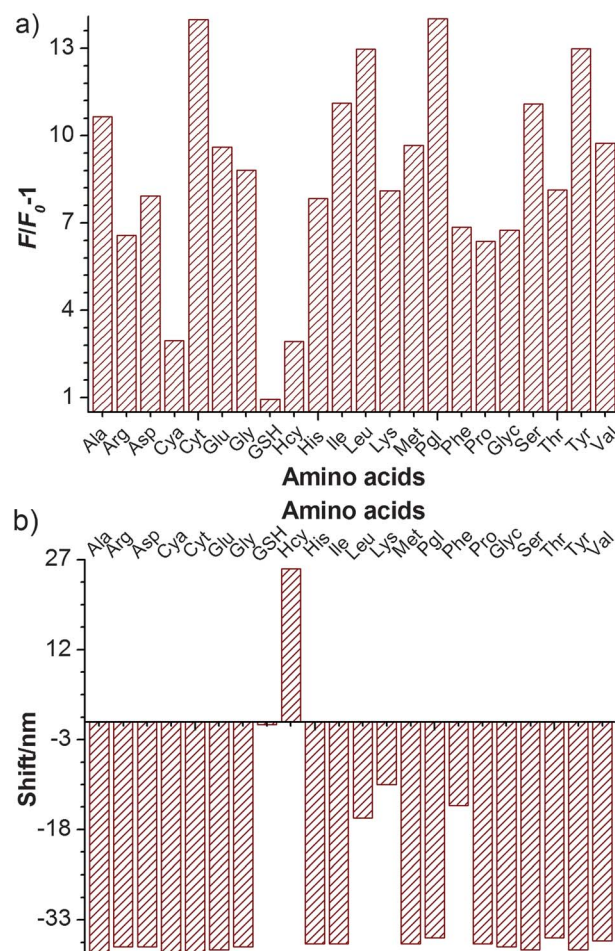


Fig. 3 FL response of DMBFDPS to the mixture of Cys in the presence of various analytes (Ala, Arg, Asp, Cys, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Ser, Thr, Tyr, Val, Pro, Hcy, Glyc, Cys, Cyt, Cya, GSH, and glucose or Glyc) in the defined buffer, [DMBFDPS] = 25 μ M, [analyte] = 2.5 mM, λ_{ex} = 356 nm. All measurements were conducted at rt after incubation for 60 min.

different Schiff-base intermediates with the free thiol group in Hcy, Cys and GSH as shown by the reactions in Scheme S1–S4.[†] These intermediates and the final adducts themselves, on one hand, are soluble in the reaction solution; and on the other hand, they interrupt the production of insoluble resulting products (aggregates). Thus the aggregation-induced emission has not been observed. The good solubility of the products allows us to measure their ¹H NMR and IR spectra (Fig. S10 and S11[†]). But the data are too complex to be clearly recognized and assigned, indicating the existence of multiple components.

In comparison with Cys, it can be considered as strong proof that Cyt had little interference with the FL response of Cys (Fig. 3a and b), because the thiol group in Cyt has been protected by a triphenylmethane group. It is noticeable that, when Hcy (2.5 mM) co-exists with Cys (2.5 mM), the addition of DMBFDPS (2.5 μM) to the mixture results in the formation of a mixture of intermediates, which subsequently proceed to a resultant mixture containing molecule **1**, **2**, and other derivatives (**3–10**, Scheme S1[†]). These derivatives have higher solubility and a lower ability to form aggregates in the buffer solution, thus showed decreased FL intensity (Fig. 3a). As shown in Fig. 3b, the addition of DMBFDPS to the mixture of Cys and Hcy leads to a red-shift of the FL peak. This observation is remarkably different from the phenomena observed for the addition of DMBFDPS to the Cys or Hcy buffer solution (Fig. 2b). The difference in spectral shift can be associated with the reactions shown in Schemes 1 and S1.[†] In this case, the intermediates rather than the resultant **1** are the main-products in 60 min. The intermediates (Schiff base structure) have larger conjugate system and higher solubility in comparison with the resultant products thus shows red-shifted and weak FL features.

