EDGE ARTICLE

Exceptional time response, stability and selectivity in doubly-activated phenyl selenium-based glutathione-selective platform†

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A phenyl-selenium-substituted coumarin probe was synthesized for the purpose of achieving highly selective and extremely rapid detection of glutathione (GSH) over cysteine (Cys)/homocysteine (Hcy) without background fluorescence. The fluorescence intensity of the probe with GSH shows a ~100-fold fluorescent enhancement compared with the signal generated for other closely related amino acids, including Cys and Hcy. Importantly, the substitution reaction with the sulfhydryl group of GSH at the 4-position of the probe, which is doubly-activated by two carbonyl groups, occurs extremely fast, showing subsecond maximum fluorescence intensity attainment; equilibrium was reached within 100 ms (UV-vis). The probe selectivity for GSH was confirmed in Hep3B cells by confocal microscopy imaging.

Introduction

Biothiols, such as cysteine (Cys), homocysteine (Hcy) and glutathione (GSH), play crucial roles in biological systems as endogenous thiols. These biothiols exist in equilibrium between their oxidized disulfide and reduced free-thiol forms in biological systems.[1] Each biothiol has its own role(s). GSH, an antioxidant enzyme, is the most abundant intracellular biothiol, composed from Cys, glutamine and glycine. GSH plays a central role in protecting the cell from oxidative damage and in maintaining biological homeostasis, which plays a significant role for cell growth and function among other biothiols (*e.g.* Cys, and Hcy).[2] Although GSH is concentrated in the liver, it protects the whole body from various toxins produced by the body itself as a result of normal metabolic processes, and exposure to external toxins, such as environmental, illicit drug use, *etc.* Abnormal levels of GSH lead to oxidative stress responsible for, or observed in, premature aging and other conditions such as Alzheimer's disease (AD), Parkinson disease

(PD), cancer, cystic fibrosis, AIDS, osteoporosis, cardiovascular disease and sickle cell anemia.[3] Hence, numbers of research groups are involved in seeking to achieve the selective determination of GSH in biological systems; this is required for better understanding of its role in biological systems and in early diagnosis of disease.

Due to the significant role of biothiols (Cys, Hcy and GSH), a number of reports have accounted for the detection of these species at the molecular level through the use of small synthetic probes.[1e,f,4] Based on the unique reactivity of biothiols, numerous recent fluorescent probes have been developed and reported that can distinguish between biothiols and amino acids. The aldehyde group has been used in the selective detection of Cys and Hcy over GSH through forming thiazolidines and thiazinane, respectively.[5]

Aldehyde groups have also been exploited for their use in the detection of amino acids generally, through forming an iminium group, which shows a red-shifted absorption wavelength, compared to the aldehyde moiety.[6] Acrylate (a 1,4-Michael acceptor) and beta-halide propionate (nucleophilic substitution) groups were used to discriminate Cys over Hcy and GSH by taking advantage of kinetic differences in intramolecular cyclization and concomitant release from the fluorophore moiety.[7] Also, 1,4-Michael additions have been used for the detection of biothiols.[8] However, it is still challenging to design a highly selective fluorescent probe for a particular biothiol because of the similarity in structure and reactivity between the species.

Recently, there have appeared reports for the selective sensing of GSH over Cys and Hcy.[9–12] Nucleophilic substitution reactions by strong nucleophilic sulfhydryl groups, exploited for initial reactivity and for events, such as native chemical ligation,[10] intramolecular displacement,[11] and intramolecular

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Established Strategy for Detection of Biothiols
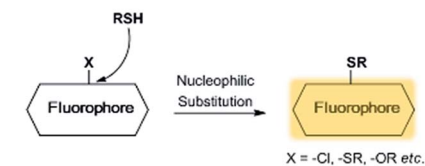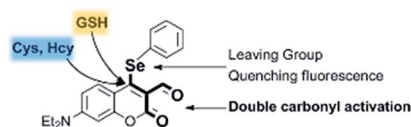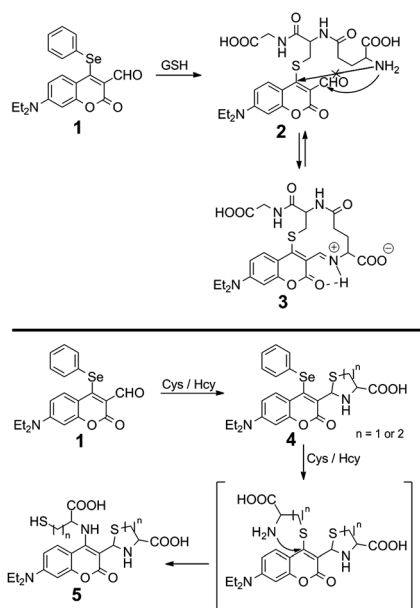

