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A novel 4-hydroxypyrene-based "off–on" fluorescent probe with large Stokes shift for detecting cysteine and its application in living cells

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ABSTRACT

This paper reports a novel fluorescent probe 4-acrylatepyrene (**PYAC**) based on 4-hydroxypyrene, which can effectively detect cysteine (Cys). The probe **PYAC** uses acrylate moiety as a recognition site and has relatively high selectivity and sensitivity for Cys with the detection limit of 0.062 μM. After treatment with Cys, **PYAC** exhibits "off–on" switching property and large Stokes shift (171 nm). Due to nucleophilic addition and specific intramolecular cyclization, it exhibits higher selectivity for Cys than other amino acids and common ions, including homocysteine (Hcy) and glutathione (GSH) with similar structures to Cys. The recognition mechanism has been characterized by high-performance liquid chromatography (HPLC) and nuclear magnetic resonance spectroscopy (^{1}H NMR). Anti-interference test and pH influence test display it is suitable for detecting Cys in living cells. Finally, the probe **PYAC** has been successfully applied to cell imaging with negligible cytotoxicity.

Introduction

Biothiols play vital roles in human physiological activities, among which the representative biothiols are cysteine (Cys), homocysteine (Hcy) and glutathione (GSH). These three biothiols share similar chemical structures and they all have important but different roles in physiology [1–6]. For example, abnormal Cys levels will lead to slow growth, skin lesions, liver injury and edema, etc. [7]. High serum Hcy levels are associated with hip fracture and cardiovascular disease [8], GSH is an antioxidant that protects cells from reactive oxygen species (ROS) [9]. Therefore, it is very important and necessary to develop a method capable of specifically detecting and distinguishing these three representative biothiols. In recent years, a variety of assays for biothiols detection have been developed, which includes high-performance liquid chromatography (HPLC) [10], titration analysis [11], mass spectrometry [12] and potentiometry [13,14]. Although these methods have various unique advantages, they also have some obvious shortcomings such as expensive instruments, complicated operations during pretreatment, and most importantly, these methods cannot be applied to detect biothiols in living cells. Hence, the fluorescent probe capable of solving the above disadvantages has attracted attention of researchers.

Fluorescent probe does not require expensive equipment compared to the conventional methods described above, so it is a convenient method to choose for substance detection and test. More importantly, most fluorescent probes are capable of detecting target analytes in living cells with obvious fluorescent phenomenon. At the same time, a large amount of fluorescent probes show high selectivity and sensitivity, low detection limit, and strong anti-interference ability in various detection experiments. Recently, there have been many reports on the detection of biothiols fluorescent probes [15–20]. To date, many fluorescent probes for biothiols detection have been developed based on different mechanisms [2,21,22], but most of them have difficulty in distinguishing Cys from Hcy and GSH because they have similar structure and reactivity. In the detection of biothiol, the kinetic difference between acrylate group and Cys, Hcy, GSH conjugate addition/cyclization reaction can be applied to the targeted detection of Cys. This strategy has been successfully employed to the design and exploitation of new fluorescent probes for Cys based on some well-known chromophores such as anthraquinone [23], benzothiazole [24], coumarin [25], rhodamine [26] and others [27–29], which shows significant fluorescence changes after treatment with Cys instead of other biothiols. As one of the most important chromophore of fluorescent probes, pyrene-based probes have been paid everlasting attentions because they usually exhibit excellent photochemical and photophysical properties [30]. Up to date, almost all the reports on pyrene-based probes are 1-substituted derivatives of pyrene due to that the 1-position is the commonly reactive active

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site of pyrene, which usually emits blue fluorescence. However, the inactive position-substituted pyrene-based probes are very limited and should be worthy of much developing, which may exhibit significantly different properties.

