The development of a fluorescence turn-on sensor for cysteine, glutathione and other biothiols. A kinetic study


Biothiols (RSH) are compounds involved in many essential cellular functions. Among these, the tripeptide glutathione (GSH), γ-glutamyl-cysteinyl-glycine, is the most abundant low-molecular-mass non-protein thiol compound in cells. Other low molecular mass thiols that contribute to the intracellular thiol pool are precursors for the synthesis of GSH: the aminoacids homocysteine (Hcy) and cysteine (Cys); and the first product of GSH degradation; the dipeptide cysteinyl-glycine (Cys-Gly). Diverse reports have demonstrated that abnormal levels of these biothiols can cause a number of health problems. Thus, development of optical probes for their detection has been an active research area in recent years. Among the available techniques to detect and quantify biothiols (Cys, Hcy and GSH) fluorescence methods have been extensively pursued due to their simplicity, sensitivity, and versatility.

In this work, we focus on the interaction between these biothiols and coumarin-based compounds containing a chalcone-like moiety. Specifically, we describe here (E)-3-cinnamoyl-7-methoxy-2H-chromen-2-one (ChC1) and (E)-3-(3-(2-hydroxyphenyl)acryloyl)-7-methoxy-2H-chromen-2-one (ChC2) as fluorescence probes for the detection of Cys, Hcy and GSH, and, for the first time, Cys-Gly, mediated by a Michael addition reaction (Scheme 1).

The probes ChC1 and ChC2 were readily synthesized in four steps from commercial precursors (Scheme 1; Supplementary data). We then proceeded to study the fluorescence response of these probes to 2-mercaptoethanol (ME), as a model compound for biothiols (Figs. S3(A) and S4; Supplementary data). Figure S3(B) shows the turn-on fluorescence of ChC1 upon addition of GSH (1 mM/HEPES buffer). The formation of the ChC1-GSH adduct is apparently complete within 50 min. ChC1, excited at 340 nm, emitted with λmax = 430 nm and quantum yield Φ = 0.00083. Upon addition of 10 equiv of Cys or GSH the quantum yield increased to 0.0013 and 0.0010, respectively, with no band shift. A similar experiment with ChC2 (λmax = 435 nm, Φ = 0.0014) led to the increase of the quantum yield to 0.0022 and 0.0016, respectively.

That these changes are due to a Michael addition was corroborated by 1H NMR spectroscopy of the probes in the absence or presence of ME. As shown in Figure 1, upon addition of ME the vinyl proton resonances (H₆ and H₇ at δ 7.80 and 7.74 ppm, respectively) disappeared, and concurrently two new triplets, assigned to the thioether methylene protons H₆ and H₇, emerged at δ 4.74 and 4.46 ppm, respectively, a result that is consistent with the formation of the ChC1–ME adduct.

The selectivity of ChC1 and ChC2 toward biothiols was investigated by incubating these probes with different species including non-sulfur amino acids (Asp, Lys, Ser, Hys, Pro, Phe, and Val). As shown in Figure 2 for the case of ChC1, none of these compounds
interferes to any obvious extent with the detection of biothiols, even at a 100:1 molar ratio with respect to the probe. In addition, when GSH is co-incubated with N-ethylmaleimide (NEM), a sulfhydryl alkylating reagent, the fluorescence response of ChCl decreases considerably.

We then studied the kinetic profiles of the reactions of ChCl and ChC2 with the tested biothiols under pseudo-first-order conditions (large excesses of the thiols). The observed rate constants (k_{obsd}) obtained at 30.0 °C are shown in the Supplementary data (Table S1 and S2). For the purpose of comparison the relative second-order rate constants were calculated. Table 1 shows a comparison of the nucleophilic rate constants (kN) for the reactions of ChCl and ChC2 with the tested biothiols. It can be seen that in both cases the reactivity increases in the sequence Cys > GSH > Hcy > Cys-Gly. This order is in agreement with the basicity of the sulfhydryl group of each thiol (pK_a values). The only exception is for Cys which, being one of the less basic thiols, is the most reactive compound. However, a similar result has been found very recently by other authors.8i,j

