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Coumarin-Based Turn-On Fluorescence Probe for Specific Detection of Glutathione over Cysteine and Homocysteine

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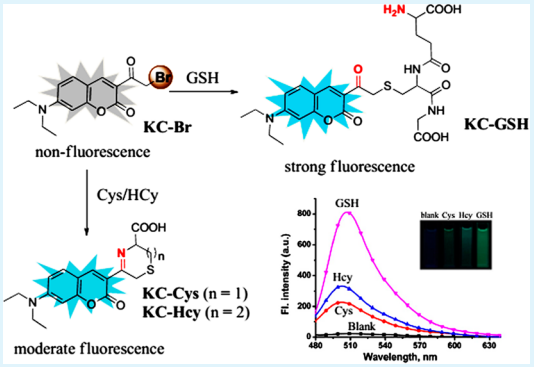
Supporting Information

ABSTRACT: We have prepared a turn-on fluorescent probe for biothiols based on bromoketo coumarin (**KC-Br**). The emission intensity of the coumarin chromophore is modulated by both the heavy atom effect and internal charge transfer (ICT) process. The probe **KC-Br** is intrinsically nonfluorescent; however, after being reacted with thiols, the bromide moiety is substituted by the −SH group, which elicits a significant fluorescence increase. We surmised the free $-NH_2$ group would further react with carbonyl in the Cys/Hcy-substituted intermediate product yielding to Schiff base compound **KC-Cys**/**KC-Hcy**, but not in compound **KC-GSH**. The ICT effect has a stronger influence in compound **KC-GSH** than that in compound **KC-Cys**/**KC-Hcy**, resulting in compound **KC-GSH** having a stronger fluorescence. Thus, the probe has a good selectivity for GSH over other various biologically relevant species and even two other similar biothiols (Cys/Hcy) and could image glutathione (GSH) in living cells. We expect the design concept presented in this work would be widely used for the design of fluorescent probes for distinguishing among biothiols.



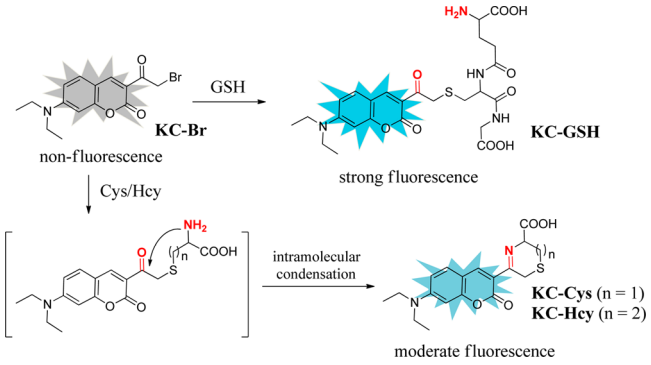
KEYWORDS: *bromoketo coumarin, fluorescent probe, specific detection, glutathione, fluorescence imaging*

1. INTRODUCTION

The small molecular weight biothiols such as cysteine (Cys), homocysteine (Hcy), and glutathione (GSH) play essential roles in human physiology.[1] GSH, the most abundant among the small intracellular molecular thiols,[2] serves many cellular functions, including maintenance of intracellular redox activities, xenobiotic metabolism, intracellular signal transduction, gene regulation, and liver damage.[3−6] However, an abnormal level of GSH directly links many diseases, including cancer, Alzheimer's disease, and cardiovascular disease.[7] So, detecting GSH has attracted a great deal of attention.

Fluorescence imaging plays an important role in monitoring biomolecules because of it is high spatiotemporal resolution in living systems.[8,9] Although many fluorescent thiol probes have been developed,[10−16] owing to the similar structures and chemical properties of these biothiols, it is very difficult to distinguish GSH/Cys/Hcy from each other. Thus, only a few fluorescent probes for discriminatively detecting glutathione from cysteine/homocysteine have been reported.[17−21] Comparing to Cys and Hcy, the distance between the sulfhydryl group (−SH) and amido group ($-NH_2$) in GHS is much longer. In this contribution, we employed this feature to construct a GSH over the Hcy/Cys fluorescent probe based on bromoketo coumarin (**KC-Br**, Scheme 1). The probe **KC-Br** is intrinsically nonfluorescent because the coumarin chromphore fluorescence is quenched by the heavy atom effect of the bromide moiety.[22] However, after reacting with thiols, the bromide moiety might be substituted by the −SH group, which elicits a significant fluorescence increase. What's more, we surmised the free $-NH_2$ group would further react with carbonyl in the Cys/

