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A highly sensitive, cell-membrane-permeable fluorescent probe for glutathione

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ABSTRACT

Glutathione (GSH) is a primary intracellular antioxidant. Here, we developed a novel, highly sensitive fluorescent probe for GSH, designated DNs-HMRG, whose fluorescence is regulated by two distinct switching mechanisms, intramolecular spirocyclization and photo-induced electron transfer (PeT). DNs-HMRG showed good cell permeability, and a rapid increase in fluorescence intensity was observed when it was applied to living cells. Further, taking advantage of the fact that the intracellular GSH level in tumor tissue is higher than that in normal tissue, we employed this probe for rapid (within a few tens of seconds) in vivo detection of tiny tumor nodules (less than 1 mm in diameter) in tumor-bearing mice. This probe is expected be a powerful tool in various biological applications, especially studies on redox status.

As a primary intracellular antioxidant, glutathione (GSH) plays an important role in controlling the redox state of living cells. Decrease in intracellular GSH caused by oxidative stress is related to many diseases,[1–3] while up-regulation of intracellular GSH has been reported in cancer cells, contributing to their resistance to radiation or chemotherapy.[4,5] Therefore, the real-time detection of intracellular GSH level in living cells is an issue of interest. For this purpose, various fluorescent probes for GSH have been developed so far by conjugating fluorophores with the strongly electron-withdrawing 2,4-dinitrobenzenesulfonyl (DNs) group.[6–11] The activation chemistry of these fluorogenic probes involves nucleophilic attack of thiol on the DNs group, regenerating the original fluorophore.[12] Among these probes, rhodamine green (RG)-based probes possess favorable characteristics for biological applications, such as high fluorescence quantum yield, high resistance to photo-bleaching, and high compatibility with the general microscope filter setup. However, RG-based probes normally have two reactive sites, and thus require two steps to obtain strongly fluorescent products, which results in a reduction in the speed and efficiency of fluorescence activation. Although a mono-substituted derivative has been reported, no cellular application has yet been described.[6–11]

We recently developed a new fluorescent scaffold, hydroxymethylrhodamine green (HMRG), which enables rapid and sensitive detection of target molecules, and retains the advantageous characteristics of RG. Further, HMRG-based probes tend to show higher cellular permeability than RG-based probes, owing to the hydrophobic nature of HMRG. Therefore, we aimed to develop a new fluorescent probe for biological thiols based on the HMRG scaffold. We synthesized DNs-HMRG by conjugating a DNs moiety to an amino group of HMRG (Scheme S1). We confirmed that DNs-HMRG showed pH-dependent absorption and emission profiles (Table 1 and Fig. S1), suggesting that DNs-HMRG has three possible forms depending on the pH value (Scheme S2). The calculated pK_a value of the sulfonamide group was 3.1, and the pK_{cycl} value (pH value at which the extent of spirocyclization is sufficient to reduce the absorbance of the compound to one-half of the maximum absorbance) was 7.0.

Therefore, at the physiological pH of 7.4, DNs-HMRG exists mainly in spirocyclic-closed form, which is colorless and non-fluorescent. Further, the fluorescence of the small amount of co-existing spirocyclic-open form is quenched by intramolecular photo-induced electron transfer (PeT), as the DNs group has a sufficiently low LUMO energy level. Thus, the two fluorescence quenching mechanisms, intramolecular spirocyclization and PeT, together efficiently quench the fluorescence of DNs-HMRG. However, upon reaction with GSH, the sulfonylamide bond is rapidly cleaved in one step to afford HMRG, which exists in highly fluorescent open form owing to its pK_{cycl} value of 8.1[13] (Fig. 1a, Scheme 1, Fig. S2). The increase in fluorescence intensity of 1 μM DNs-HMRG

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Table 1
Photochemical properties of DNs-HMRG

Absorption maximum (nm)	Emission maximum (nm)	Fluorescence quantum yield	Molar extinction coefficient	pK_{cycl}
510 [a]	532 [a]	0.002[a]	20000[a]	7.0

[a] Measured in 0.2 M sodium phosphate buffer, pH 5.0.