Present Research

Fig. 1 Contemporary strategy for fluorescent thiol detection.

cyclization,[12] have allowed for the discrimination of GSH over Cys and Hcy. Although a few probes for the detection of thiols have been reported for real-time detection,[13] to the best of our knowledge there is no probe for the selective detection of GSH demonstrating real-time detection as of yet. Considering the current state-of-the-art and these points of consideration, we have developed fluorescent probe **1**, which has two independent potentially reactive sites, aldehyde and phenylselenide, for the selective detection of GSH (Fig. 1). Recently, our group has explored several fluorescent probes containing selenium as the reactive center to detect important biological analytes.[14] Here, we chose to incorporate a phenylselenide group at the coumarin 4-position; this group is able to quench fluorescence *via* photo-induced electron transfer (PET) and is also able to behave as a leaving-group. The proximal aldehyde group plays a dual role: it enhances the electrophilicity of the 4-position as a Michael acceptor and enables a cyclization reaction with the sulfhydryl and primary amine groups of the biothiols (Scheme 1). Below,

we discuss the preparation, screening, biological studies and general suitability of probe **1**.

Results and discussion

Probe **1** was synthesized from 4-chloro-7-(diethylamino)-2-oxo-2*H*-chromene-3-carbaldehyde (**7**)[12b] by treatment with PhSeZnBr[15] (Scheme 2). The structure of **1** was characterized by spectroscopic techniques (multinuclear NMR spectral data, mass spectrometry; Fig. S3–S7†). Analyte screening was performed, which included various biothiols (Cys, Hcy, GSH) and amino acids (Pro, Thr, Val, Leu, Asn, Trp, Ile, Ala, His, Glu, Tyr, Lys, Met, Asp, Phe, Arg, Gly, Gln, Ser); the analytes were used to treat the solution (DMSO/10 mM PBS pH 7.4, 1 : 3, v/v) in the presence of 20 μM of **1**. A new shoulder at ~518 nm appeared, consistent with the addition of GSH to **1**, with a simultaneous decrease of the absorption at ~471 nm. On the other hand, upon treating Cys and Hcy, the changes in the absorption spectra were different. A new absorption peak at ~380 nm was obtained, the original absorption peak for **1** simultaneously decreased; no change in the absorption at ~518 nm was observed in the case of other amino acids (Fig. 2, left). A new absorption band (518 nm) and the absorption maxima for **1** (471 nm) were monitored. Only the solution of **1** with GSH led to enhanced fluorescence under λ_{ex} = 471 nm (Fig. S14†) and 518 nm (Fig. 2, right) without background fluorescence. The solution of **1** under the same conditions with other amino acids, including Cys and Hcy, were non-emissive (λ_{ex} = 471 and 518 nm).

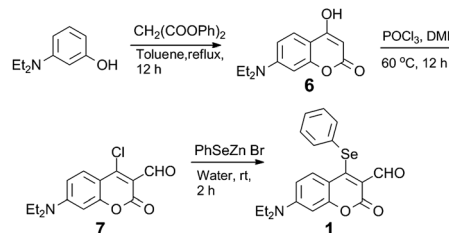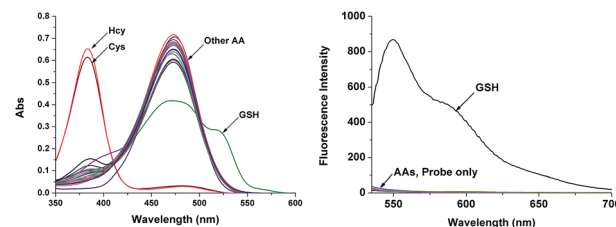Titrations of **1** with GSH were then more carefully studied. Spectral determinations with compound **1** and various

Scheme 2 Synthesis of **1**.