Scheme 1. Proposed Turn-On Fluorescent Probe for GSH over Cys/Hcy



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Hcy-substituted intermediate product yielding the Schiff base compound **KC-Cys**/**KC-Hcy** containing the C═N group. The electron-withdrawing ability of the C═N group is weaker than that of the C═O group, so the process would weaken the internal charge transfer (ICT) effect in coumarin dye. It might result in the final product exhibiting weaker fluorescence than carbonyl coumarin. In constrast with Cys/Hcy, the intramolecular condensation reaction might not occur in the GSH substitution product because the free $-NH_2$ group is far from the carbonyl group. Thus, the compound **KC-Br** might specifically detect GSH over Cys/Hcy as a turn-on sensor via modulation of the heavy atom effect, which works in coordination with the ICT process.

2. EXPERIMENTAL SECTION

2.1. Materials and Instruments. The compound **KC-Br** was prepared via the method reported by our laboratory.[22] Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification. Redistilled water was used for preparing all test solutions. The used instruments in this work were listed in the Supporting Information.

2.2. Determination of the Fluorescence Quantum Yield. Fluorescence quantum yields (Φ_f) for compound **KC-Br** were determined by using quinine sulfate (Φ_f = 0.58, in 0.1 M H_2SO_4 aqueous solution) as the fluorescence standard.[23] The quantum yields were calculated using the following equation.

$$\Phi_{f(X)} = \Phi_{f(S)}(A_S F_X / A_X F_S)(n_X / n_S)^2$$

where A is the value of absorbance at the excitation wavelength; F is the value of area in the corrected emission spectrum; and n is the refractive index of the solvents used. Subscripts S and X represent the standard substance and the test sample, respectively.

2.3. HeLa Cell Culture and Fluorescence Imaging of GSH in Living Cells. HeLa cells were cultured in DMEM (Dulbecco's modified Eagle's medium) containing 10% FBS (fetal bovine serum) in 5% CO_2 and 95% air atmosphere at 37 °C.

HeLa cells were incubated with 5.0 μM **KC-Br** for 20 min in 5% CO_2 and 95% air atmosphere. The control group of living cells was preincubated with 1 mM *N*-ethylmaleimide (NEM) for 20 min, and then the probe **KC-Br** (5 μM) was added and incubated for another 20 min. Subsequently, the cells were imaged using a confocal microscope (OLYMPUS FV1000, TY1318) with the 405 nm excitation filter and emission channel of 490−520 nm (green channel).

3. RESULTS AND DISCUSSION

3.1. Sensing Comparison of the Probe KC-Br to GSH/Cys/Hcy. With compound **KC-Br** in hand, we first evaluated the capability of the probe to sense Cys, Hcy, and GSH, respectively, in aqueous buffer. The titration of Cys, Hcy, and GSH to the probe **KC-Br** (10 μM) was, respectively, performed in 25 mM PBS buffer (pH 7.4) with 2% DMF. As expected, the free probe **KC-Br** is almost nonfluorescent excited at 454 nm. However, addition of Cys, Hcy, or GSH induced a significant increase of fluorescent intensity at around 505 nm (Figure 1). We found that the increase of fluorescence intensity induced by GSH is clearly larger than that induced by Cys or Hcy. Addition of Cys/Hcy/GSH induced 7-, 10-, and 37-fold enhancement of emission intensity, and the fluorescence quantum yields (Φ_f) were measured at 0.038, 0.043, and 0.131, respectively, using quinine sulfate (Φ_f = 0.58, in 0.1 M H_2SO_4 aqueous solution) as a standard.

