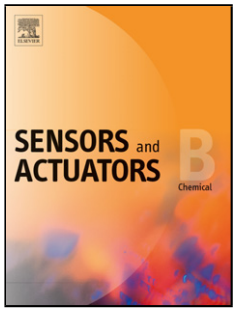

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Development of a novel benzothiadiazole-based fluorescent turn-on probe for highly selective detection of glutathione over cysteine/homocysteine

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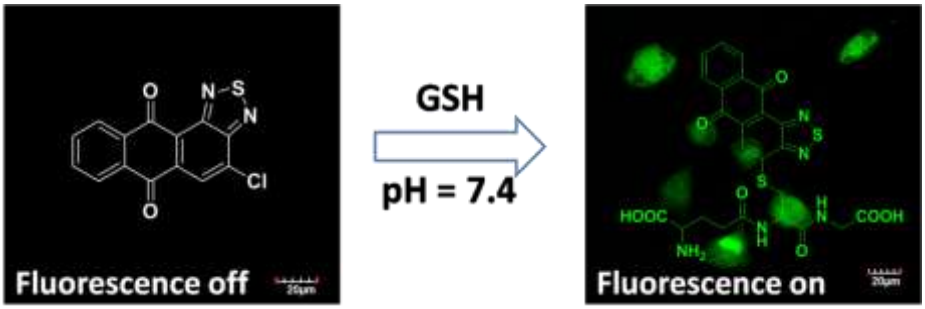
Graphical Abstract



Highlights

- A novel fluorescent turn-on probe for efficient detection of GSH over Cys/Hcy was developed.
- The sensing behavior of the probe to biothiols was completely different from most of the previous reported NBD-based fluorescent probes.
- This probe can be successfully used for detecting GSH in living cells.

ABSTRACT: Biothiols, such as glutathione (GSH), cysteine (Cys), homocysteine (Hcy), plays crucial roles in biological systems due to their physiological and pathological functions. So it is of great interest to develop new fluorescence sensing systems for distinguishing between them. Herein we report a novel fluorescent turn-on probe for selective sensing of GSH over Cys/Hcy with high selectivity and sensitivity. The sensing behavior of ATD-Cl toward GSH and Cys/Hcy is completely different from most of the previous reported NBD-based fluorescent probes. The detection limit of ATD-Cl for GSH is found to be 89 nM. Cell imaging experiments demonstrate that ATD-Cl possesses excellent cell permeability and can be employed to image GSH in living MCF-7 cells.

Keywords: Benzothiadiazole derivatives; Glutathione detection; Fluorescent turn-on sensor; Smiles rearrangement; Bioimaging.

Introduction

As one of the most important and abundant intracellular non-protein biothiol, glutathione (GSH) has been proved that it can be served to maintain intracellular redox homeostasis, signal transduction, gene regulation and xenobiotic metabolism [1]. Owing to the existence of GSH, the damage of cellular components caused by reactive oxygen species (ROS) is avoided effectively [2]. Furthermore, many diseases including cancer, Alzheimer's and other ailments have been associated with the abnormal levels of GSH [3-5]. As a consequence, monitoring the fluctuation of GSH concentrations becomes critical in bioanalysis and medical diagnosis.

Currently, a wide variety of methods such as liquid chromatography-mass spectrometry (LCMS), high performance liquid chromatography, gas chromatography-mass spectrometry (GCMS) and fluorescence spectrometry have been developed for the detection of biothiols. Among these methods, fluorescent probes have drawn considerable interest as an effective tool due to its simple operation, high selectively and sensitivity [6-15]. However, to the best of our knowledge, it is still a challenging and interesting issue to distinguish GSH from Cys/Hcy because of their similar molecular structure and chemical reactivity [16-20]. Therefore, developing a probe for highly selective and sensitive detection of cellular GSH is desperately needed [21, 22].

Generally, NBD-based probes can make a sensitive off-on fluorescence response to Cys/Hcy over GSH. [23-40]. In this work, through introducing the electron-withdrawing o-phenylenedione group into the skeletons of benzothiadiazole, we reported a new type of fluorescent turn-on probe 4-chloro-anthra[1,2-c] [1,2,5]thiadiazole-6,11– dione (ATD-Cl) for detecting the GSH with high selectively and sensitively. Interestingly, the sensing behaviors of ATD-Cl to biothiols was completely different from most of the previous reported NBD-based fluorescent probes. The probe showed high sensitivity and an enhancement off-on response to GSH over Cys/Hcy. Additionally, the probe was successfully applied for imaging cellular GSH with non-detectable cytotoxicity.

