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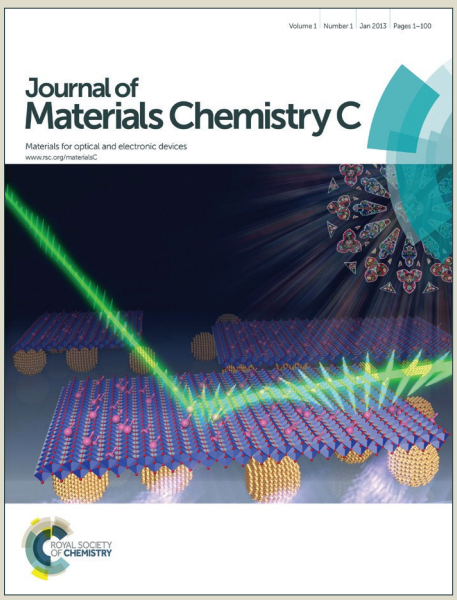

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ARTICLE

AIE and ESIPT Based Kinetic 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 salicyladazine based fluorescent probe showed high selectivity, and sensitivity for biothiols. With the aid of different reactivity, a kinetic resolved method was successfully applied to distinguish different biothiols both in the 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 cells 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 body is also closely related to various diseases including cancer, AIDS, and liver damage et al.[4] Cys is an important amino acid plays an important role in the formation of protein function. Lower level of Cys in 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. Aggregation-induced emission (AIE) based fluorophore has no emission in a low concentration solution but has strong emission in the aggregate state.[12] If designed probe reacts with biothiols, for example, *via* Michael addition, the addition product will affect the solubility of 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 kinetic-resolved fluorescent probe, which can efficiently discriminate Cys, GSH, Hcy, and H_2S. This probe is based on classic "AIE + ESIPT" platform salicyladazine with acrylate moiety (**AIE-S**) as the receptor and fluorescence quencher. When the hydroxyl group in salicyladazine 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 fluorescent emission. Interestingly, probe **AIE-S** showed significant difference in its reaction kinetic with biothiols, offering a unique method to identify Cys from other biothiols. After probe **AIE-S** was treated with biothiols for 15 min, only Cys induced a strong fluorescent signal. After 40 min, strong fluorescent emissions were observed with both Cys and GSH but not with Hcy or H_2S. Moreover, all biothiols induced similar fluorescent 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). Fluorescent spectra were measured using a RF-5301/PC (Shimadzu)

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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. Pure product A was obtained as a yellow powder (223 mg, 75% yield). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 11.77 (s, 2 H), 8.60 (s, 2 H), 7.25(d, *J* = 6.0 Hz, 2 H), 6.56-6.52 (m, 4 H), 3.86 (s, 6 H). ^{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 with a syringe. After stirring for 30 min, the solution was warmed to rt for 3h. Then, 20 mL 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, 2 H), 8.03 (d, *J* = 9.0 Hz, 2 H), 6.89 (dd, J_1 = 2.4 Hz, J_2 = 9.0 Hz, 2 H), 6.73 (d, *J* = 17.4 Hz, 2 H), 6.39 (q, J_1 = 10.5 Hz, J_2 = 17.4 Hz, 2 H), 6.09 (dd, J_1 = 1.2 Hz, J_2 = 10.2 Hz, 2 H), 3.87 (s, 6 H). ^{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

Optical spectra of probe AIE-S and 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 fluorescent spectra were recorded upon the addition of various analytes.

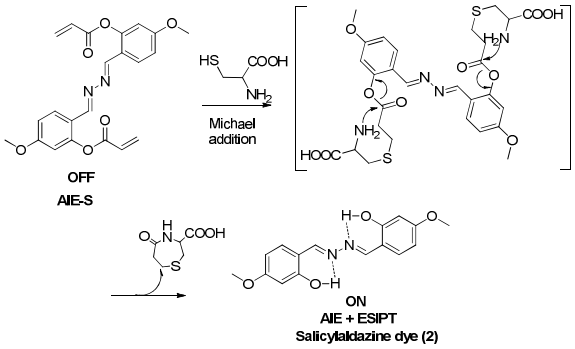
Cell imaging experiments

HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS (Fatal Bovine Serum), with 100 mg/mL penicillin and 100 mg /mL streptomycin to prevent bacterial contamination, in 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 living cells imaging experiment of 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 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.

