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An AIE and ESIPT based kinetically resolved fluorescent probe for biothiols†

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A new aggregation-induced emission and excited-state intramolecular proton transfer based fluorescent probe, containing a salicylaldazine moiety as a platform, displayed an excellent light-up ratio and a large Stokes shift for the detection of biothiols (cysteine, homocysteine, and glutathione). The salicylaldazine based fluorescent probe showed high selectivity, and sensitivity for biothiols. With the aid of different reactivities, a kinetically resolved method was successfully applied to distinguish different biothiols both in solution and cells.

Introduction

Biothiols such as cysteine (Cys), homocysteine (Hcy), and glutathione (GSH) are very important small molecules and play important roles in various physiological processes.[1] In living systems, Cys and Hcy are vital molecules for promoting cell and tissue growth. GSH as a regulator keeps the redox balance.[2] For example, the oxidized state of GSH is glutathione (GSSG), which plays critical roles in keeping cellular homeostasis and various physiological functions.[3] The level of these biothiols in the body is also closely related to various diseases including cancer, AIDS, and liver damage.[4] Cys is an important amino acid that plays an important role in protein function. A lower level of Cys in the body can cause various diseases such as slow growth, hair depigmentation, edema, lethargy, liver damage, and muscle/fat loss.[5] In this regard, developing sensitive and convenient methods for the detection of biothiols is very important.

Fluorescent probes have been actively investigated as an effective technique for the detection of bioactive species.[6] As described above, owing to the biological importance of these biothiols, various thiol-responsive fluorescent probes have been developed.[7] The mechanism of reported fluorescent probes for the detection of biothiols falls into the following categories: Michael addition, cyclization reaction with aldehyde, deprotection of sulphonamide and sulfonate esters, and disulfide exchange reaction.[8] Even though there have been some successful examples to discriminate Cys, HCy, and GSH,[9–11] designing a selective fluorescent probe for one of these three biothiols is still a challenging task. An aggregation-induced emission (AIE) based fluorophore has no emission in a low concentration solution but has strong emission in the aggregate state.[12] If the designed probe reacts with biothiols, for example, *via* Michael addition, the addition product will affect the solubility of the molecule in water, which will further change the fluorescence intensity. AIE and ESIPT-based platforms have shown ever-increasing interest in recent years.[12,13]

Herein, we report a new kinetically-resolved fluorescent probe, which can efficiently discriminate Cys, GSH, Hcy, and H_2S. This probe is based on the classic "AIE + ESIPT" platform salicylaldazine with the acrylate moiety (**AIE-S**) as the receptor and fluorescence quencher. When the hydroxyl group in the salicylaldazine dye is protected, the excited-state proton transfer from the hydroxyl group (proton donor) to nitrogen (proton acceptor) will be blocked, and **AIE-S** will show a weak fluorescence emission. Interestingly, the probe **AIE-S** showed a significant difference in its reaction kinetics with biothiols, offering a unique method to identify Cys from other biothiols. After the probe **AIE-S** was treated with biothiols for 15 min, only Cys induced a strong fluorescence signal. After 40 min, strong fluorescence emissions were observed with both Cys and GSH but not with Hcy or H_2S. Moreover, all biothiols induced similar fluorescence intensities about 4 h later.

Experimental

Materials

2-Hydroxybenzaldehyde, hydrazine monohydrate, cesium carbonate, and anhydrous dimethylformamide were all purchased from Sigma-Aldrich. All other chemicals and solvent were purchased from Daejung, Korea.

Equipment and methods

UV-visible absorption spectra were obtained using a UV-visible spectrometer (Scinco 3000 spectrophotometer). Fluorescence spectra

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were measured using a RF-5301/PC (Shimadzu) fluorescence spectrophotometer at 25 °C. ^{1}H and ^{13}C spectra were measured using a Bruker ARX 300 NMR spectrometer. The molecular mass was acquired using ion trap time-of-flight mass spectrometry (MS-TOF). The pH values of buffers were adjusted using a Sartorius PB-10 basic pH meter. Deionized water was used to prepare all aqueous solutions.