Please, insert Scheme 1 here.

Experimental Section

Reagents and Apparatus

All reagents and solvents for the experiments were purchased from commercial suppliers and used without further purification. All of the reactions were magnetically stirred and monitored by thin-layer chromatography (TLC) using UV light. Ultrapure water (18.2 M cm-1) was used to prepare all solutions in this work. UV-vis absorption and fluorescence emission spectra were acquired on a Shimadzu UV-2700 spectrophotometer and a Shimadzu RF-5301 spectrofluorimeter respectively. All the spectra were measured in a quartz cuvette with 10.0 mm path length (volume: 3.5 ml). The excitation wavelength was 465 nm for all fluorescence measurements and the excitation/emission slit width set at 10 nm. NMR experiments were performed with a BRUKER 400 spectrometer with tetramethylsilane (TMS) as internal standard. Bruker ultrafleXtreme MALDI-TOF/TOF and ThermoExactive Plus were used for the mass analysis. Cells images were imaged on Olympus FV1000 laser scanning confocal microscope.

Cell Culture and Fluorescence Imaging

MCF-7 cells were cultured in DMEM medium supplemented with 10% (v/v) fetal bovine serum and penicillin/ streptomycin (100 μg/ml) in an atmosphere of 5% CO2 at 37 °C. For living cell imaging, three groups were studied as follows: (1) the cells were only incubated with ATD-Cl; (2) the cells were preincubated with N-ethylmaleimide (NEM) at a concentration of 0.5 mM for 30 min and then incubated with ATD-Cl (10 μM) for 30 min; (3) the cells were preincubated with NEM (0.5 mM) for 30 min and then incubated with ATD-Cl (10 μM) for 30 min, followed by treatment with GSH, Cys or Hcy (100 μM) for another 30 min. Before imaging, the cells were washed three times with PBS buffer. Emission was collected at green channel (530-590 nm) with 488 nm excitation.

Synthesis of 4-chloroanthra[1,2-c][1,2,5]thiadiazole-6,11-dione (ATD-Cl)

The synthetic procedure of the targeted compound ATD-Cl was shown in the Supporting Information. ^{1}HNMR (400 MHz, $CDCl_3$): δ 8.61 (s, 1H), 8.38 (dd, J = 7.0, 1.7 Hz, 1H), 8.31 (dd, J = 7.2, 1.6 Hz, 1H), 7.91-7.84 (m, 2H); HRMS: Calcd for $C_{14}H_5ClN_2O_2S$ $[M+H]^+$: 300.9833; Found: 300.9828. The observed characterization data (^{1}H NMR and HRMS) was consistent with that previously reported in the literature [41].

Results and Discussion

Probe design

The electron-withdrawing characteristic of –Cl group could effectively block the intramolecular charge transfer (ICT) process and made fluorescent dye exhibit weak fluorescence emission. Once –Cl group was substituted by the electron-donating group, fluorescent dye was very likely to emit strong fluorescence due to the enhanced ICT. Encouraged by this, probe ATD-Cl was designed for the discrimination of GSH from Cys/Hcy. In the design, the chlorine moiety of ATD-Cl as a recognizing site was easily replaced by the sulfhydryl of biothiols (Cys, Hcy and GSH) through nucleophilic substitution reaction to generate high fluorescence sulfur-substituted ATD derivatives (S-ATD-Cys, S-ATD-Hcy and S-ATD-GSH). The obtained intermediate product S-ATD-Cys/S-ATD-Hcy but not S-ATD-GSH could be further converted to weak fluorescence amino-substituted ATD derivatives (N-ATD-Cys/ N-ATD-Hcy) via S-N Smiles rearrangement.

Optical properties of ATD-Cl

ATD-Cl was synthesized according to the previous literature [41, 42]. The chemical structure of ATD-Cl was well characterized by nuclear magnetic resonance (NMR) and high-resolution mass spectrum (HRMS). With probe ATD-Cl in hand, we first examined the sensing ability of ATD-Cl toward GSH/Cys/Hcy/NaSH by fluorescence spectra in PBS buffer (10 mM, pH 7.4, containing CTAB 1mM) at 37°C.