Recently, we designed and synthesized a new pyrene-based 4-hydroxypyrene with hydroxyl at the inactive 4-position of pyrene by multistep reactions and found that it emitted strongly yellow fluorescence with a large Stokes shift, suggesting it can be employed as a new probe precursor. In this work, a novel 4-hydroxypyrene-based "off–on" fluorescent probe **PYAC** for Cys with a large Stokes shift has been designed and developed by a simple reaction between 4-hydroxypyrene and acryloyl chloride. The probe **PYAC** has high sensitivity and good selectivity for Cys. The probe **PYAC** has been successfully applied to cell imaging experiments and can detect Cys in living cells with negligible cytotoxicity. Therefore, the probe **PYAC** has great potential for application.

Results and discussion

Design and synthesis of probe PYAC

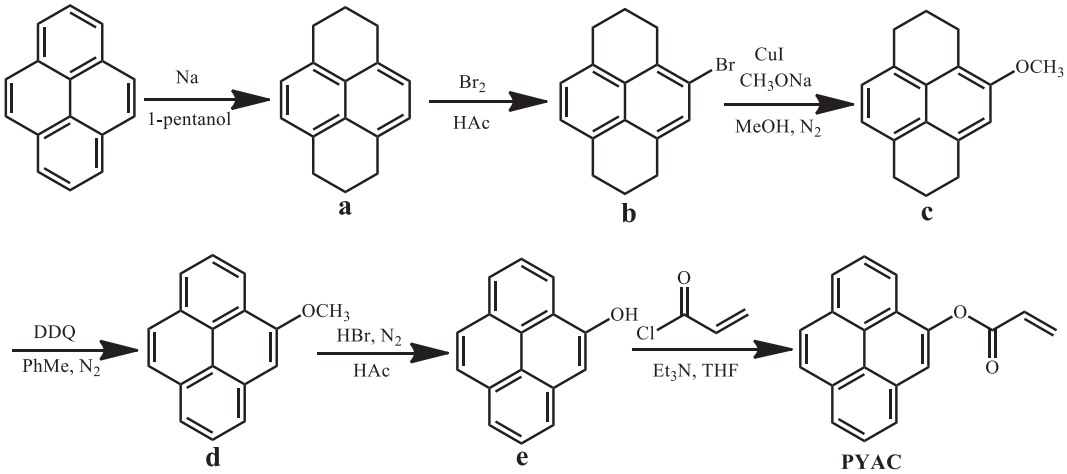
Pyrene is a traditional and classical chromophore. Generally, the reported studies on pyrene-based fluorescent probes focused on its 1-substituted derivatives since 1-position is easily be replaced and modified. However, the researches on other positions are always ignored due to the synthetic difficulty. Recently, a new 4-substituted pyrene-based derivative 4-hydroxypyrene (**e**) has been synthesized by the following indirectly multistep synthetic strategy (Scheme 1). Firstly, pyrene is hydrogenated to 1,2,3,6,7,8-hexahydropyrene which can be easily transformed to its 4-bromo-substitued pyrene-based compound **b** [31]. Secondly, intermediate **b** is transformed to compound **c** by the typical methoxylation catalyzed by CuI with moderate conversion (51%) but high selectivity (95%), therefore the material **b** can be separated to the repeated reaction and the real yield of this step is very high (ca. 90%). Thirdly, compound **d** can be easily prepared by dehydrogenation oxidation of compound **c** tin the presence of DDQ with high yield of 90%. Finally, the expected 4-hydroxylpyrene (**e**) can be synthesized by the classical demethylation of methoxyl group with nearly 100% yield. The finally probe **PYAC** is designed and easily synthesized in high yield by the reaction of compound **e** with acryloyl chloride in dry THF considering 4-hydroxypyrene unique. All the intermediates and the final product are fully characterized by ^{1}H NMR, ^{13}C NMR and MALDI TOF mass spectrometry techniques (Figs. S4–S21).