Interestingly, we found that GSH is more reactive than Hcy in the Michael addition to ChCl and ChC2, in contrast to other probes which have proved to be less reactive toward GSH in comparison with Hcy.8i However, a very recent paper describes a coumarin-substituted chalcone probe that reacts efficiently, via activation by an intramolecular hydrogen bond, with GSH and Cys but only slightly with non-thiol natural amino acids.9 In addition, a comparison of the nucleophilic rate constants (kN) for the reactions with all four biothiols (Table 1) shows that under our experimental conditions ChC2 is 12 to 60-fold more reactive toward thiolate ions than ChCl.

Table 1

<table>
<thead>
<tr>
<th>Biothiols</th>
<th>pK_a</th>
<th>kN/s·M⁻¹</th>
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<tr>
<td></td>
<td></td>
<td>ChCl</td>
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<tr>
<td>Cysteinyglycine</td>
<td>7.00</td>
<td>0.34 ± 0.02</td>
</tr>
<tr>
<td>Cysteine (Cys)</td>
<td>8.10</td>
<td>5.17 ± 0.31</td>
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<tr>
<td>Homocysteine (Hcy)</td>
<td>8.25</td>
<td>0.49 ± 0.04</td>
</tr>
<tr>
<td>Glutathione (GSH)</td>
<td>8.72</td>
<td>1.56 ± 0.22</td>
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</table>

Figure 1. Change in the partial ¹H NMR spectrum of ChCl (20 mM) upon addition of ME (1.8 equiv) in DMSO-d₆ at 25 °C. (A) ChCl only; (B) ChCl + ME, (10 min).

Figure 2. Fluorescence responses of ChCl (20 µM; λ_ex 340 nm) in the presence of amino acids (2 mM), GSH (2 mM) or GSH plus NEM (500 µM).

![Scheme 1. Reaction of ChCl and ChC2 with tested biothiols (RSH).](image)
We propose that the higher reactivity of ChC2 than ChCl toward thiolate ions might be a consequence of proton donation by the hydroxy group of ChC2 to the vinyl carbon neighboring the carbonyl group, thus making the reaction site more prone to attack by the thiolate. While this manuscript was in preparation, it was reported that (E)-7-(diethylamino)-3-(3-(2-hydroxyphenyl)acryloyl)-2H-chromen-2-one, which bears an o-hydroxyl group in the same relative position as ChC2, showed an only threefold enhancement of its reaction rate with GSH as compared to (E)-3-cinnamoyl-7-diethylamino-2H-chromen-2-one, the corresponding analogue of ChCl. Those authors concluded that the participation of the hydroxyl group in this Michael addition was unlikely and therefore the presence of this group made little difference. In our hands, ChC2 proved to be remarkably more reactive than ChCl, particularly toward Cys-Gly, Hcy, and GSH, warranting future studies of the detailed reaction mechanism.

A practical application of ChCl and ChC2 was to evaluate the effect of N-acetylcysteine (NAC), an agent capable of stimulating the synthesis of glutathione, on the fluorescence of both probes in human neuroblastoma SH-SYSY cells. This cell line was treated for 12 h with NAC (30 mM) after which the cells were washed and incubated with the probes. The fluorescence was measured using a microplate fluorescence reader and by epifluorescence microscopy (Figs. 3A and 4A). The results showed that the fluorescence was significantly increased after NAC treatment. Similar results were observed when the cells were loaded with the probes and Cys (5 mM) was subsequently added, incubating for 5 min (Figs. 3B and 4B). The data represent mean ± SEM; n = 6, P < 0.01.

In conclusion, we have prepared and characterized two new fluorescence turn-on sensors (ChC1 and ChC2) that exhibit a highly selective response to biothiols both in vitro and in vivo. Unexpectedly, introduction of an ortho-hydroxyl group on the cinnamoyl...
moiety resulted in large rate enhancements of the Michael addition reactions that underlie the fluorescence response of our probes.

Acknowledgments

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Supplementary data


References and notes