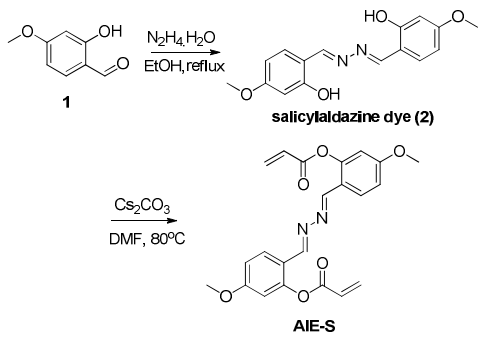
Results and discussion

Probe design and Synthesis

Probe **AIE-S** was designed based on salicylaldazine, in which the hydroxyl group is protected by 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. The similar reaction mechanism has also been reported before.[14] The synthetic procedure of 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 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 supporting information.



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



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

Optical properties

The optical spectra of 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*.

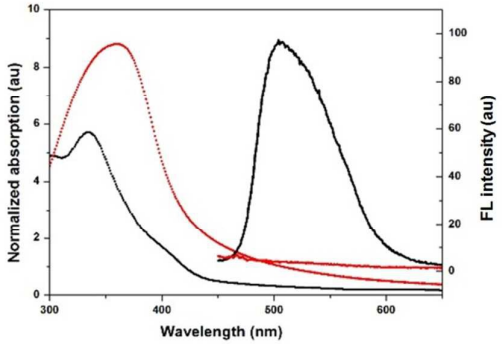


Fig. 1. UV–vis absorption (dashed line) and FL (solid line) spectra of **AIE-S** (red) and salicylaldazine dye **2** (black) in DMSO–water (1:99, *v*/*v*). The concentration of **AIE-S** and salicylaldazine dye were 10 μM and 30 μM, respectively.

Optical spectra of salicylaldazine dye **2** were recorded in 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, fluorescence was almost completely quenched in pure DMSO solution. As the ratio of DMSO in solution gradually decreases, the fluorescent intensity increased slightly with a maximum emission at the wavelength 505 nm from f_d = 100 to 20 (v/v). Between f_d = 80 to 1 (v/v), the fluorescent intensity increased dramatically from 23 to 578 and is consistent to the phenomenon of AIE.

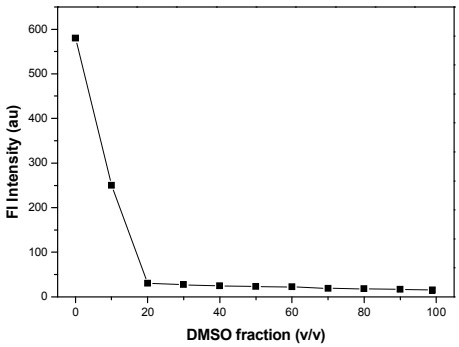


Fig. 2 Plot of relative FL intensity (I/I_0) versus the solvent composition of the DMSO/water mixture of salicylaldazine dye **2**.

Detection of Biothiols

Before studying probe **AIE-S** for the detection of biothiols, the effect of pH on salicylaldazine dye **2** was examined. As shown in Fig. S1, a strong fluorescence signal was observed between pH ~4-8. With increasing pH value continues, the fluorescent intensity decreased, because of the deprotonation of the salicylaldazine dye's hydroxyl group. The fluorescent intensity is relatively stable in the pH range 4-8 (< ± 4%). As shown in Scheme 1, biothiols react with 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 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 ~505 nm. In order to get more information about the reaction, a kinetic experiment was also examined. As shown in Fig 3b, in the absence of Cys, fluorescent intensity did not change for ~50 min, supporting that the probe was stable enough under this condition. When probe **AIE-S** was mixed with Cys in PBS buffer, a significant enhancement in the fluorescent intensity was observed at 505 nm. In 25 min, the fluorescence intensity reached its maximum. Two different ways of measuring the time-dependent fluorescent kinetic spectra provided consistent data, indicating that probe **AIE-S** could be used as a highly sensitive OFF-ON probe for Cys in PBS buffer with 1% DMSO at room temperature.

