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Fluorescence Imaging of Cellular Glutathione Using a Latent Rhodamine

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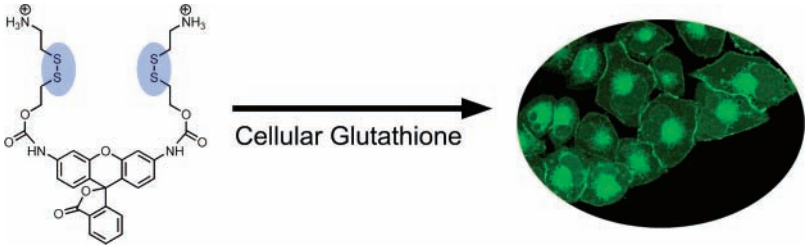
ABSTRACT



Glutathione is a crucial component of the redox homeostasis of cells, and altered levels have been linked to human pathologies. We constructed a latent fluorophore (RhoSS) that responds to cellular thiols in vitro and in cyto following intracellular reduction by glutathione to yield rhodamine 110. Importantly, RhoSS was demonstrated to respond to changing levels of glutathione in cells. This compound represents a class of rationally designed latent fluorophores with exciting potential for monitoring cellular thiols.

Fluorescent molecules that are responsive to the intracellular environment of cells have proven indispensable for the rapid and accurate detection of elements essential for homeostasis. However, only a small number of rationally designed fluorophores capable of detecting intracellular organic biomolecules exist.[1] A number of these molecules are particularly important to the oxidative state of cells, thus making them integral parts of both normal and aberrant cellular processes.

Due to the crucial role of reduced cysteines in the regulation, structure, and function of proteins, cells have evolved a complex and tightly regulated system to maintain cytoplasmic thiols in their reduced state. The most abundant cellular thiol is the cysteine containing tripeptide, glutathione (GSH), whose concentration ranges from 1 to 15 mM.[2] Deviations from optimum cellular ratios of reduced (GSH) and oxidized (GSSG) glutathione can lead to a number of human pathologies. Increased levels of GSSG have been associated with heart disease, cancer, stroke, and several neurological disorders.[3] Conversely, several forms of drug resistance have been correlated with elevated levels of GSH.[4] Therefore, fluorophores capable of detecting intracellular GSH would be useful tools in cell biology. Herein, we report the design and synthesis of a latent rhodamine that becomes highly fluorescent upon the reduction of its disulfides by cellular glutathione.

To date, a number of inventive detection methods for thiols and thiol-containing peptides have been described for in vitro studies.[5] Yet, examples of convenient fluorescent probes for the live imaging of cellular GSH have been scarce.[6] Miller and co-workers have recently described a novel xanthene-based fluorophore,[6a] while Hanson and co-workers[6b] elegantly designed a GFP-variant for the detection of GSH. However, most other available probes are based on nonspe-

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cific electrophiles that covalently modify cellular components in an irreversible manner (often generating GSH−thioether adducts) and thus have the tendency to be strongly oxidizing.[7] We believe that a disulfide-based probe would be more selective toward cellular thiols and would minimally perturb the total content of thiols/disulfides.

We were inspired by disulfide-linked bioconjugates which, after being reduced inside of cells, undergo a chemical transformation to generate the original unmodified drug.[8] Our design consists of a probe, **RhoSS**, with two strategically placed disulfide bonds (Figure 1a). The reduction of these