Fluorescence spectra titration

The dependence of **PYAC** on the concentration of Cys in the fluorescence spectra was investigated. When the probe was titrated using Cys, a new emission band at 512 nm increased significantly. Fluorescence reaches maximum when Cys concentration was increased to 20 μM (Fig. 1a). At the same time, the linear relationship in the Cys concentration of 2–20 μM is excellent $R^2 = 0.99661$ (Fig. 1b), this means that the probe **PYAC** can quantitatively detect Cys. According to the IUPAC definition, the detection limit of the probe **PYAC** is calculated by the following formula: $C_{DL} = 3S_a/K_b$, where S_a is the standard deviation of the blank solution and K_b is the slope of the calibration curve. According to the formula, the detection limit is calculated to be 6.2×10^{-8} M. This is much lower than the normal Cys concentration level (30–200 mM) [32], indicating that the **PYAC** probe is highly sensitive for detecting Cys in biological systems.

Selectivity and competition studies

The selectivity and anti-interference of fluorescent probes are important indicators for evaluating their performance. In order to detect the selectivity of **PYAC**, the natural amino acids Hcy, GSH, Ser, His, Ala, Asn, Asp, Gln, Arg, Glu, Met, Phe, Trp, Tyr, Lys (200 μM), metal ion Na^+, Mg^{2+}, K^+, Ca^{2+} (200 μM) and Cys (20 μM) was separately added to a ACN–PBS buffer solution (pH = 7.4, 3:7 v/v) containing 10 μM **PYAC**. As shown in Fig. 2a, significant fluorescence enhancement was observed only at 512 nm after the addition of Cys. The effect of other amino acids and metal ions on fluorescence intensity is negligible, including Hcy and GSH. This indicates that **PYAC** has good selectivity for Cys. Then, a competitive experiment was conducted to investigate the response of **PYAC** to Cys in the presence of other analytes. Add 20 μM Cys to the above buffer solution to measure the fluorescence, and the results are shown in Fig. 2b. The black bars indicate the fluorescence of **PYAC** in the presence of various analytes without the addition of Cys, and the red bars indicate the fluorescence of **PYAC** in the presence of various analytes after the addition of Cys. The results show that the probe **PYAC** has high selectively to Cys even in the presence of other amino acids, and there is no adverse



Scheme 1. Synthetic routes of compound **PYAC**.

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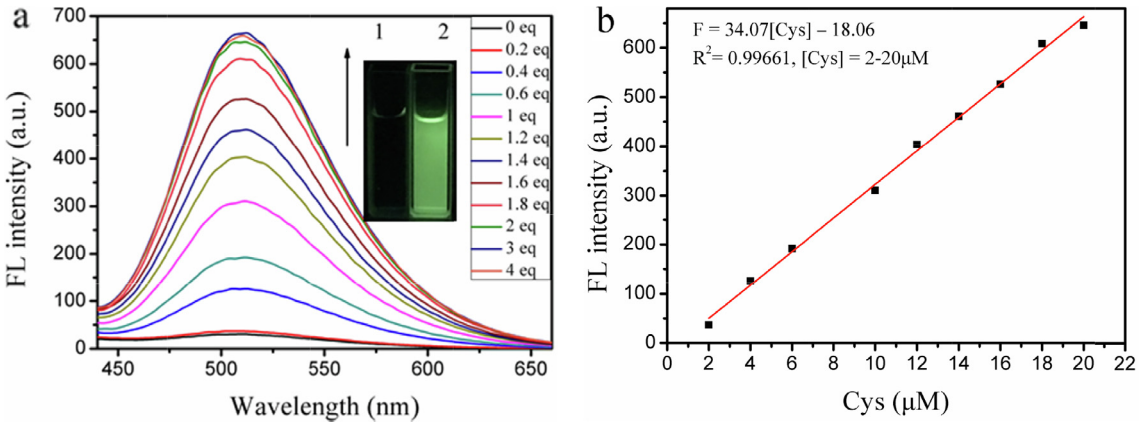

Fig. 1. (a) The fluorescence spectrums of probe (10 μM) in the presence of various concentrations of Cys (0–4 equiv) in ACN–PBS buffer solution (pH = 7.4, 3:7 v/v) (λ_{ex} = 365 nm, slit: 5 nm/5 nm). Inset: Comparison of fluorescence in the absence of (Cuvette1) and presence of (Cuvette2) Cys (20 μM) in a solution containing 10 μM probe **PYAC** under 365 nm UV illumination. (b) Plot of the fluorescence intensity of probe at 512 nm as a function of the concentration of Cys in ACN–PBS buffer solution (pH = 7.4, 3:7 v/v) (λ_{ex} = 365 nm).