The fluorescent response to GSH

GSH shows a significant quenching effect on the FL responses of the probe molecule to Cys. This is rational because the intermediates and the resultant products have good solubility in aqueous solution and can hardly form aggregates (Schemes S2 and S3[†]) and thereby have low FL intensity (Fig. 3a and b). This observation triggered a new idea. That is to use the mixture of Cys and DMBFDPS as a FL probe to detect GSH in the DMSO/buffer solution *via* a FL turn-off mechanism, which likes the retro-titration methodology used in analytical chemistry. Accordingly, we monitored the changes in FL, UV-vis absorption spectra and the appearance of the mixtures of Cys (2.5 mM) and GSH at different concentrations (Fig. 4, S12 and S13[†]). The FL peak (470.5 nm) intensity for the reference sample (25 μM free DMBFDPS in the defined buffer solution) was measured to be 10, while the FL peak (424 nm) intensity of mixture containing equivalent Cys and DMBFDPS without GSH was 358. Upon adding GSH into the buffer solution containing Cys, emission goes down rapidly. Along with the decrease of the $F/F_0 - 1$ value from 34.8 without GSH to 0.4 with 2.5 mM GSH, the emission peak red-shifts from 424 to 484.5 nm. The FL quenching slows down at 1 mM GSH [40 equivalent (equiv. for short) to DMBFDPS] and the quenching factor is about 100 for the solution without GSH. Meanwhile, the change of FL peak is in good accordance with

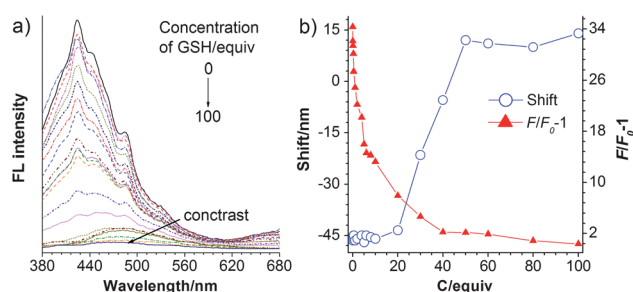


Fig. 4 FL spectra of DMBFDPS and Cys in the presence of different GSH concentrations in the mixture of defined buffer. [Cys] = 2.5 mM, [DMBFDPS] = 25 μM, λ_{ex} = 356 nm. All measurements were conducted at rt 60 min later.

the trend of FL intensity (Fig. 4b). In addition, as shown in Fig. S13,[†] the reverse detection of GSH was visually observed by FL quenching and the turbid-to-clear transition of the solutions.

FL response of probe to Cys in deproteinized human plasma

As mentioned above, the normal level of Cys is 240–360 μM in human plasma, and the above results give the clue that this probe could be applied as a potential indicator of Cys deficiency (Fig. S4). To confirm this assumption, deproteinized human serum was used in the following investigation. The removal of proteins is to lessen the effect of viscosity of the fluid on the emission behavior of the fluorescent probe according to the mechanism of the AIE phenomenon.^{18a} Even though the FL intensity recorded for the contrast sample (without the probe molecule in the deproteinized human plasma) is higher than that recorded for the probe in HEPES buffer/DMSO solution due to the higher viscosity of the deproteinized plasma (Fig. 5), the FL intensity of the probe molecule in the mixture of the human serum containing 250 μM spiked Cys (10 equiv.) is still lower than the contrast sample and the FL response does not reverse to positive until the amount of Cys approaches the normal level