The use of CTAB not only increased the solubility of the probe by forming micelles, but also speeded up the reaction between the probe and biothiols by improving the nucleophilic reactivity of biothiols [43-45]. As shown in Figure 1, the free ATD-Cl exhibited almost no fluorescence at 558 nm ($Ø_{fl}$=0.0016, using Rhodamine B as a standard). Interestingly, upon addition of GSH/Cys/Hcy/NaSH to the solution of ATD-Cl respectively, only GSH showed a remarkable fluorescence enhancement at 558 nm of approximately 17-fold (Figure 1). In contrast, Cys/Hcy/NaSH caused slight fluorescence intensity increase, suggesting ATD-Cl could selectively detect GSH over Cys/Hcy/NaSH.

Please, insert Figure 1 here.

Then the fluorescence titration was carried out to evaluate the sensitivity of the probe toward GSH. As illustrated in Figure 2, upon addition of various amounts of GSH, the fluorescence emission of ATD-Cl at 558 nm increased gradually and reached a plateau in the presence of 60 μM of GSH. A good linear relationship (R^2 = 0.993) was obtained between the fluorescence intensity at 558 nm and the concentration of GSH over a wide range from 0 μM to 20 μM. The limit of detection for GSH was estimated to be 89 nM based on 3σ/K, which indicated the probe could be potentially used to monitor GSH with high sensitivity in chemical and biological systems (Table S1).

Please, insert Figure 2 here.

To evaluate the sensitivity, the time-dependent fluorescence response of ATD-Cl in the presence of GSH was investigated. As shown in Figure 3A and S1, the fluorescence intensity of ATD-Cl was saturated within 30 min in the presence of GSH (60 μM). The pseudo-first-order reaction kinetic constant (K_{obs}) was calculated to be 0.11 min^{-1} (Figure S2). In comparison, upon addition of Cys, Hcy and NaSH

respectively, the fluorescence intensity of ATD-Cl was slightly changed within 30 min. Thus, the response time of 30 min was used for the subsequent experiment to make sure that GSH could react with ATD-Cl completely. In addition, the effect of pH on the sensing response of ATD-Cl toward GSH was also investigated. As demonstrated in Figure 3B, negligible fluorescence changes of ATD-Cl were observed under different pH values ranging from 3.0 to 12.0. Instead, it was found that ATD-Cl displayed significant fluorescence enhanced response for GSH in the pH range of 5.0-12.0, while the fluorescence intensity of ATD-Cl reached its maximum at around pH 8.0. Overall, these results suggested that ATD-Cl could be employed as a good candidate for detection of GSH in physiological pH range.

Please, insert Figure 3 here.

As we all know, good selectivity and anti-interference are two important performance considerations for designing fluorescence probe. Given this, we next investigated the selectivity of ATD-Cl toward GSH over other biologically relevant analytes. As can be seen from Figure S3, upon addition of common amino acids (Ala, Arg, Asp, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val, Glu, Gly, Cys, Hcy), only GSH induced significant fluorescence turn-on at 558 nm, while other amino acids did not trigger detectable fluorescence changes. Additionally, no appreciable increase in fluorescence intensity of ATD-Cl was observed after treating with the representative anions (HS^-, Br^-, Cl^-, F^-, HPO_4^{2-}, NO_3^-, SO_4^{2-}), cations (Al^{3+}, Fe^{3+}, K^+, Mg^{2+}, Na^+, Zn^{2+}) and reactive oxygen species (H_2O_2, ClO^-) (Figure S4). Furthermore, competitive experiments were conducted to ensure whether probe ATD-Cl was capable of detecting GSH in the presence of potential interfering species. As shown in Figure 4, the coexistence of potential interfering species showed no significant influence in the detection of GSH. Therefore, probe ATD-Cl could detect GSH with high selectivity even in the presence of other interfering species.

Please, insert Figure 4 here.