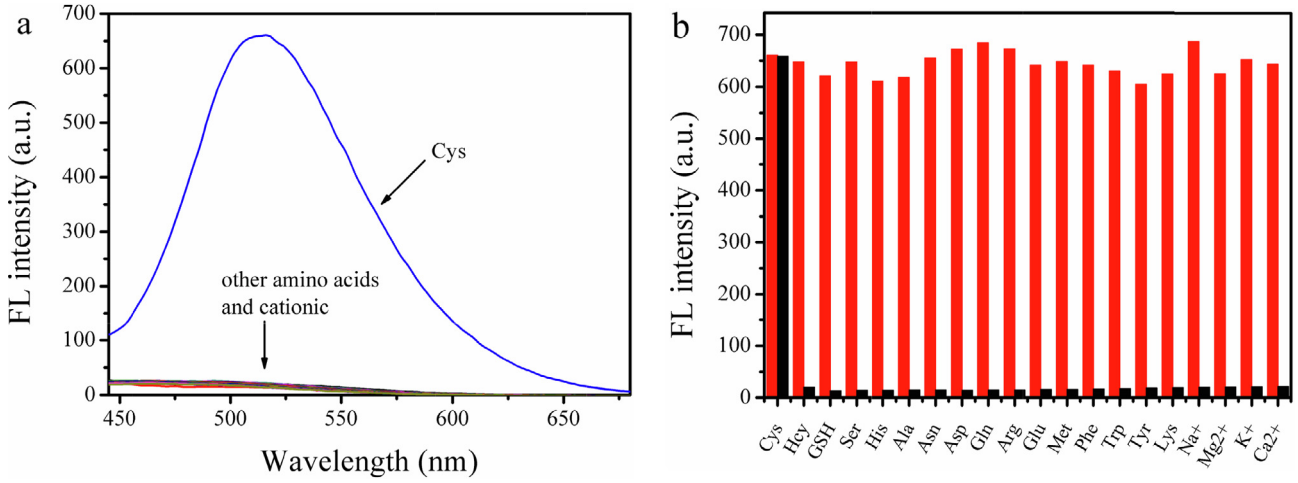

Fig. 2. (a) Fluorescence intensity of probe **PYAC** (10 μM) in ACN–PBS (pH = 7.4, 3:7 v/v) buffer solution in the presence of various analytes. (b) Fluorescence intensity of **PYAC** in ACN–PBS buffer solution (pH = 7.4, 3:7 v/v) at 512 nm in the presence of Cys (20 μM), 200 μM of other amino acids and cationic (Hcy, GSH, Ser, His, Ala, Asn, Asp, Gln, Arg, Glu, Met, Phe, Trp, Tyr, Lys, Na^+, Mg^{2+}, K^+, Ca^{2+}) (black bar). Fluorescence intensity of **PYAC** in the presence of the above analyte and additional 20 μM Cys (red bar). λ_{ex} = 365 nm, Slit: 5.0 nm/5.0 nm.

interference from other species. The above results indicate the probe **PYAC** has good selectivity and sensitivity to Cys.

pH effect

Under alkaline aqueous conditions, two Cys molecules are prone to chemical reactions and produce cysteine, and the acrylate group is easily decomposed under such conditions. So Cys can only exist under neutral or slightly acidic conditions, which requires the probe to remain stable under the same conditions. The effect of pH on the fluorescence intensity of **PYAC** (10 μM) in the presence and absence of Cys has been investigated. As shown in Fig. 3, in the absence of Cys, there was almost no change in fluorescence intensity from pH 1 to 9, indicating that the probe **PYAC** is very stable over a relatively wide pH range. After the addition of 20 μM Cys, the fluorescence intensity of the probe did not change under strong acidic conditions but it was significantly enhanced and stable under neutral conditions. Since the physiological environment of the human body is weakly alkaline and the probe **PYAC** works well under this condition, pH = 7.4 is selected as the experimental parameter.