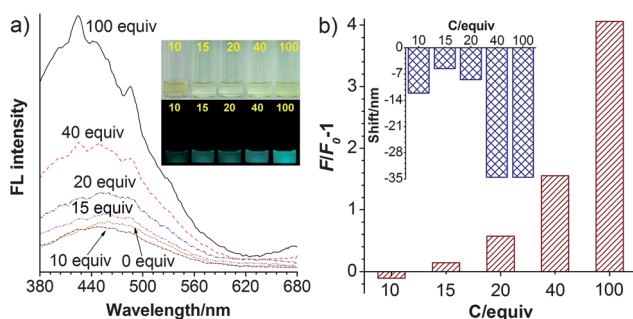


Fig. 5 (a) FL spectra of DMBFDPS with different Cys concentrations (equiv.) in the mixture of DMSO and deproteinized human plasma (6/4 in volume, pH 7.4), [DMBFDPS] = 25 μM, λ_{ex} = 356 nm. Inset: FL photographs of DMBFDPS and Cys in the above mixtures, taken under ambient (top) and UV-light (λ_{ex} = 365 nm, bottom). (b) $F/F_0 - 1$ versus Cys concentration (C) in the above mixtures. Inset: shift of FL peak *versus* Cys concentration. F_0 and F are the peak intensity when Cys concentration is 0 and other values. All measurements were carried out at rt after incubation for 3 h.

(375 μM , or 15 equiv.). This concentration can be defined as the threshold of FL turn-on. Coincidentally, the FL peak wavelength exhibits a monotonous blue-shift from 460 to 424 nm upon increasing the Cys concentration. The FL enhancement with spiked 2.5 mM (100 equiv.) Cys is about 4-fold and the blue-shift of the emission peak is about 36 nm (inset of Fig. 5b). The corresponding UV-visible spectra show little changes in absorption features (Fig. S14†). Meanwhile, the appearance change can be easily witnessed by the naked eye (from light-yellow transparent solution to turbid suspension) and under a UV lamp (enhanced and apparent blue-shift FL) (inset of Fig. 5a). Hence, this probe should be a candidate biological sensor for Cys detection and recognition.

Conclusions

In summary, we have developed a novel FL probe that is capable of discriminately and simultaneously detecting Cys, Hcy and GSH. The probe molecule is composed of two aldehyde groups as reactive functionalities and an AIE active DMTPS core as the signaling moiety. Due to the aldehyde functionalities, this specially designed probe can selectively react with Cys and Hcy to form thiazinane and thiazolidine derivatives in the presence of diverse amino acids, protected Cys and glucose. Due to the DMTPS core, the resulted thiazinane and thiazolidine derivatives display the expected AIE properties, or they are non-emissive in DMSO solution but become highly emissive in suitable mixture of DMSO and HEPES buffer. Because the reactions of the probe molecule with Cys and Hcy undergo distinct kinetics, which are determined by the formation of 5-membered thiazinane and 6-membered thiazolidine rings, the time of FL turn-on for probe-Cys system (~ 120 min) is much shorter than that for probe-Hcy system (~ 3 days). Under the same conditions and in a limited time (e.g. 60 min), the interaction of Cys with probe molecule can lead to an evident blue-shift of FL peak from 479 to 424 nm and a pronounced FL enhancement, but Hcy cannot. Relying on the differences in kinetics, Cys can be easily and discriminately detected over Hcy by the observation of FL responses. GSH shows great interference with the detection of Cys and Hcy. In the presence of a proper amount of GSH, the FL of the mixture of Cys and probe molecule in DMSO/HEPES buffer solution is heavily quenched. The experiment on the addition of a stoichiometric amount of GSH to the prepared mixture containing equivalent Cys and probe molecule suggests that GSH can be quantitatively detected by the FL spectroscopic titration

method. The threshold of the FL turn-off concentration for GSH is measured to be 1 mM.

This is an unprecedented report of using a single fluorescent probe to discriminately detect Cys, Hcy and GSH by FL turn-on and turn-off strategies. The discrimination relies on the reaction-dependent fluorophore aggregation, or the solubility of adducts of the probe molecule and analytes. The present strategy is intrinsically a fluorescent titration, which combines the high sensitivity of FL spectroscopy and the reliability of precipitate titration methodology. Fortunately, the threshold concentration of Cys (375 μM , at which the FL is turned-on) coincides with the upper margin of the deficient Cys levels in human plasma, and the primary investigation of the FL response to deproteinized human plasma indicates that this FL probe is a promising one for the discriminatory detection of Cys over Hcy and GSH on a clinical level.