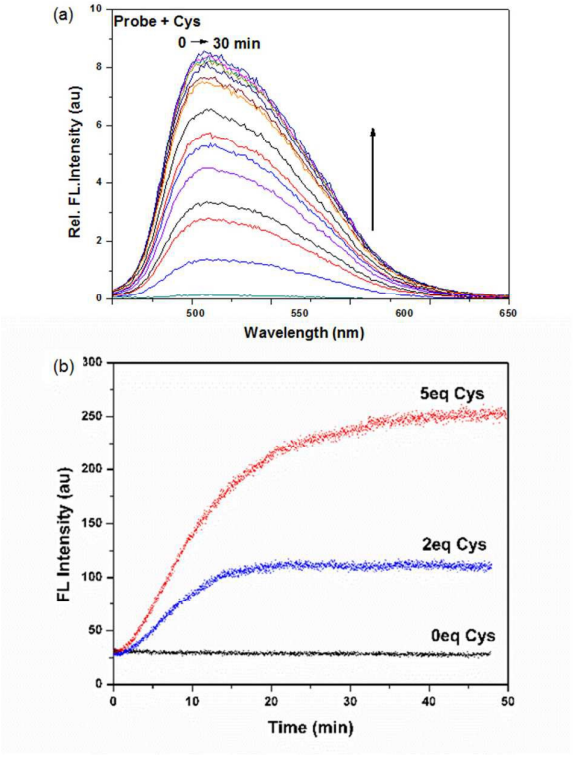


Fig. 3 (a) Fluorescent spectral changes of probe **AIE-S** (10 μM) upon addition of Cys (50 μM) in PBS buffer (10 mM, pH 7.4) with 1% DMSO

at 25 °C; (b) Time-dependent of fluorescence kinetics spectra of 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.

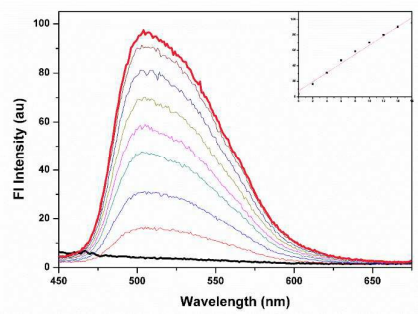


Fig. 4 Fluorescence spectra of 10 μM **AIE-S** in the presence of different amounts of Cys (from 0 to 16 μM) in PBS buffer (10 mM, pH = 7.4, 1% DMSO), λ_{ex} = 340 nm. Inset: the fluorescence intensity at 505 nm as a function of Cys concentration.

The linear relationship was observed between I_{505} and [Cys] in the range 1-16 μM (Fig. 4). The relationship between emission at 340 nm and Cys concentration was calculated as y =7.59 + 5.92*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 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 fluorescent intensity changes were observed for Hcy, GSH, and Na_2S. The fluorescent intensity response of **AIE-S** with GSH slightly decreased in 20 min, then increased quickly, and reached to almost the same fluorescent intensity with Cys after 50 min. For Hcy, the fluorescent intensity increased slowly for 50 min, and only a quarter of the fluorescence intensity observed was compared to that with Cys. After 4 h, similar fluorescent intensities were observed for all the three biothiols (Cys, GSH, and Hcy) (Fig. S2). 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 kinetic rate of the intramolecular adduct/cyclization reactions. The seven-membered ring formation by Cys is 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 fluorescent enhancement was delayed from 20 to 40 min (Fig. S3). 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.

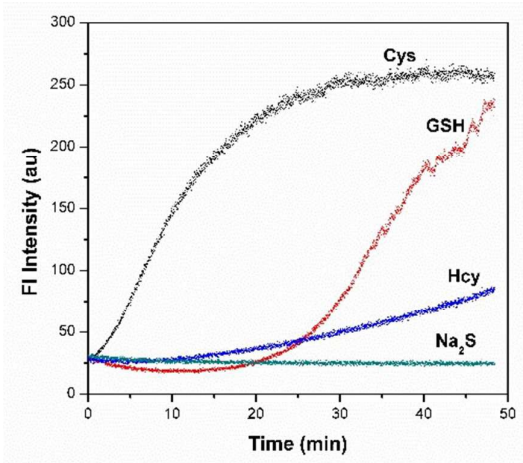


Fig. 5 Time-dependent fluorescence kinetics spectra of probe **AIE-S** (10 μM) upon addition of Cys, GSH, Hcy, and Na_2S (100 μM) in DMSO–PBS buffer (10 mM, pH 7.4, 1:99, v/v) at 25 °C. All the reactions were monitored every 0.02 s at 505 nm. λ_{ex} = 340 nm.

Next, the selectivity of probe **AIE-S** to different amino acids was investigated in water solution with 1% DMSO in 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 slight change in their fluorescent 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 (Figs. S4 and S5). 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 probe **AIE-S** solution in 20 min. None of these anions could induce any significant fluorescent changes, indicating that probe **AIE-S** is a highly selective fluorescent probe for Cys.