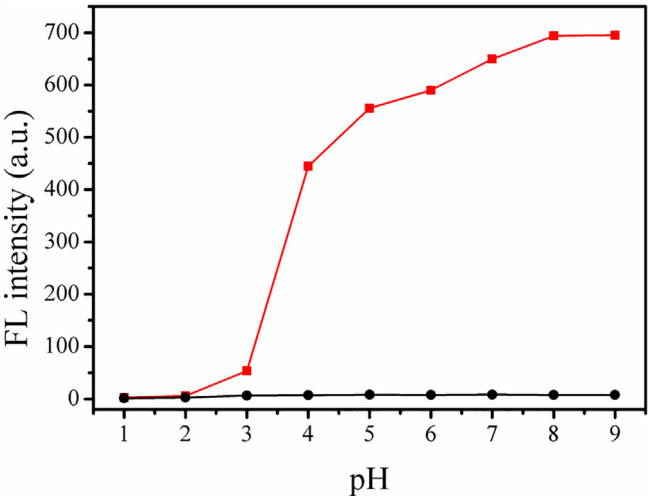

Fig. 3. Fluorescence intensity of **PYAC** with (red dot) or without Cys (black dot) in solutions with different pH values. Test condition: Cys (20 μM), ACN–PBS (3:7, v/v), λ_{ex} = 365 nm, Slit: 5.0 nm/5.0 nm.

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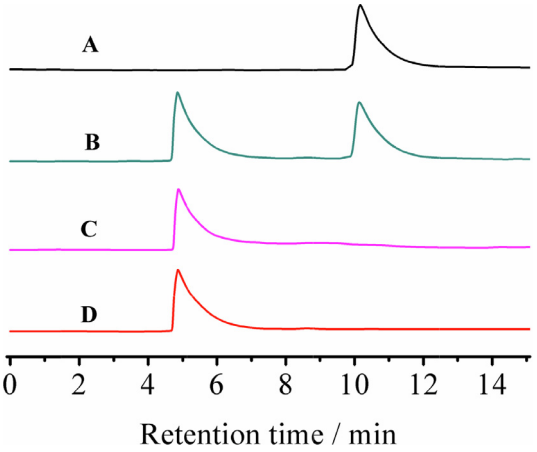


Fig. 4. HPLC chromatogram of probe **PYAC**, compound **e**, **PYAC** + Cys (sample A: **PYAC** only, sample B: **PYAC** + 2 equiv Cys, sample C: **PYAC** + 5 equiv Cys, sample D: compound **e** only).

Recognition mechanism

To investigate the detection mechanism, probe **PYAC**, compound **e**, and the reaction product of probe **PYAC** with Cys were analyzed by HPLC (Fig. 4). A new peak with a retention time of 4.7 min appeared when 2 equiv of Cys was added to the probe (Fig. 4B). When 5 equiv of Cys were added, the peak at 9.9 min completely disappeared, leaving only a peak at 4.7 min (Fig. 4C). This indicates that the probe **PYAC** is converted to compound **e** upon detection of Cys. At the same time, **PYAC** and Cys were added to acetonitrile–H_2O and stirred at room temperature for 2 h, then the product is named as product **1**. The product **1** was isolated by column chromatography and then characterized by ^{1}H NMR (Fig. S22). The result showed that the product **1** was the compound **e**. Then, by comparing the ^{1}H NMR spectrum of compound **e** in pure DMSO and DMSO–water mixed solution, it is found that the protons on the hydroxyl group will dissociate in the presence of water (Fig. S3). Based on the above experiments and related literatures in recent years [33–36], the detection mechanism of probe **PYAC** was proposed as shown in Scheme 2.