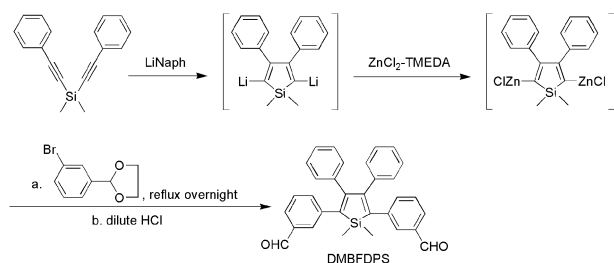
Experimental

Chemicals and materials

THF was distilled from sodium benzophenone ketyl under dry nitrogen immediately prior to use. Dimethyl sulfoxide (DMSO) is chromatographically pure purchased from Alfa Aesar. *p*-Toluene sulfonic acid and magnesium sulphate were purchased from Sinopharm Chemical Reagent Co., Ltd. 3-Bromobenzaldehyde was obtained from Acros Organics Co. Ethylene glycol was purchased from Alfa Aesar. Dichloro-(*N,N,N',N'*-tetramethylenediamine)zinc ($\text{ZnCl}_2\text{-TMEDA}$), HEPES, alanine, arginine, aspartic acid, cysteine (Cys), glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tyrosine, valine, proline, homocysteine (Hcy), phenylglycine and glutathione, glucose and protected Cys (Fmoc-Cys(Trt)-OH and *N*-acetyl-L-Cys) were purchased from Aldrich Chemical Co. Bis(triphenyl phosphine)-palladium(II) dichloride ($\text{Pd}(\text{II})$) were purchased from ABCR GmbH&Co.KG. All other chemicals and reagents were commercially available and used as received without further purification. Human plasma (EDTA as an anticoagulant) was obtained from a local hospital and stored at -20°C until analysis. Distilled water was used in all experiments. Di(phenylethynyl)silanes were prepared by lithiation of phenylacetylene followed by reaction with dichlorosilanes.¹⁹

General characterization

^1H and ^{13}C NMR spectra were measured on a Bruker AV 400 spectrometer in deuterated chloroform and deuterated acetone using tetramethylsilane (TMS; $\delta = 0$) as the internal reference. FTIR spectra were recorded on a Bruker Vector 22 spectrometer. The element analysis was carried out on Thermo Finnigan Flash EA 1112. The melting point was determined by differential scanning calorimetry (DSC) measurement conducted on Perkin-Elmer DSC 7 under N_2 atmosphere at a heating/cooling rate of 10 K min^{-1} . UV-visible (UV-vis) absorption spectra were measured on a Varian CARY 100 Bio UV-visible spectrophotometer. Fluorescence (FL) spectra were recorded on a Perkin-Elmer LS 55 spectrofluorometer.



Scheme 2 Synthesis of the fluorescent probe 1,1-dimethyl-2,5-bis(*meso*-benzaldehyde)-3,4-diphenylsilole (DMBFDPS).

Synthesis of fluorescent probe of 1,1-dimethyl-2,5-bis(*meso*-formylphenyl)-3,4-diphenylsilole (DMBFDPS)

The targeted silole derivative, *i.e.* DMBFDPS was prepared from di(phenylethynyl)-silane according to the synthetic routes shown in Scheme 2.²⁰