Scheme 1 Proposed mechanism of **1** with biothiols.

Fig. 2 Absorption and emission spectra of **1** (20 μM) with 10 equiv. of an amino acid (Pro, Thr, Val, Leu, Asn, Trp, Ile, Ala, His, Glu, Tyr, Lys, Met, Asp, Phe, Arg, Gly, Gln, Ser) or biothiol (Cys, Hcy, GSH) in solution (DMSO/10 mM PBS pH 7.4, 1 : 3 v/v) incubated for 5 min at r.t. λ_{ex}: 518 nm.
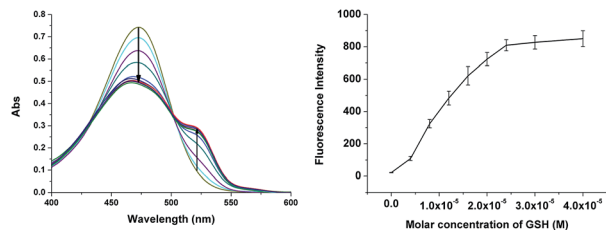
Fig. 3 Absorption and emission spectral changes of **1** (20 μM) with various concentrations of GSH (0–40 μM) in solution (DMSO/10 mM PBS pH 7.4, 1 : 3, v/v) incubated for 5 min at r.t. λ_{ex}: 518 nm.

concentrations of GSH (0–40 μM) were performed (Fig. 3) in which both absorption and emission spectra were recorded (20 μM, DMSO/10 mM PBS pH 7.4, 1 : 3, v/v). The absorption and fluorescence intensity, and concentration of GSH were linearly proportional and became saturated at 24 μM (1.2 equiv.). The detection limit was determined to be 2.7×10^{-7} M.

Our hypothesis, thus, is that the 4-position of **1**, which is doubly-activated by two carbonyl groups, undergoes faster substitution with a strong nucleophilic sulfhydryl group such as that for GSH. Indeed, **1** shows very rapid analyte detection. The time-dependent fluorescent analysis was performed with 10 equiv. of GSH. The extremely fast emissive spectral change reached saturation involving a maximum intensity within 1 s (Fig. 4). Stopped-flow with UV-vis spectroscopy then allowed us to track two absorption wavelengths (471, 518 nm) at very short times, affected by the addition of GSH to **1**. The equilibrium of absorption was reached within 100 ms (Fig. 5), suggesting that **1** undergoes an extremely fast reaction with GSH due to the doubly-activated α,β-unsaturated system.
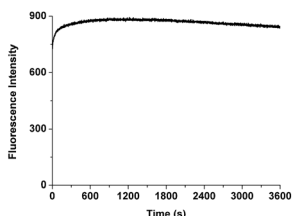

Fig. 4 Time-dependent emission spectral changes of **1** (20 μM) with 10 equiv. of GSH in solution (DMSO/10 mM PBS pH 7.4, 1 : 3, v/v). λ_{ex}: 518 nm. λ_{em}: 550 nm.

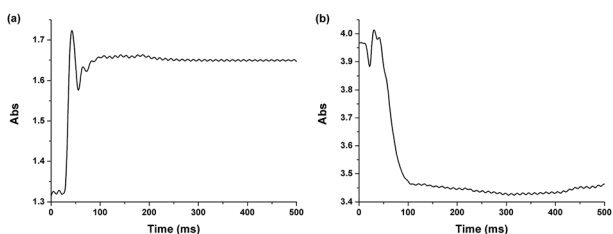**Fig. 5** Absorption changes of **1** (200 μM) with 1.0 equiv. of GSH (200 μM) by stopped-flow spectroscopy (average of 4 experimental trials). (a) λ = 518 nm, (b) λ = 471 nm.