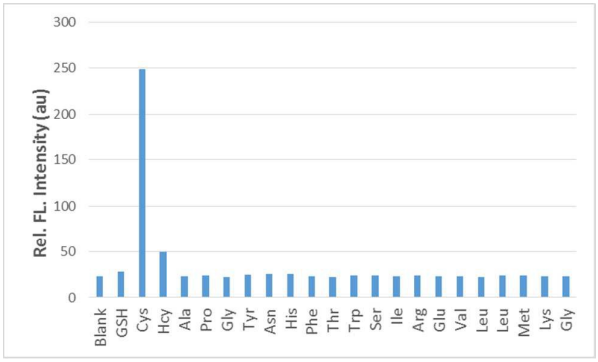


Fig. 6 Selectivity of **AIE-S** towards Cys. Fluorescence response of **AIE-S** (10 μM) at 505 nm in the presence of different amino acids (1 mM) in a PBS/DMSO mixture (99:1, v/v) at an excitation wavelength of 340 nm.

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

with the physiology or proliferation of the cells under the tested concentration range.

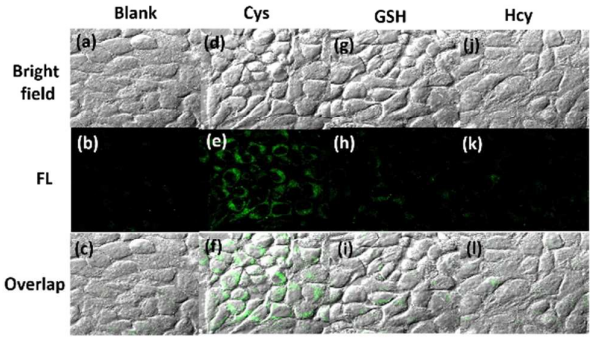


Fig. 7 Imaging of biothiols using probe **AIE-S** in living HeLa cells. Top: bright field images. (a) HeLa cells were incubated with probe AIE-S (5 μM) for 20 min. (d), (g), and (J) HeLa cells were pre-incubated with probe **AIE-S** for 20 min and then treated with Cys, GSH, and Hcy (10 μM) for 20 min.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

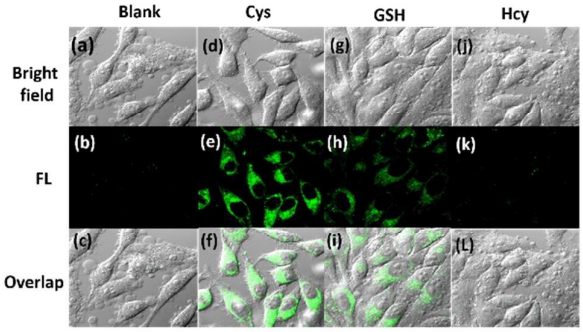


Fig. 8 Imaging of biothiols using probe **AIE-S** in living HeLa cells. Top: bright field images. (a) HeLa cells were incubated with probe **AIE-S** (5 μM) for 20 min. (d), (g), and (J) HeLa cells were pre-incubated with probe **AIE-S** for 40 min and then treated with Cys, GSH, and Hcy (10 μM) for 20 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.

Probe **AIE-S** was then used to image biothiols in living cells. As shown in Fig. 7, when HeLa cells were treated only with **AIE-S** for 20 min, no fluorescence was observed. When HeLa cells were incubated with probe for 20 min then treated with Cys for (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 week green fluorescence. As described in the previous experiment, the GSH would have a similar fluorescent intensity in ~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 as above except for GSH. When GSH was added to the probe pretreated with Hela cells for 40 min, a strong green fluorescent was detected, indicating that after incubating for 40 min, both Cys and GSH showed a strong green fluorescent but Hcy 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 probe **AIE-S** was treated with biothiols for 15 min, only Cys induced a strong fluorescent signal. After 40 min, strong fluorescent 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 the living cells.

Acknowledgements

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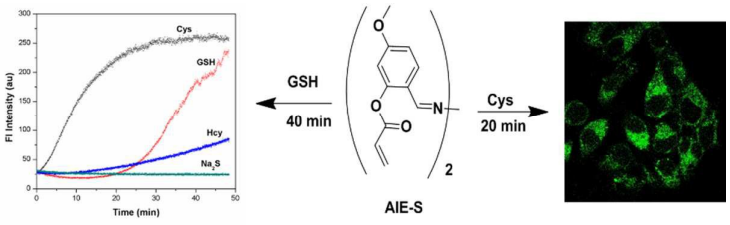

A new fluorescence probe based on AIE and ESIPT was developed. With the aid of different reactivity, a kinetic resolved method was successfully applied to distinguish different biothiols both in the solution and cells.