Synthesis and characterization

Synthesis of compound A. To a solution of 2-hydroxy-4-methoxybenzaldehyde (300 mg, 1.97 mmol) in ethanol (20 mL), hydrazine monohydrate (50 mg, 48 μL, 0.98 mmol) was added. Then, the mixture was heated to reflux for 4 h. The precipitate was collected and washed twice with ethanol. The pure product A was obtained as a yellow powder (223 mg, 75% yield). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 11.77 (s, 2H), 8.60 (s, 2H), 7.25 (d, J = 6.0 Hz, 2H), 6.56–6.52 (m, 4H), 3.86 (s, 6H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 163.86, 162.82, 161.76, 133.50, 111.10, 107.50, 101.20, 55.51.

Synthesis of compound AIE-S. To a solution of compound **A** (160 mg, 0.53 mmol) in anhydrous DMF (5 mL), cesium carbonate (347.2 mg, 1.07 mmol) was added and stirred at room temperature (rt) for 30 min. Acryloyl chloride (192.9 mg, 2.13 mmol) was added slowly at 0 °C using a syringe. After stirring for 30 min, the solution was warmed to rt for 3 h. Then, 20 mL of water was added to the reaction solution and stirred for 10 min. The obtained precipitate was filtered and washed twice with H_2O before drying under vacuum. Product B was obtained as a light yellow powder (200 mg, 92% yield). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.64 (s, 2H), 8.03 (d, J = 9.0 Hz, 2H), 6.89 (dd, J_1 = 2.4 Hz, J_2 = 9.0 Hz, 2H), 6.73 (d, J = 17.4 Hz, 2H), 6.39 (q, J_1 = 10.5 Hz, J_2 = 17.4 Hz, 2H), 6.09 (dd, J_1 = 1.2 Hz, J_2 = 10.2 Hz, 2H), 3.87 (s, 6H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 164.1, 162.6, 156.6, 151.4, 133.3, 129.5, 127.5, 119.1, 113.0, 108.1, 55.7. HRMS(ESI): m/z $[M + 1]^+$ calcd for $C_{22}H_{20}N_2O_6$: 409.1321; found: 409.1364.

Solutions preparation and optical measurements

The optical spectra of the probe AIE-S and the compound were measured in a mixture of DMSO–H_2O (v/v = 1/99) at an excitation wavelength of 340 nm. The stock solution of analytes including biothiols Cys, Hcy, GSH, and other amino acids such as Phe, Gln, Trp, Thr, Met, Glu, Ile, Gly, Tyr, Arg, Ser, Ala, Asp, Leu, and His (1 mM of each) as well as anions F^-, Cl^-, Br^-, I^-, NO_3^-, NO^{2-}, SCN^-, CO_3^{2-}, SO_4^{2-}, SO_3^{2-}, HS^- and S^{2-} (50 mM of each, sodium salts) were prepared in ultrapure water. The stock solution of probe 1 (1 mM) was prepared in HPLC grade DMSO. UV-vis or fluorescence spectra were recorded upon the addition of various analytes.

Cell imaging experiments

HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS (Fatal Bovine Serum), with 100 mg mL^{-1} penicillin and 100 mg mL^{-1} streptomycin to prevent bacterial contamination, in a 5% CO_2, water saturated incubator at 37 °C, and then seeded in a 12-well culture plate for one night before cell imaging experiments. For the living cell imaging experiment of a probe, the cells were incubated with 5 μM probe AIE-S (with 1% DMSO, v/v) for 20 min at 37 °C and washed twice with prewarmed PBS and then imaged immediately. For imaging of biothiols using probe AIE-S treated experiments, HeLa cells were pretreated with the 5 μM probe for 20 min at 37 °C, washed twice with prewarmed PBS, and then incubated with 10 μM biothiols at 37 °C. Cell imaging was then performed after washing cells with PBS buffer.