Proposed sensing mechanism

To get insight into the sensing mechanism, control experiments of the reactions between ATD-Cl and N-acetylcysteine, 2-mercaptoacetate or Cysteamine were carried out. Similar turn-on fluorescence response to GSH was found after adding N-acetylcysteine or 2-mercaptoacetate to the solution of ATD-Cl other than Cysteamine (Figure S5). Perhaps the reason for this was that S-N Smiles rearrangement reaction of the sulfur-containing intermediates via nucleophilic substitution reaction between N-acetylcysteine or 2-mercaptoacetate and ATD-Cl would not occur owing to the lack of a free amino group. Additionally, three model compounds were prepared based on the nucleophilic substitution reaction of ATD-Cl with n-butylamine, dibutylamine and butanethiol (Figure S6). As we expected, the compound ATDS emitted strong fluorescence centred at 558 nm, which matched well with S-ATD-GSH. However, the compounds ATDN1and ATDN2 showed weak fluorescence signals accompanied with longer wavelength shift of fluorescence maximum from 558 nm to 598 nm. As shown in Figure 1B and S5, after treatment with Cys, Hcy or Cysteamine, a weak fluorescence emission at 598 nm was also observed. Through analysis of molecular orbitals (HOMO and LOMO) obtained by DFT calculations at the B3LYP/6-311G (d, p) level, the energy gaps of S-ATD-GSH and N-ATD-Cys were calculated to be 2.928 eV and 2.853 eV respectively, which further provided strong evidence for the fluorescence measured results (Figure S7). The reaction products of ATD-Cl with GSH/Cys/ NaSH were also confirmed by MS spectrum (Figure S8). With addition of GSH to a solution of ATD-Cl, the peak at m/z 572.08 (calcd 571.08 for $C_{24}H_{21}N_5O_8S_2$) was obtained, which was in accordance with $[ATD\text{-}GSH + H]^+$. In comparison, after reaction with Cys or NaSH, the peaks appeared at m/z 386.02 (calcd 385.02 for $C_{17}H_{11}N_3O_4S_2$) or m/z 296.98 (calcd 297.99 for $C_{14}H_6N_2O_2S_2$) attributed to $[ATD\text{-}Cys + H]^+$, $[ATD\text{-}SH - H]^-$ respectively. All the above results indicated that probe ATD-Cl could discriminate GSH from Cys/Hcy by means of the fluorescence spectral differences caused by intramolecular S-

N Smiles rearrangement.

Cellular imaging

Inspired by the excellent performance of ATD-Cl, we then examined the utility of ATD-Cl for fluorescence imaging GSH in living cells by confocal laser scanning microscopy (CLSM). In view of potential toxicity of CTAB to the cells, cell-imaging experiments with probe ATD-Cl were carried out without addition of CTAB. MCF-7 cells incubated with ATD-Cl (10 μM) for 30 min displayed bright fluorescence in the green channel (530-590 nm) (Figure 5a), showing that ATD-Cl possessed good cell permeability and had the capability to imaging the basal intracellular GSH. However, when the cells were preincubated with N-ethylmaleimide (NEM, a widely used thiol blocking reagent) at a concentration of 0.5 mM for 30 min and then incubated with ATD-Cl (10 μM) for 30 min, the cells showed hardly any fluorescence in the green channel (Figure 5b). By contrast, when the NEM-preincubated MCF-7 cells were incubated with ATD-Cl (10 μM) for 30 min, followed by treatment with GSH, Cys or Hcy (100 μM) for another 30 min, only GSH induced a remarkable fluorescence increase in the green channel (Figure 5c). Furthermore, the cytotoxicity of ATD-Cl with different concentrations was also investigated by CCK-8 assay (Figure S9) [46-47]. The results revealed that about 90 % of cells were still surviving after 12 h at concentration below 10 μM, indicating ATD-Cl was of low cytotoxicity to the living cells. Overall, the observed fluorescence changes of MCF-7 cells demonstrated that ATD-Cl was successfully used as a new fluorescent probe for specific detection of GSH in living cells.

Please, insert Figure 5 here.

Conclusion

In conclusion, we have described a novel turn-on fluorescent sensor ATD-Cl, which selectivity detection of GSH over Cys/Hcy with a low detection limit of 89 nM.