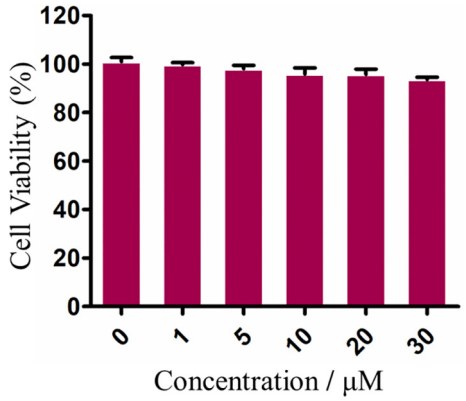
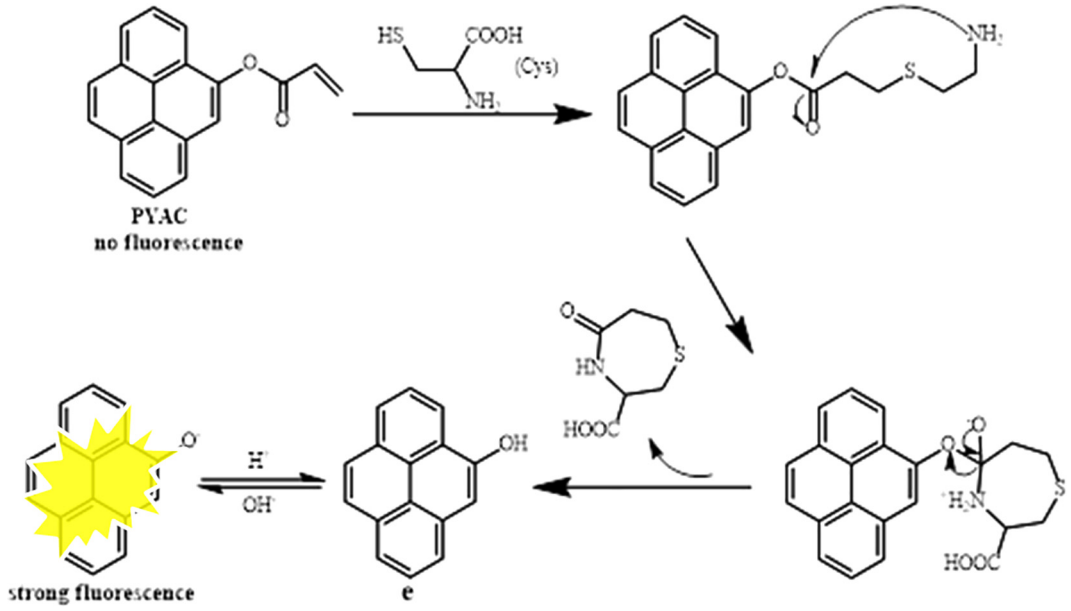


Fig. 5. Toxicity test for HeLa cells at different concentrations of probe **PYAC**.

Cell imaging experiment

The above test results indicate that the probe was capable of detecting Cys in cells, so cell imaging experiments are ready to perform. Firstly, the toxicity of the probe to cells was tested using the MTT method. Probes at concentrations of 0 μM, 1 μm, 5 μM, 10 μM, 20 μM, and 30 μM were separately added to HeLa cells for incubation, and survival was analyzed one day later. Even with probe concentrations as high as 30 μM, cell viability was still very high, indicating that the probe is less toxic to cells (Fig. 5). Then, cell imaging experiment was performed, and fluorescence of the probe itself was extremely weak, but after the probe was added to HeLa cells, the cells showed fluorescence. In the control group, the cells was treated with NEM at 100 μM and 300 μM, respectively, and then added the probe **PYAC**. After 1 h of incubation, the cells were photographed. *N*-ethylmaleimide (NEM) is a widely used thiol-blocking agent, cells treated with NEM at a concentration of 100 μM were still found to have weak fluorescence. Cells treated with NEM at a concentration of 300 μM showed no fluorescence (Fig. 6). This indicates that the probe is capable of detecting Cys in living cells.