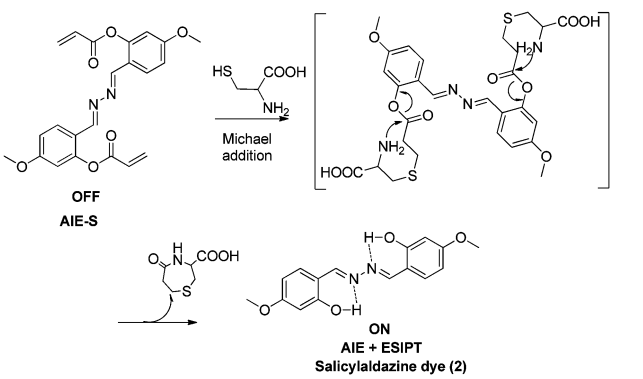
Results and discussion

Probe design and synthesis

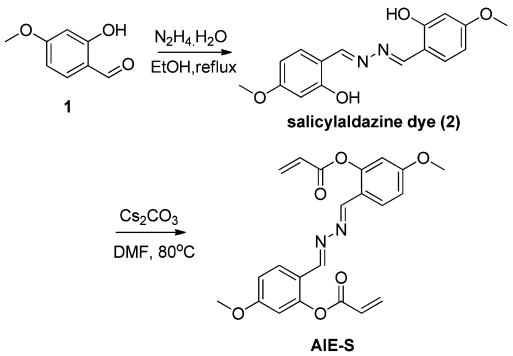
The probe **AIE-S** was designed based on salicylaldazine, in which the hydroxyl group is protected by the acryloyl group. When **AIE-S** was treated with biothiols, two-step reactions were involved, as shown in Scheme 1. The thiol moiety in Cys first reacts with the acryloyl group *via* Michael addition, followed by a spontaneous intramolecular cyclization reaction to release the salicylaldazine dye. After the deprotection of the hydroxyl group, the salicylaldazine dye can restore the intermolecular hydrogen bonds, which would further light up a long-wavelength emission. A similar reaction mechanism has been reported before.[14] The synthetic procedure of the probe **AIE-S** is illustrated in Scheme 2. Fluorophore **2** was obtained by the condensation reaction between 2-hydroxy-4-methoxybenzaldehyde **1** and hydrazine monohydrate in 75% yield. Then, salicylaldazine dye **2** reacted with acryloyl chloride in anhydrous DMF with Cs_2CO_3 as the base to afford the probe **AIE-S** in 92% yield. The structures were confirmed by ^{1}H NMR, ^{13}C NMR, and HRMS. The detailed data can be found in the ESI.†

Optical properties

The optical spectra of the probe **AIE-S** and salicylaldazine dye **2** were recorded in PBS buffer with 1% DMSO at room temperature. As shown in Fig. 1, **AIE-S** shows a maximum absorption at 340 nm, and the fluorescence is quenched upon excitation at 340 nm. In contrast, salicylaldazine dye **2** displayed a strong fluorescence emission at a wavelength of 505 nm. This observable change guarantees that the probe **AIE-S** will display high sensitivity to biothiols both *in vivo* and *in vitro*.



Scheme 1 A plausible mechanism of the response of the probe **AIE-S** to Cys.



Scheme 2 Synthetic route for the probe **AIE-S**.

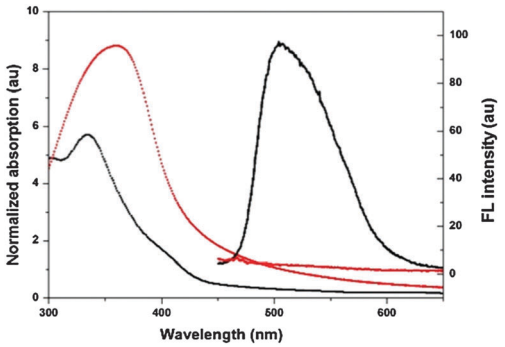


Fig. 1 UV-vis absorption (dashed line) and FL (solid line) spectra of **AIE-S** (red) and salicylaldazine dye **2** (black) in DMSO–water (1 : 99, v/v). The concentrations of **AIE-S** and salicylaldazine dye were 10 μM and 30 μM, respectively.