To help unravel the mechanism and structure of **1** with biothiols, model compounds **8** and **9** were synthesized (Fig. 6 and S8–S13†) and their reactivity studied. Compounds **8** and **9** have the same atoms at the 4-position of coumarin as would be expected through the reaction of Cys/Hcy and GSH with **1**, respectively. The absorption and emission spectra of **8** were recorded under the same conditions as previously (DMSO : 10 mM PBS, pH 7.4, v/v, 1 : 3) (Fig. S15†). The spectra show very similar photophysical properties with the proposed structure **5**, bearing a nitrogen atom at the 4-postion of the coumarin. Similar S_{Ar} substitution and replacement have also been reported.[11] High resolution mass spectrometry (HRMS) data of **1** with Cys (1 and 10 equiv.) were recorded. Mass spectra of **1** with 1 equiv. of Cys showed a correct isotopic pattern for selenium and was in good agreement with the calculated mass value of **4** (Fig. S17†). The solution containing 10 equiv. of Cys indicated the formation of both **4** and **5**, which does not have an isotopic pattern of selenium (Fig. S18†). Based on these results, the 4-position of coumarin is believed to undergo a substitution reaction and S,N-displacement. The aldehyde undergoes cyclization to form thiazolidine. Intermediates **4** and **5** would be the expected structures of **1** after reaction with Cys/Hcy.

In the case of the reaction of **1** with GSH, S,N-displacement would not be possible with GSH due to an unfavorable macrocyclic transition state.[11b] Structure **3** was proposed for the reaction of **1** with GSH; this motif is consistent with how coumarin-aldehydes react with amino acids to form iminium ions, previously reported by Glass et al.[6] When coumarin-aldehyde forms an iminium group, it shows a red-shifted absorbance and emission (see pH-dependent studies below). This red-shifted wavelength was attributed to the hydrogen bond between the carbonyl and the iminium group. Based on these reports, compound **9** was synthesized and its photophysical properties examined because **9** was expected to show similar photophysical properties to **2**. The absorption spectrum of **9** is very similar to that of **1**. There was no absorption peak at ~520 nm as shown in **1** with GSH. It is demonstrated that the absorption peak enhancement at ~520 nm does not originate from the relevant features of structure **2**, which undergoes a substitution reaction only. Compound **9** was treated with biothiols under the same conditions to help rationalize the absorption peak at ~520 nm. When GSH is added to **9**, the original maximum absorption peak (~480 nm) decreased and simultaneous enhancement of ~520 nm was observed, consistent with what is known for **1** and GSH (Fig. S16†). Furthermore, the observed mass spectra of the reaction mixture, **1** with GSH, supports that GSH underwent substitution and intramolecular cyclization (calc.: 533.1706, obtained: 533.1724 $[\mathbf{3} + \text{H}]^{+}$); also,
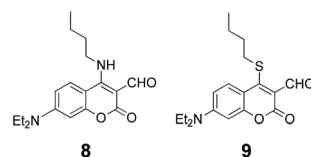

Fig. 6 Model compounds **8** and **9**.

the selenium isotopic pattern has disappeared (Fig. S19†). These results strongly support that GSH undergoes nucleophilic substitution at the 4-position of the coumarin and intra-molecular cyclization with aldehyde through forming an iminium-group.

Considering red-shifted wavelengths and hydrogen bonding between iminium and carbonyl oxygen, density functional theory (DFT) calculations (B3LYP/6-31g*,[16] G09) were performed for **1**, **2** and **3** to visualize the optimized structures and calculate molecular orbital energy levels. The results of the optimized structures show that **3** forms a stable hydrogen bond between the carbonyl group and iminium, which induces a red-shifted wavelength (Fig. S20†). The calculated frontier orbital energy difference for **3** is also smaller than that for **1** and **2** (Fig. S21†).

To demonstrate the effects of pH on **1**, the fluorescence emission of **1** was checked with a wide range of pH under λ_{ex} = 518 nm. It shows that pH does not affect the fluorescence of **1**. Furthermore, GSH (10 equiv.) was added to solutions at various pH values in the presence of 20 μM of **1** (Fig. 7). In the pH range of 4–8, **1** showed a strong response for GSH. The results indicated that **1** is stable within the overall range of pHs tested and works properly under physiological conditions.