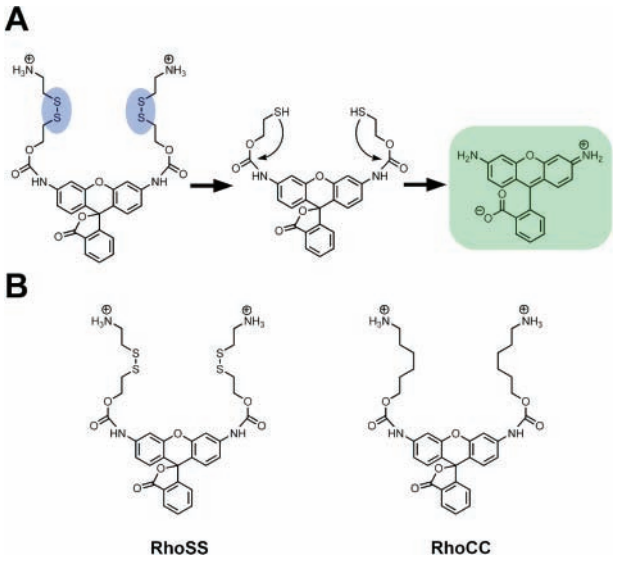
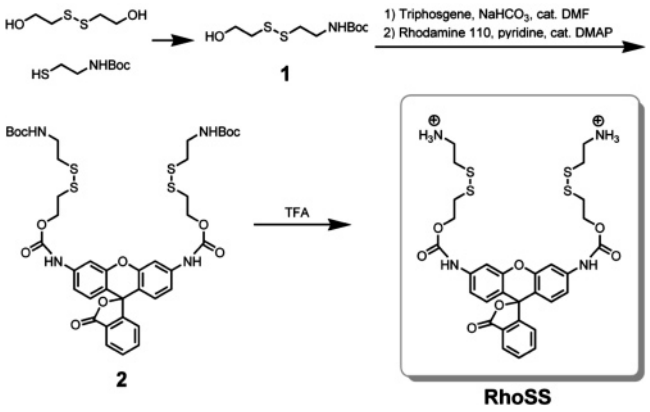


Figure 1. Structure of **RhoSS** and **RhoCC**. (a) Mechanism of unmasking for **RhoSS**. (b) Structures of **RhoSS** and control compound **RhoCC**.

bonds by thiols in solution or intracellular GSH would reveal nucleophilic sulfhydryl groups that would cause the breakdown of the neighboring carbamate bonds, thereby unmasking the rhodamine 110 (Rh110). The absence of the disulfide bonds in the control compound **RhoCC** should make it insensitive to reducing conditions and would allow us to monitor the cellular stability of the carbamate moiety (Figure 1b). Previous derivatives of caged Rh110 have been utilized for the detection of proteases,[9] cellular esterases,[10] DT diaphorase activity,[11] and allyloxycarbonyl (alloc) deprotection in cells.[12] Two important features of our caged rhodamines were the site of modification and the inclusion of amino groups in the added moieties for aqueous solubility. By modifying both aniline nitrogens on rhodamine, the carboxylate forms an internal lactone that should endow more efficient cell permeability due to the loss of the negative charge—a process that would be reversed upon uncaging within the cell.

Scheme 1. Synthesis of **RhoSS**



The synthesis of **RhoSS** began with a disulfide exchange between 2-(Boc-amino)ethanethiol and 2,2′-dithiolethanol (Scheme 1). The hydroxyl group on compound **1** was activated as a chloroformate, followed by treatment with Rh110 to afford compound **2**. The amino protecting groups on **2** were removed using trifluoroacetic acid to produce **RhoSS**. Additionally, the control compound **RhoCC** (Figure 1b) was synthesized and purified in a similar manner to **RhoSS** (see Supporting Information).

Initial in vitro experiments were performed to determine if **RhoSS** was responsive to reducing conditions. We demonstrated that **RhoSS** is stable in aqueous buffer because very low fluorescence was observed when the reducing agent was excluded from the solution (Figure 2, Figure 3a).

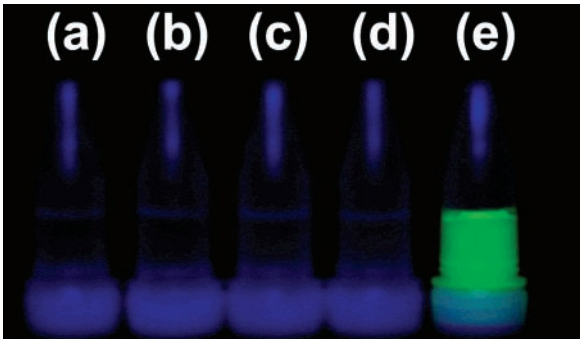


Figure 2. Image of fluorescence unmaking of Rh110 in vitro. (a) Buffer alone. (b) Buffer with 10 mM DTT. (c) Buffer with 40 μM of **RhoSS**. (d) Buffer with 40 μM of **RhoCC** and 10 mM DTT. (e) Buffer with 40 μM of **RhoSS** and 10 mM DTT. Tris-HCl Buffer 80 mM, pH 8.0, 37 °C, 1 h. Irradiated with long-wavelength UV lamp (366 nm).