The optical spectra of salicylaldazine dye **2** were recorded in a DMSO/H_2O mixture with different DMSO fractions (f_d), showing the relationship between the solvent polarity and the extent of salicylaldazine dye aggregation. As shown in Fig. 2, the fluorescence was almost completely quenched in pure DMSO solution. As the ratio of DMSO in solution gradually decreases, the fluorescence intensity increased slightly with a maximum emission at the wavelength 505 nm from f_d = 100 to 20 (v/v). Between f_d = 80 and 1 (v/v), the fluorescence intensity increased dramatically from 23 to 578 and is consistent with the phenomenon of AIE.

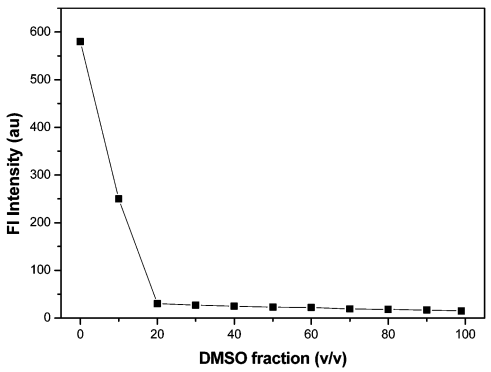


Fig. 2 Plot of relative FL intensity (I/I_0) *versus* the solvent composition of the DMSO/water mixture of salicylaldazine dye **2**.

Detection of biothiols

Before studying the probe **AIE-S** for the detection of biothiols, the effect of pH on salicylaldazine dye **2** was examined. As shown in Fig. S1 (ESI†), a strong fluorescence signal was observed between pH ~ 4 and 8. With increasing pH value continues, the fluorescence intensity decreased, because of the deprotonation of the salicylaldazine dye's hydroxyl group. The fluorescence intensity is relatively stable in the pH range of 4–8 (< ±4%). As shown in Scheme 1, biothiols react with the probe **AIE-S** *via* Michael addition, followed by a spontaneous intramolecular cyclization, which induced the ester bond cleavage. Theoretically, all biothiols (Cys, Hcy, and GSH) will react with **AIE-S**. As shown in Fig. 3a, there is almost no fluorescence for the probe itself. After the addition of Cys to the probe solution for ~30 min, a significant green fluorescence signal gradually increased at the maximum wavelength of ~505 nm. In order to get more information about the reaction, a kinetic experiment was also performed. As shown in Fig. 3b, in the absence of Cys, the fluorescence intensity did not change for ~50 min, supporting that the probe was stable enough under this condition. When the probe **AIE-S** was mixed with Cys in PBS buffer, a significant enhancement in the fluorescence intensity was observed at 505 nm. In 25 min, the fluorescence intensity reached its maximum. Two different ways of measuring the time-dependent fluorescence kinetic spectra provided consistent data,