The synthesis of DMBFDPS followed the procedure described in literature.²⁰ A solution of lithium naphthalenide (LiNp) was made by stirring a mixture of naphthalene (2.10 g, 16 mmol) and lithium granular (0.21 g, 30 mmol) in THF (20 mL) at room temperature for 12 h under nitrogen atmosphere. The resultant dark green mixture was transferred to a pre-deoxygenated two necked flask *via* a cannula as soon as possible. A solution of di(phenylethynyl)silane (1.04 g, 4 mmol) in THF (10 mL) was added dropwise to the solution of LiNp, and the resultant mixture was stirred for 3 h at room temperature. After the solution was cooled to -10°C , ZnCl_2 -TMEDA (4.00 g, 16 mmol) and 40 mL of THF were added. The fine suspension was stirred for 1 h and then 160 mg Pd (II) and 2-(3-bromophenyl)-1,3-dioxolane (1.83 g, 8 mmol) in THF (20 mL) were added quickly to this system. Afterward the mixture was refluxed at 85°C for 12 h. 1 M HCl aqueous solution (50 mL) was added and then after stirring for 30 min, the mixture was extracted with dichloromethane. The organic layer was washed successively with brine, and dried over magnesium sulphate. After filtration, the solvent was evaporated under reduced pressure, and the residue was purified by silica-gel column chromatography using petroleum ether ($60-90^{\circ}\text{C}$)/ethyl acetate = 10 : 1 (v/v) as eluent, $R_f = 0.30$. Pale yellow green solid of DMBFDPS was obtained in 73.4% yield (1.39 g). ^1H NMR (400 MHz, CDCl_3): δ (TMS, ppm) 9.89–9.71 (s, 2H), 7.69–7.55 (d, 2H), 7.48–7.39 (s, 2H), 7.33–7.22 (t, 2H), 7.20–7.10 (d, 2H), 7.09–6.93 (m, 6H), 6.90–6.71 (d, 4H), 0.58–0.45 (s, 6H). ^{13}C NMR (80 MHz, CDCl_3): δ (TMS, ppm) 192.7, 155.5, 141.2, 141.0, 138.0, 136.5, 135.0, 130.5, 130.4, 130.1, 129.0, 127.9, 127.0, -3.8 . IR (KBr): $\nu = 3061$ (w), 2959 (w), 2824 (w), 2724 (w), 1699 (s), 1586 (s), 1479 (m), 1435 (w), 1385 (w), 1302 (m), 1253 (w), 1206 (w), 1158 (m), 1081 (w), 1020 (w), 862 (w), 788 (s), 697 (s), 651 (w), 578 (w) cm^{-1} . Anal. calcd for $\text{C}_{32}\text{H}_{26}\text{O}_2\text{Si}$: C, 81.66; H, 5.57. Found: C, 81.655; H, 5.51. mp: 165°C . UV (THF): λ_{max} (ϵ_{max}): 356 nm (10^{-5} mol L^{-1}).

Preparation of the nano-aggregates

Stock solution of DMBFDPS in DMSO with a concentration of 0.25 mM was prepared respectively. Aliquots (1 mL) of the stock solutions were transferred to 10 mL volumetric flasks. After adding appropriate amounts of DMSO, 10 mM HEPES buffer solution (pH 7.4) was added dropwise under vigorous stirring to furnish 25 μM solutions with defined fractions of buffer ($f_b = 0-80$ vol%). Spectral measurements of the resultant solutions or aggregate suspensions were performed immediately.

Detection of Cys in DMSO/10 mM HEPES (6/4 in volume, pH 7.4)

The appropriate amount of Cys was dissolved in 4 mL 10 mM HEPES and then 5 mL chromatographically pure DMSO was added into the mixture, finally 1 mL DMSO solution of DMBFDPS (0.25 mM) was added under vigorous stirring to afford the detection system with 25 μM of probe. The mixture

was allowed to stand at RT for 60 min and underwent the necessary measurement.

Preparation of the plasma sample and the detection of Cys in the human serum

Human plasma (EDTA as an anticoagulant) was obtained from the local hospital and was stored at -20°C until analysis. When used, the plasma sample was thawed and deproteinized by adding 9 mL of acetone to 3 mL of plasma sample. After vortex-mixing for 30 s, the mixture was centrifuged at 8000 rpm for 8 min (Mikro 22R refrigerated centrifuge, Hettich, Germany) to precipitate protein, and the supernatants obtained were concentrated by evaporation to *ca.* 1/3 of the total volume, under nitrogen flow. And then the solution was recentrifuged at 10 000 rpm for another 8 min, the resultant supernatants were stored at $4-8^{\circ}\text{C}$ for further use. 1 mL of the deproteinized human plasma was doped with Cys to afford stock solution of human plasma with Cys solution (12.5 mM, 2.5 mM). The stock solution was prepared to the appropriate concentration, and the detection of Cys in the deproteinized plasma was carried out *via* a similar procedure to the above mentioned synthesis of thiazolidine in the DMSO/10 mM HEPES (6/4 in volume, pH 7.4).

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