The probe also showed excellent selectivity and sensitivity toward GSH over other biologically relevant analytes. In particular, the probe was successfully applied to image GSH in living MCF-7 cells by by confocal laser scanning microscopy. Taken altogether, this work provided a promising fluorescent probe for selective detection of GSH over Cys/Hcy.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at

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Figures:

Scheme 1. (A) The chemical structure of Cys, Hcy, GSH and NBD-based fluorescent probes for Cys/Hcy. (B) Probe ATD-Cl for sensing of GSH over Cys/Hcy.

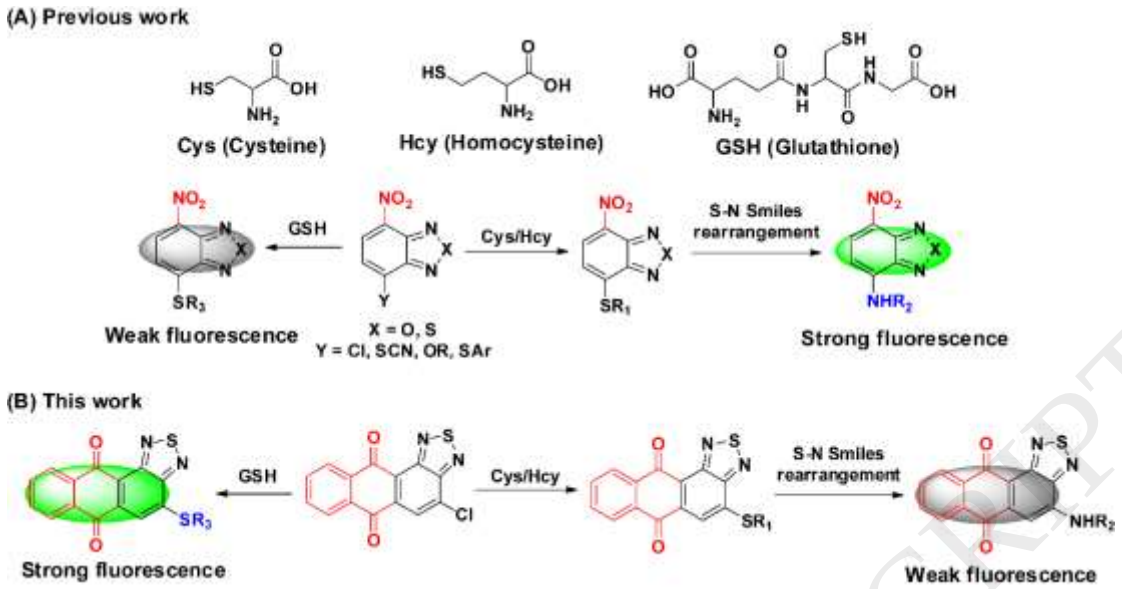
Figure 1. Fluorescence spectra of ATD-Cl (10 μM) upon addition of GSH, Cys, Hcy and NaSH (150 μM each) in PBS buffer (10 mM, pH 7.4, containing 1 mM CTAB) at 37 ℃. E_x = 465 nm, d_{em} = d_{ex} = 10 nm.

Figure 2. (A) Titration graph of the fluorescence response of ATD-Cl (10 μM) upon gradual addition of various amounts of GSH (0-60 μM). Each spectrum was obtained after 30 min addition. E_x = 465 nm, d_{em} = d_{ex} = 10 nm. (B) Fluorescence intensity at 558 nm of ATD-Cl (10 μM) as a function of GSH concentrations. Inset: plot of the fluorescence intensity at 558 nm of ATD-Cl versus GSH concentrations.

Figure 3. (A) Time-dependent fluorescence response of ATD-Cl (10 μM) (black) after treatment with 60 μM of GSH (red), Cys (blue), Hcy (pink), NaSH (green). (B) Effect of pH on the fluorescence intensity of ATD-Cl (10 μM) in the absence (black) or presence (red) of 60 μM of GSH. E_x = 465 nm, d_{em} = d_{ex} = 10 nm.

Figure 4. The selectivity of ATD-Cl for GSH. Fluorescence intensity at 558 nm of ATD-Cl (10 μM) in the prescence of 150 μM GSH and 150 μM interfering species.