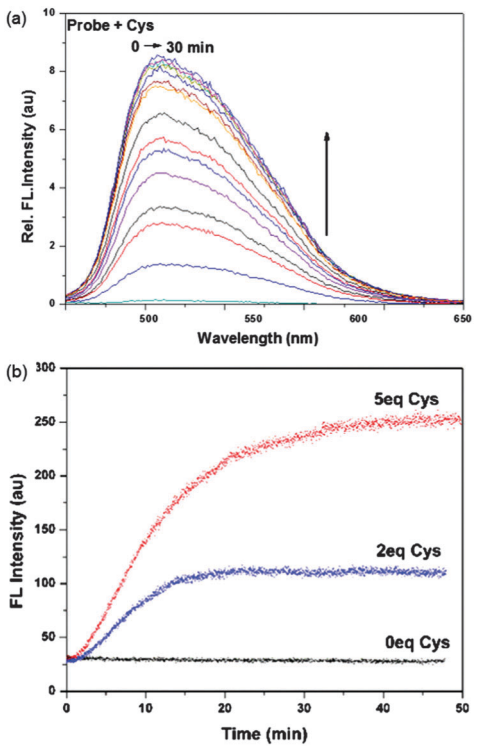


Fig. 3 (a) Fluorescence spectral changes of the probe **AIE-S** (10 μM) upon addition of Cys (50 μM) in PBS buffer (10 mM, pH 7.4) with 1% DMSO at 25 °C; (b) time-dependence of the fluorescence kinetic spectra of the probe AIE-S (10 μM) upon addition of Cys (0, 20, and 50 μM) in DMSO-PBS buffer (10 mM, pH 7.4, 1 : 99, v/v) at 25 °C. All the reactions were monitored every 0.05 s at 505 nm. λ_{ex} = 340 nm.

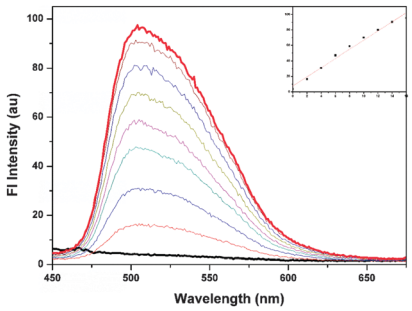


Fig. 4 Fluorescence spectra of 10 μM **AIE-S** in the presence of different amounts of Cys (from 0 to 16 μM) in PBS buffer (10 mM, pH = 7.4, 1% DMSO), λ_{ex} = 340 nm. Inset: The fluorescence intensity at 505 nm as a function of Cys concentration.

indicating that the probe **AIE-S** could be used as a highly sensitive OFF–ON probe for Cys in the PBS buffer with 1% DMSO at room temperature.

A linear relationship was observed between I_{505} and [Cys] in the range of 1–16 μM (Fig. 4). The relationship between the emission at 340 nm and the Cys concentration was calculated as $y = 7.59 + 5.92 \times x$, where y is the fluorescence intensity at 340 nm and x is the concentration of Cys. The linear range of the method was in the concentration range of 1–20 μM Cys with a correlation coefficient (R^2) of 0.985.

Next, the time-dependent fluorescence kinetic spectra of **AIE-S** to Hcy, GSH, and Na_2S were also tested (Fig. 5). Surprisingly, in 20 min, only the one treated with Cys reached the plateau. In contrast, negligible fluorescence intensity changes were observed for Hcy, GSH, and Na_2S. The fluorescence intensity response of **AIE-S** with GSH slightly decreased in 20 min, then increased quickly, and reached almost the same fluorescence intensity as Cys after 50 min. For Hcy, the fluorescence intensity increased slowly for 50 min, and only a quarter of the fluorescence intensity was observed compared to that with Cys. After 4 h, similar fluorescence intensities were observed for all three biothiols

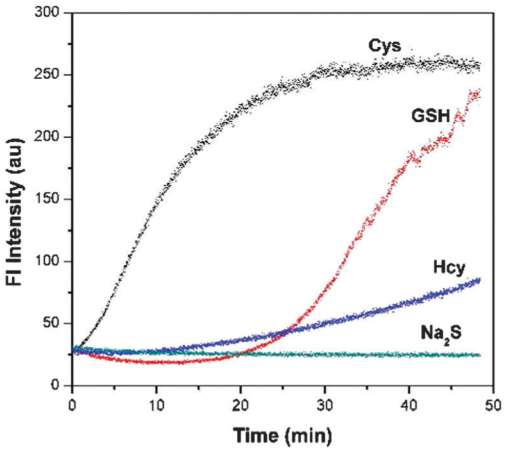


Fig. 5 Time-dependent fluorescence kinetic spectra of the probe **AIE-S** (10 μM) upon addition of Cys, GSH, Hcy, and Na_2S (100 μM) in DMSO–PBS buffer (10 mM, pH 7.4, 1 : 99, v/v) at 25 °C. All the reactions were monitored every 0.02 s at 505 nm. λ_{ex} = 340 nm.