To confirm that **1** has passed through the cellular membrane and is interacting with GSH in living cells, several biological studies were carried out. Human liver carcinoma Hep3B cells were treated with various concentrations of **1** (0 μM, 2 μM, 5 μM, and 10 μM) for 30 min to determine the optimum concentration for probe treatment (Fig. S22†). From the photophysical test results (Fig. 2 and S14†), a 488 nm argon laser was chosen for the excitation to demonstrate the reaction between **1** and intracellular GSH. Fluorescence emission was detected at a concentration of 2 μM, but 10 μM was considered optimal as the signal was valid within the major population of cells. Upon treatment of **1** (10 μM), a strong green fluorescence emission was observed, indicating that the reaction of **1** with intracellular GSH proceeds (Fig. S22,† 10 μM). Using live cell imaging techniques, it was shown that the fluorescence signal became noticeable within 5 min, confirming the rapid interaction between **1** and GSH (ESI, Movie file†). The results demonstrated that **1** permeates into living cells and reacts with intracellular GSH.

Additional experiments were performed to better support the conclusion that fluorescence signals were due to the interaction between **1** and GSH in the cells. A thiol-specific reagent
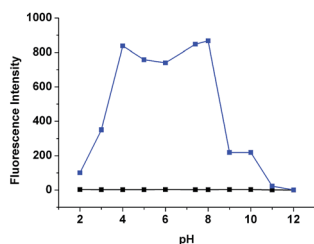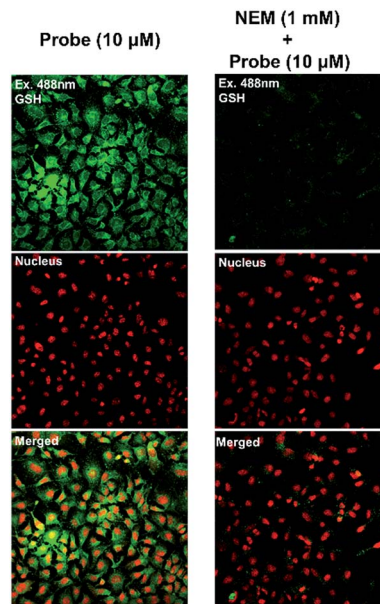
Fig. 8 Confocal microscopy image of Hep3B cells pre-treated with 1 mM of NEM for 30 min and then treated with **1** for 30 min.
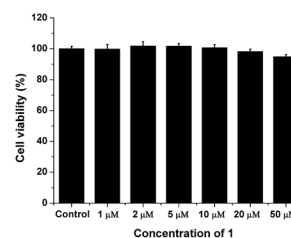
Fig. 9 Concentration-dependent cell viability assays. Hep3B cells were pre-incubated with various concentrations of **1**; their proliferation rates were measured with a WST-1 assay kit.

N-ethylmaleimide (NEM) at a concentration of 1 mM was pre-treated in Hep3B cells for 30 min; the cells were further incubated with **1** for another 30 min. The confocal microscopy image of NEM-treated cells showed a significant decrease in the fluorescence signal, suggesting the specific reactivity of **1** with intracellular GSH (Fig. 8).

In order to evaluate the biocompatibility of the probe, cell viability testing was carried out. Hep3B cells were pre-incubated with various concentrations of **1** for 1 h followed by WST-1 cell proliferation assays (Fig. 9). This assay resulted in no significant decrease in viability of the cells incubated with 50 μM of **1**, compared with untreated cells; thus, the probe has low cytotoxicity and may be appropriate for real biological applications.

Conclusions

In conclusion, a new probe has been developed for the highly selective and rapid detection of GSH in live cells; selectivity over Cys/Hcy without background fluorescence was demonstrated. The fluorescence intensity was ~100-fold greater, compared with other amino acids, including Cys and Hcy. Importantly, the

Fig. 7 Fluorescence intensity changes by **1** (20 μM, black) and **1** (20 μM) with 10 equiv. of GSH (blue) under various different pH environments. λ_{ex}: 518 nm. λ_{em}: 550 nm.

probe reached maximum fluorescence intensity within ~1 s and reached equilibrium of absorption within ~100 ms. Confocal microscopy imaging of living cell systems indicate the probe detects GSH in Hep3B cells rapidly and specifically. Furthermore, cell viability testing showed low cytotoxicity of the probe and its potential for biological uses.

Acknowledgements

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