Figure 5. Bright field (top row), green channel (second row), merged (third row) images of MCF-7 cells. (a_1, a_2, a_3) cells incubated with ATD-Cl (10 μM); (b_1, b_2, b_3) cells were preincubated with NEM (0.5 mM) and then incubated with ATD-Cl (10 μM); (c-e) NEM-preincubated cells were incubated with ATD-Cl (10 μM), followed by treatment with 100 μM of (c) GSH, (d) Cys or (e) Hcy.



Scheme 1

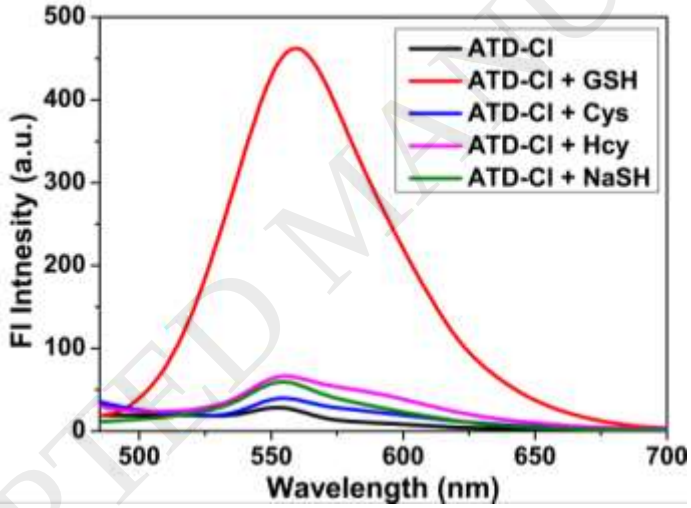


Fig. 1

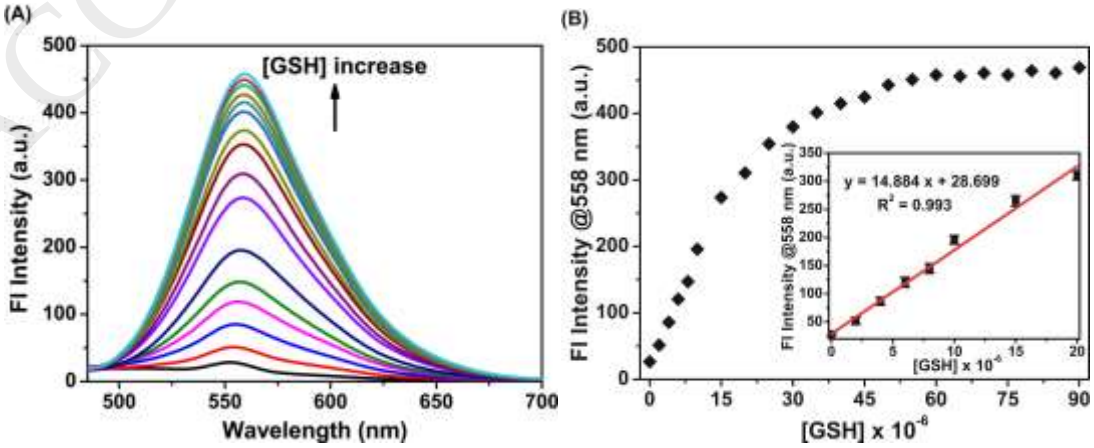


Fig. 2

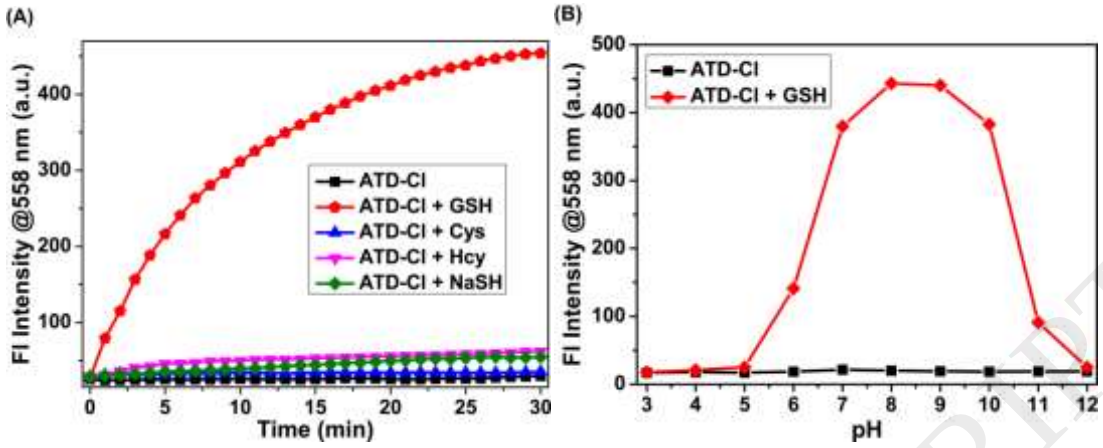


Fig. 3

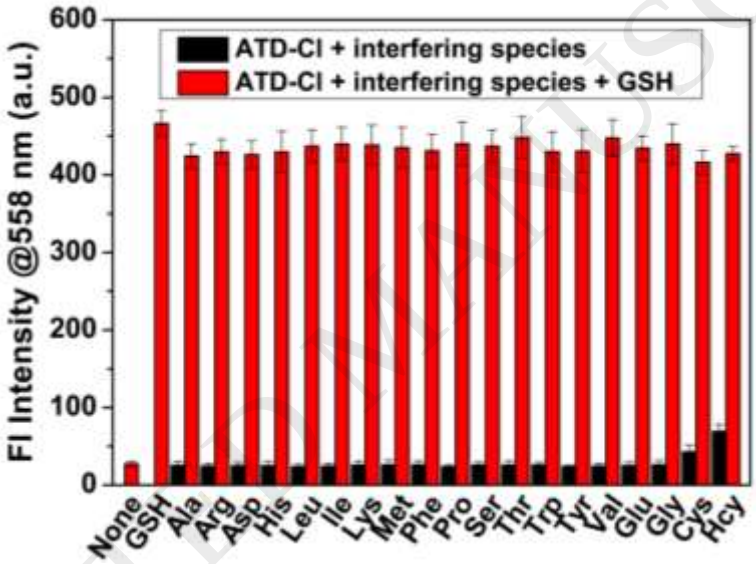


Fig. 4

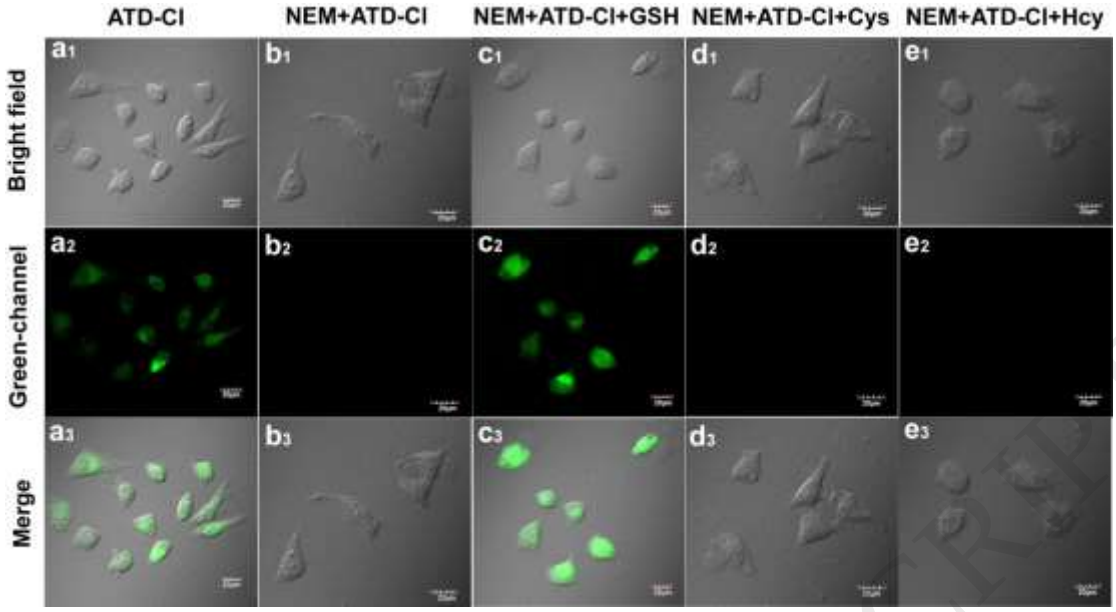


Fig. 5