(Cys, GSH, and Hcy) (Fig. S2, ESI†). In contrast, Na_2S cannot induce the second intermolecular cyclization reaction; therefore, there was not any significant fluorescence change. The huge kinetic difference between Cys and Hcy can be attributed to the kinetic rate of the intramolecular adduct/cyclization reactions. The seven-membered ring formation by Cys is a kinetically favored intramolecular cyclization reaction compared to the eight-membered ring by Hcy. For GSH, the product of the first step is the Michael adduct.

Further kinetic experiments of GSH showed that if the concentration of GSH was decreased from 100 μM to 20 μM, the fluorescence enhancement was delayed from 20 to 40 min (Fig. S3, ESI†). Therefore, the kinetically resolved AIE and ESIPT based **AIE-S** probe is sensitive enough to discriminate different biothiols especially in the case of low concentration.

Next, the selectivity of the probe **AIE-S** to different amino acids was investigated in water solution with 1% DMSO over 25 min. (Fig. 6). The blank solution of **AIE-S** showed almost no fluorescence. Upon the addition of Cys, a selective fluorescence enhancement was observed. In contrast, Hcy and GSH induced only a slight change in their fluorescence emissions. In addition, there was not any significant change upon the addition of other amino acids. Other common metal cations and anions were also tested (Fig. S4 and S5, ESI†). Potential interfering anion species such as Cl^-, Br^-, I^-, NO_3^-, NO_2^-, AcO^-, $C_2O_4^{2-}$, PO_4^{3-}, CO_3^{2-}, SO_4^{2-}, SO_3^{2-}, S^{2-}, HS^-, SCN^-, IO_4^-, and ClO_4^- were also added to the probe **AIE-S** solution over 20 min. None of these anions could induce any significant fluorescence changes, indicating that the probe **AIE-S** is a highly selective fluorescent probe for Cys.

Prior to the cell imaging experiments, the cytotoxicity of the probe **AIE-S** was first tested by MTT assays (Fig. S6, ESI†). The cell viability test did not show any difference between the cells treated with **AIE-S** and untreated cells, indicating that the probe **AIE-S** would not interfere with the physiology or proliferation of the cells under the tested concentration range.

The probe **AIE-S** was then used to image biothiols in living cells. As shown in Fig. 7, when HeLa cells were treated with only **AIE-S** for 20 min, no fluorescence was observed. When HeLa cells were incubated with the probe for 20 min and then treated

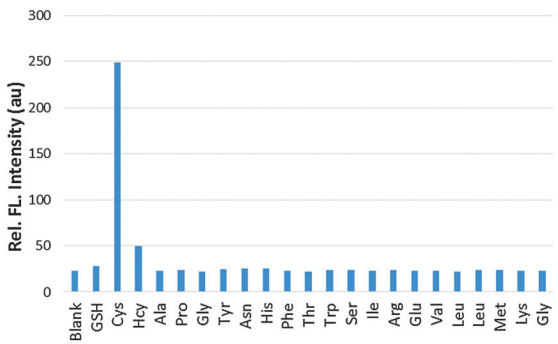


Fig. 6 Selectivity of **AIE-S** towards Cys. Fluorescence response of **AIE-S** (10 μM) at 505 nm in the presence of different amino acids (1 mM) in a PBS/DMSO mixture (99 : 1, v/v) at an excitation wavelength of 340 nm.