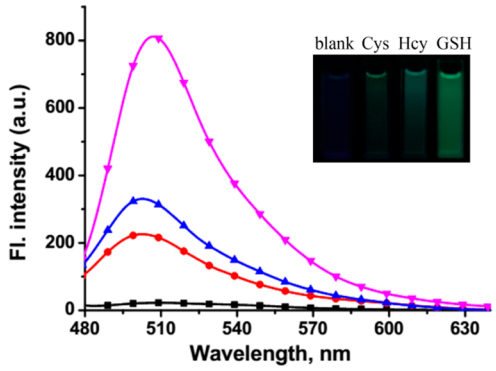

Figure 1. Fluorescence emission spectra (λ_{ex} = 454 nm) of free **KC-Br** (■), or with great excess Cys (●), Hcy (▲), and GSH (▼) in 25 mM PBS buffer (pH 7.4, containing 2% DMF). Inset: the visual fluorescence pictures of **KC-Br** in the absence or presence of Cys/Hcy/GSH (from left to right, UV lamp, 365 nm).

3.2. Sensing Mechanism Studies. To reveal the thiol-induced fluorescence turn-on response, the mixture of Cys/GSH and **KC-Br** was characterized by mass spectrometry. The molecular ion peak of **KC-Cys** (m/z = 361.0)/**KC-GSH** (m/z = 563.0) was observed (Figure 2). It demonstrated the GSH just substitutes for the bromide group of **KC-Br**, while Cys first substitutes for the bromide group and then sequentially reacts with carbonyl yielding a Schiff base product (Scheme 1). In addition, the NMR spectra (Figures S1−4, Supporting Information) and HRMS data (see Supporting Information) of the products of **KC-Br** reacting with Cys (**KC-Cys**) or GSH (**KC-GSH**) further confirm that the proposed approach is correct.

3.3. Sensing of the Probe KC-Br to GSH. Encouraged by the above desirable preliminary results, we further decided to detailedly investigate the fluorescence signals of probe **KC-Br** by detection of GSH in detail. As shown in Figure 3, the emission intensity of **KC-Br** increased gradually from 0 to 12.0 equiv of GSH at 508 nm in 25 mM PBS buffer (pH 7.4, containing 2% DMF). The fluorescence intensity reached maximum when about 8.0 equiv of GSH was added.

3.4. Stability Studies. To check the stability of the probe, the photostability of **KC-Br** in ethanol solution was examined by continuous irradiation with a xenon lamp (150 W) at the maximal absorption wavelength (454 nm). As shown in Figure 4(a), the results demonstrate that **KC-Br** had only a slight increase of fluorescence intensity after 1 h irradiation. The chemical stability was measured in the presence of various relevant reactive oxygen/nitrogen species (ROS/RNS), and the fluorescence intensity of **KC-Br** fluctuated very little (Figure 4(b)). Thus, these data indicate that the probe has good photostability and chemical stability.

3.5. Reaction Time Studies. The time plots of the emission intensities of **KC-Br** (10.0 μM) reacted with various thiols (15 equiv for Cys/Hcy and 10 equiv for GSH) were displayed in Figure 5. The maximal fluorescence signal of **KC-Br** responding to GSH was reached in 15 min.

3.6. Selectivity Studies. Then the selectivity of the probe **KC-Br** was examined. The response of probe **KC-Br** to a variety of amino acids, glucose, and related thio-containing compounds was investigated by emission spectroscopy. Probe

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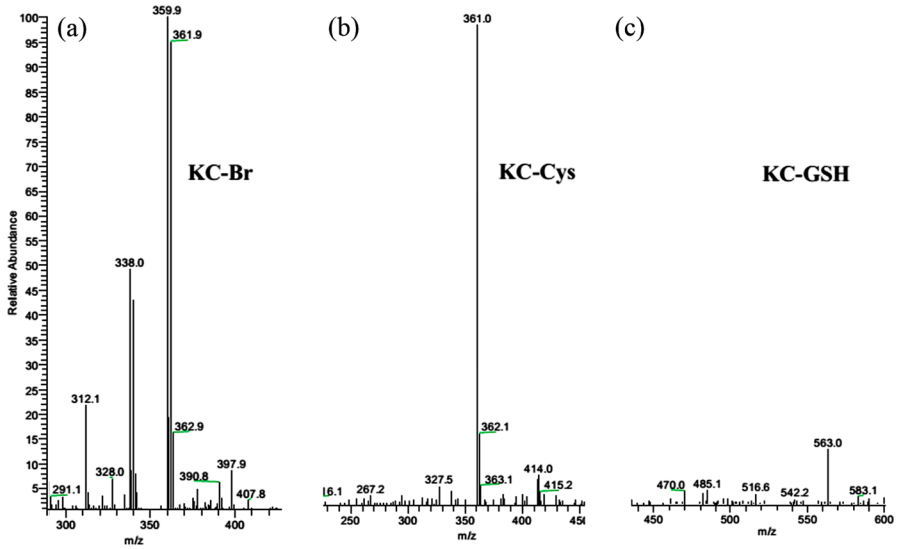

Figure 2. Partial mass spectra of (a) probe **KC-Br** or probe **KC-Br** in the presence of excess (b) Cys or (c) GSH.