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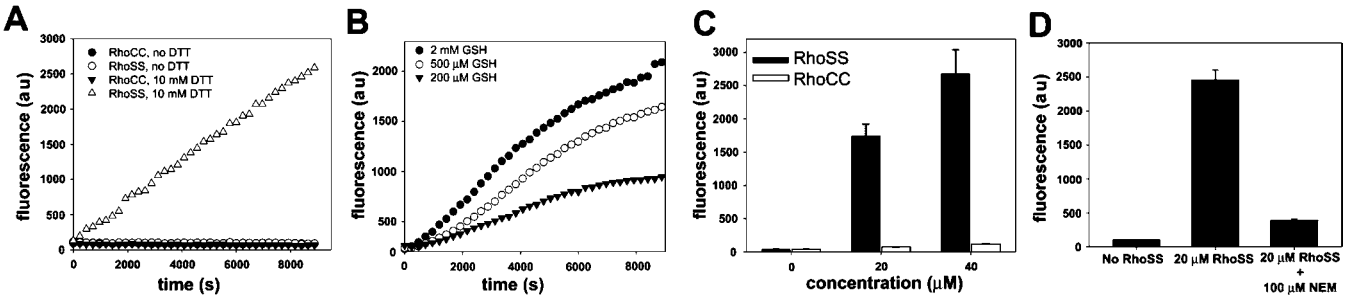

Figure 3. Fluorescence was monitored with 10 μM of **RhoSS** and 10 μM of **RhoCC** in the presence and absence of 10 mM of DTT. (b) **RhoSS** (10 μM) was incubated with varying concentrations of GSH at pH 7.4 at 37 °C. (c) Fluorescence in HeLa cells due to the incubation with **RhoSS** and **RhoCC** for 1 h at 37 °C. (d) Fluorescence in HeLa cells with **RhoSS** with and without a 30 min preincubation with the thiol-blocking agent NEM.

However, when **RhoSS** was co-incubated with DTT, fluorescence was observed to increase steadily over time. The conversion of **RhoSS** to Rh110 in this reaction was verified using HPLC (see Supporting Information). Because **RhoCC** did not display change in fluorescence in the presence of DTT (Figure 2, Figure 3a), it is apparent that the fluorescence obtained from **RhoSS** was a direct result of disulfide reduction. GSH was also found to induce the unmasking of Rh110 from **RhoSS**, and the fluoresence was found to be dependent on the concentration of the reducing agent (Figure 3b). Notably, there was a strong increase in fluorescence with physiologically relevant concentrations of GSH in solution.

We next monitored the ability of **RhoSS** to respond to cellular thiols, such as GSH, in a cell-based assay. HeLa cells were incubated with different concentrations of **RhoSS** and **RhoCC**, and cellular fluorescence was quantified using flow cytometry. A marked increase in fluorescence was observed when HeLa cells were treated with **RhoSS** that was both concentration (Figure 3c)- and time-dependent (see Supporting Information). Confocal microscopy of these cells treated with RhoSS (Figure 4b, 20 μM; and 5b, 40 μM) demonstrates strong cellular fluorescence that increases with increasing concentration. Importantly, confocal microscopy of cells with the latent fluorophore still in the media (Figure 4e), shows intense fluorescence within the cells and no fluorescence in the media, indicating that the unmasking of the fluorophore is intracellular. Minimal cellular fluorescence was observed when cells were incubated with **RhoCC** (Figures 3c and 4c), again indicating that the latent rhodamine itself shows little basal fluorescence. These data also demonstrate that the carbamate bond in the latent probes is not appreciably cleaved through an unanticipated cellular process. Moreover, **RhoSS** displayed a similar fluorescence profile when incubated with MCF-7 cells (see Supporting Information), thus demonstrating its utility in multiple cell lines.

It is possible to perturb the amount of cellular GSH by preincubating cells with the known thiol-blocking agent, *N*-ethylmaleimide (NEM).[8b] We incubated HeLa cells with NEM, followed by **RhoSS**, and measured cellular fluorescence (Figure 3d). The level of cellular fluorescence was dramatically lower after pretreatment with NEM, indicating that **RhoSS** is responsive to changing intracellular GSH levels. This same trend held with MCF-7 cells (see Supporting Information). Confocal microscopy was used to confirm these results: HeLa cells that were treated with **RhoSS** (Figure 4), **RhoCC**, or NEM, followed by **RhoSS**, demonstrated results that were consistent with those obtained using flow cytometry.