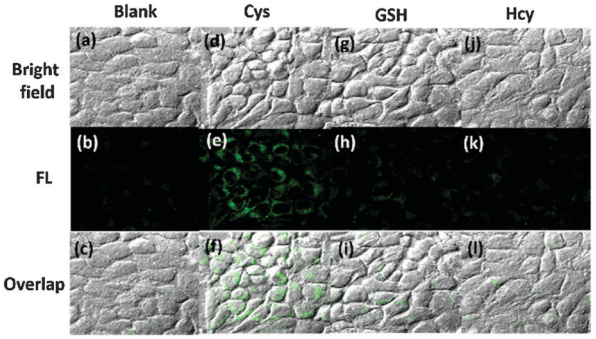


Fig. 7 Imaging of biothiols using the probe **AIE-S** in living HeLa cells. Top: bright field images. (a) HeLa cells were incubated with the probe AIE-S (5 μM) for 20 min. (d), (g), and (j) HeLa cells were pre-incubated with the probe **AIE-S** for 20 min and then treated with Cys, GSH, and Hcy (10 μM) for 20 min. Middle: (b), (e), (h), and (k) are the fluorescence images of (a), (d), (g), and (j), respectively. Bottom: (c), (f), (i), and (l) are the overlap images of bright field and fluorescence images of (a), (d), (g), and (j), respectively.

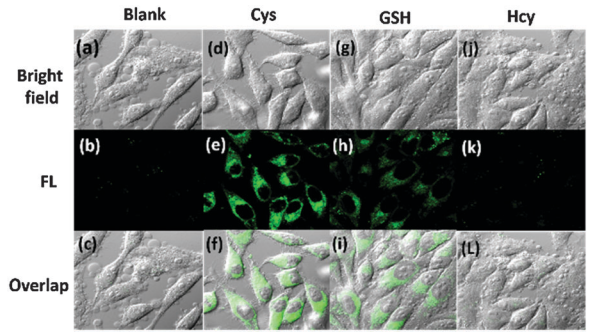


Fig. 8 Imaging of biothiols using the probe **AIE-S** in living HeLa cells. Top: bright field images. (a) HeLa cells were incubated with the probe **AIE-S** (5 μM) for 20 min. (d), (g), and (J) HeLa cells were pre-incubated with the probe **AIE-S** for 40 min and then treated with Cys, GSH, and Hcy (10 μM) for 20 min. Middle: (b), (e), (h), and (k) are the fluorescence images of (a), (d), (g), and (J), respectively. Bottom: (c), (f), (i), and (l) are the overlap images of bright field and fluorescence images of (a), (d), (g), and (J), respectively.

with Cys (10 μM) for 20 min, strong green fluorescence was clearly observed (e). In contrast, the treatment with GSH or HCy for 20 min induced only very weak green fluorescence. As described in the previous experiment, the GSH would have a similar fluorescence intensity for ~40 min. Therefore, the reaction time was extended to 40 min to that of the probe treated HeLa cells with biothiols. In this experiment, similar results were obtained to those above except for GSH. When GSH was added to the probe pretreated with Hela cells for 40 min, a strong green fluorescence was detected, indicating that after incubation for 40 min, both Cys and GSH showed a strong green fluorescence but Hcy did not. These results are extremely consistent with the previous kinetic experiment (Fig. 8).

Conclusions

In conclusion, we designed and synthesized a new AIE and ESIPT-based fluorogenic dye, displaying signal amplifications in the presence of biothiols. **AIE-S** showed a high selectivity for Cys over GSH and Hcy. More specifically, when the probe **AIE-S** was treated with biothiols for 15 min, only Cys induced a strong fluorescence signal. After 40 min, strong fluorescence emissions were observed with both Cys and GSH but not with Hcy or H_2S. The turn-on emissions of **AIE-S** by biothiols allowed us to identify these three biothiols by the kinetic process. Finally, the probe **AIE-S** was also used to image biothiols in living cells.

Acknowledgements

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