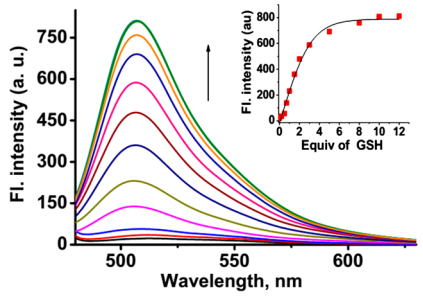

Figure 3. Fluorescence emission spectra (λ_{ex} = 454 nm) of 10 μM probe **KC-Br** with GSH (0−12 equiv) in 25 mM PBS buffer (pH 7.4, containing 2% DMF). The inset shows the changes in the fluorescence intensity of probe **KC-Br** (10 μM) at 508 nm upon addition of increasing concentrations of GSH (0−12.0 equiv).

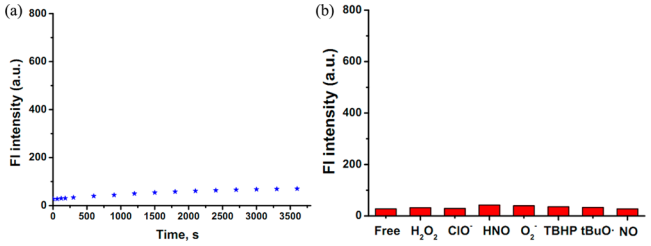

Figure 4. Changes of fluorescence intensity of **KC-Br** (10 μM, in ethanol) (a) under continuous irradiation by a xenon lamp (150 W) at the maximal absorption wavelength (454 nm) within 1 h or (b) in the absence or presence of various ROS or RNS (200 μM for H_2O_2, ClO^-, O_2^-, TBHP, tBuO•; 100 μM for HNO, NO).

KC-Br (10 μM) was treated with 100 equiv of various amino acids (including Leu, His, Phe, Ala, Gly, Val, Arg, Lys, Tyr, Glu, Ser), glucose, 10 equiv of GSH, 50 equiv of Cys and Hcy, or 100 equiv of representative interfering species (including Fe^{2+}, K^+, Mg^{2+}, Na^+, Zn^{2+}, ATP). As displayed in Figure 6, the reaction of all interfering species with the probe resulted in negligible changes of fluorescence intensity. Notably, the addition of GSH induced much greater emission enhancement than the other two homogeneous biothiols (Cys/Hcy) even at very high concentration.

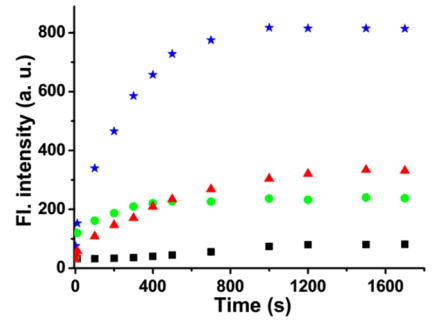

Figure 5. Reaction−time profiles of free **KC-Br** (■) and **KC-Br** in the presence of Cys (15 equiv, ●), Hcy (15 equiv, ▲), or GSH (10 equiv, ★). The fluorescence intensities at respective maximal emission wavelength were monitored in succession in PBS buffer (25 mM, pH 7.4, containing 2% DMF).

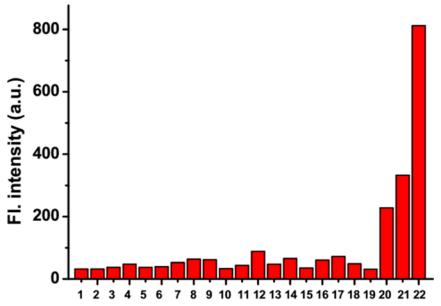

Figure 6. Fluorescence intensity (at 507 nm) of probe **KC-Br** (10 μM) responding to various analytes in aqueous solution (pH 7.4 PBS, containing 2% DMF): 1, blank; 2, Fe^{2+}; 3, K^+; 4, Mg^{2+}; 5, Na^+; 6, Zn^{2+}; 7, ATP; 8, Leu; 9, His; 10, Phe; 11, Ala; 12, Gly; 13, Val; 14, Arg; 15, Lys; 16, Tyr; 17, Glu; 18, glucose; 19, Ser; 20, Cys; 21, Hcy; 22, GSH.