Scheme 2. Reaction mechanism of probe **PYAC** for detecting Cys.

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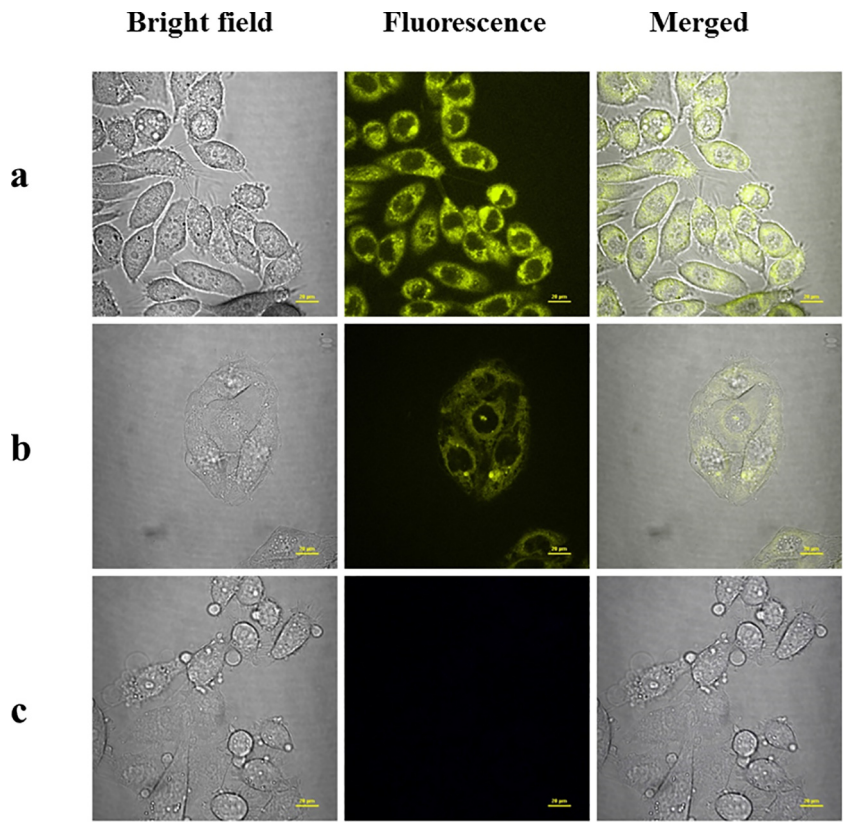

Fig. 6. Confocal microscopy images of Hela cells under different treatments. (a) Hela cells were incubated with probe **PYAC** (10 μM) for 1 h. (b) Hela cells were incubated with NEM (100 μM) for 1 h and then incubated with probe **PYAC** (10 μM) for 1 h. (c) Hela cells were incubated with NEM (300 μM) for 1 h and then incubated with probe **PYAC** (10 μM) for 1 h. λ_{ex} = 488 nm, images were collected from green (500–550 nm) channel.

Conclusion

In this paper, a new 4-hydroxypyrene-based fluorescent probe **PYAC** has been designed and synthesized with the recognizing site at the inactive 4-position of pyrene, which exhibits excellent "off–on" switching property and large Stokes shift (171 nm) after treatment with the representative biothiol Cys. The probe **PYAC** shows high selectivity and sensitivity for Cys, and the detection limit of Cys is down to 0.062 μM. Recognition mechanism was confirmed by HPLC analysis, ^{1}H NMR and MALDI-TOF-MS. Cell imaging experiments have been carried out and the results indicate that the probe **PYAC** can be used to detect endogenous Cys in living cells, and the toxicity to cells is negligible. This work provides a new and valuable method for the sensitive detection of Cys in vitro and vivo, and also shows that the inactive substituents of pyrene have promising research prospects.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tetlet.2019.151467.

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