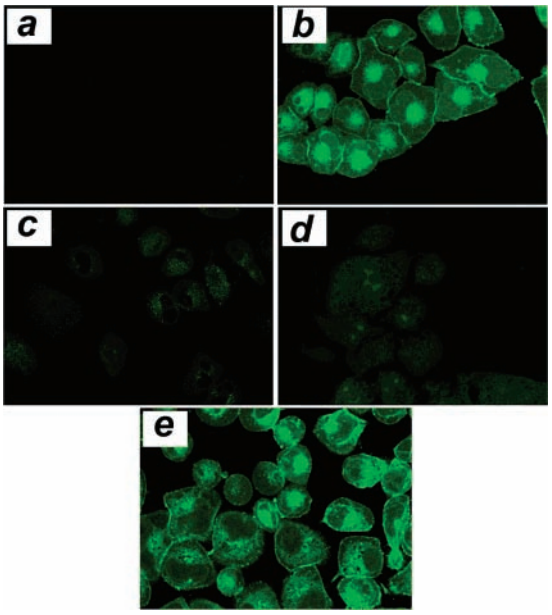

Figure 4. Confocal microscopy images of live HeLa cells. (a) No probe. (b) Treated with 20 μM of **RhoSS**. (c) Treated with 20 μM of **RhoCC**. (d) Preincubated with 100 μM of NEM and treated with 20 μM of **RhoSS**. (e) Unwashed cells treated with 40 μM of **RhoSS**. Transmittance images can be found in the Supporting Information.

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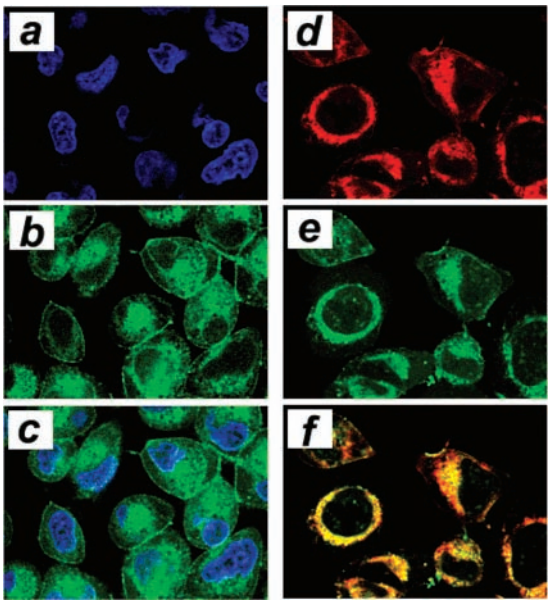

Figure 5. Confocal microscopy images of co-labeled live HeLa cells. HeLa cells were incubated for 4 h with 40 μM of **RhoSS** and 500 nM of Hoechst 33342 (a−c) or 100 nM of MitoFluor 589 (d−f). Images (a) and (d) display the Hoechst 33342 and MitoFluor 589 channels, images (b) and (e) the Rh110 channel, and images (c) and (f) display the overlay of the two sets of images.

We investigated the intracellular localization of Rh110 in HeLa cells treated with **RhoSS**, using Hoechst 33342 and MitoFluor 589 to label the nucleus and mitochondria, respectively (Figure 5). The fluorophore was generally not found to localize within the nucleus, but appears to be mainly within the mitochondria, as has been previously observed with the free fluorophore.[13] Although it is possible that the staining pattern observed here is due to the highly reducing nature of the mitochondria of cells,[14] it is also likely that the unmasked fluorophore freely redistributes itself to cellular compartments that have a propensity to accumulate Rh110.[13] Finally, we determined the cytotoxicity of RhoSS using an MTT-based assay and found that even at a concentration of 80 μM the cells were completely viable (see Supporting Information).

In conclusion, we have designed and synthesized a new disulfide-based fluorescent probe (**RhoSS**) that becomes fluorescent upon reduction by cellular thiols such as GSH in vitro and in cyto. We have shown that a variant of this probe (**RhoCC**) is not responsive to reducing conditions or the cellular environment. Furthermore, we have shown that when the intracellular levels of GSH are varied, the probe responds accordingly. Taken together, this latent fluorophore represents a new class of GSH-probes that offer exciting potential for the detection of GSH in cells.

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Supporting Information Available: Experimental details, characterization data, and additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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