3.7. pH Effect. What's more, probe **KC-Br** responds well to GSH at round physiological pH (Figure 7). The results indicated probe **KC-Br** might be suitable for detection of GSH in the biological samples.

3.8. Application of the Probe KC-Br in Living Cells. We then decided to investigate the capability of the probe **KC-Br** for imaging GSH in living cells. HeLa cells were incubated with probe **KC-Br** for 20 min, and the probe reacted with the

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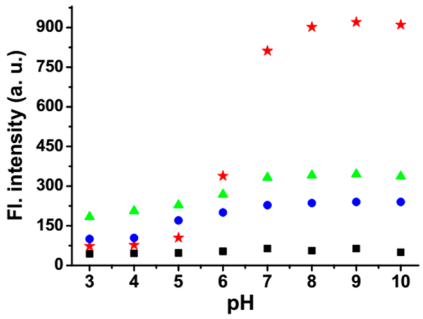

Figure 7. pH influence on the fluorescence intensity at respective maximal emission wavelength of the 10 μM probe **KC-Br** in the absence (■) or presence of 10 equiv of GSH (★), 15 equiv of Cys (●), and 15 equiv of Hcy (▲).

abundant GSH in living cells and elicited strong blue fluorescence (Figures 8a–c). However, cells were preincubated

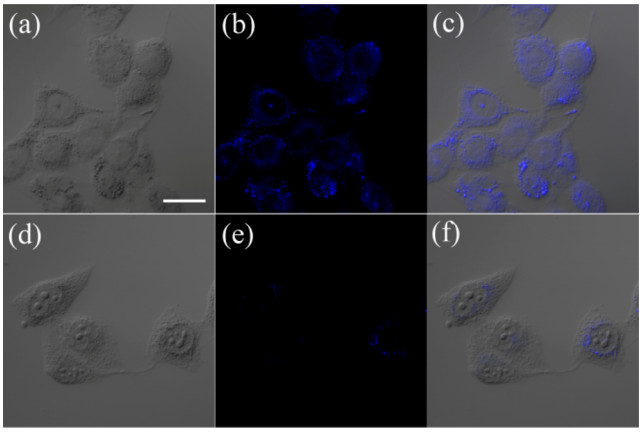

Figure 8. Confocal fluorescence images of living HeLa cells. (a) Bright-field image of cells treated with **KC-Br** (5 μM) for 20 min; (c) fluorescence image of (a); (c) overlay of the images of (a) and (b). (d) Bright-field image of cells pretreated with 1 mM NEM for 30 min, followed by addition of the probe **KC-Br** (5 μM) and incubated for 20 min; (e) fluorescence image of (d); (f) overlay of the images of (d) and (e). Images were obtained using 405 nm excitation and emission channels of 490–520 nm. Scale bar = 20 μm.

with 1 mM *N*-ethylmaleimide (NEM, a scavenger of biothiols)[24] for 30 min and then treated with 5 μM **KC-Br** for another 20 min. As shown in Figures 8d–f, the cells exhibited very faint emission in the blue channel (490–520 nm). Thus, these results revealed that **KC-Br** has a good membrane permeability and is capable of monitoring GSH in living cells.

4. CONCLUSION

In conclusion, we prepared a turn-on fluorescent probe for GSH based on bromoketo coumarin (**KC-Br**). The probe has good selectivity and sensitivity for GSH over other various biologically relevant species and even two other similar biological thiols (Cys/Hcy) and could image GSH in living cells. We expect the design concept presented in this work could be widely applied to construct fluorescent probes for distinguishing among the biothiols.

■ ASSOCIATED CONTENT

Supporting Information

Instrument, synthesis of **KC-Cys**/**KC-GSH**, and characterization spectra. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.5b01934.

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Notes

The authors declare no competing financial interest.

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