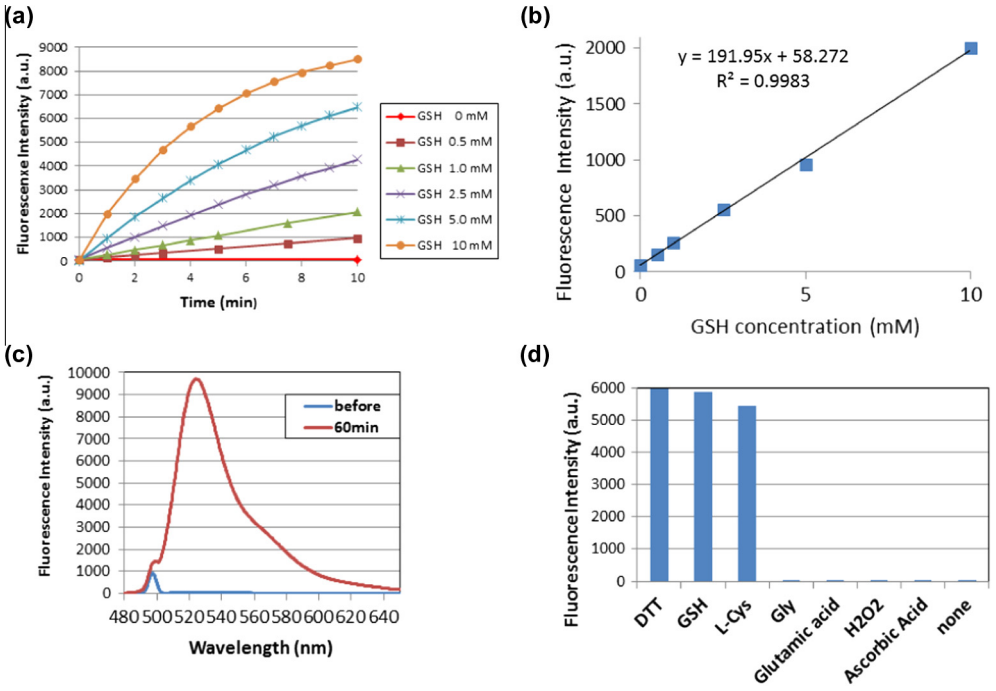
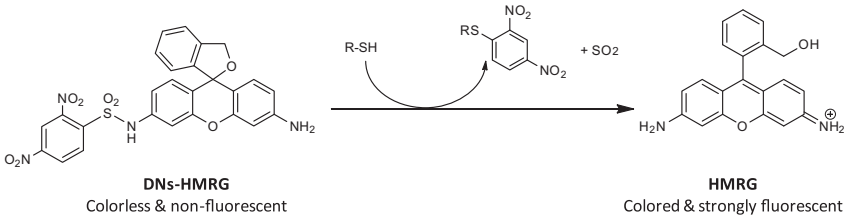


Figure 1. Fluorescence activation of DNs-HMRG with thiols. (a) Reaction-time profiles of 1 μM DNs-HMRG in the presence of various concentrations of GSH. A dose-dependent increase of fluorescence intensity was observed. (b) Plot of fluorescence intensity of 1 μM DNs-HMRG after 1 min reaction with GSH against GSH concentration. The observed fluorescence intensity was proportional to the added GSH concentration around the physiological concentration range. (c) Fluorescence intensity of DNs-HMRG (1 μM) was increased more than 7000-fold after reaction with 10 mM GSH for 60 min. Measured in 0.2 M phosphate buffer (pH 7.4) Ex: 495 nm. (d) Response of DNs-HMRG to various substrates. DNs-HMRG (1 μM) was incubated with 10 mM analyte for 60 min. Recorded in 0.2 M phosphate buffer (pH 7.4) Ex: 495 nm/Em: 520 nm.



Scheme 1. Mechanism of DNs-HMRG activation by thiols.

after reaction with 10 mM GSH for 60 min is up to 7000-fold, which, to our knowledge, is one of the largest activation ratios so far achieved. Further, the fluorescence increase is proportional to the added GSH concentration over the range around the physiological concentration (Fig. 1b). The selectivity of the fluorescence activation for thiol was confirmed by treating the probe with various biologically relevant analytes and measuring the fluorescence signals at 520 nm (Fig. 1c and d).

In order to evaluate whether DNs-HMRG has sufficient cellular permeability and can be activated by intracellular biothiols in living cells, we applied DNs-HMRG to cultured SHIN3 cells (derived from human ovarian cancer) and the fluorescence increase was monitored under a confocal microscope. We observed a rapid fluorescence increase in the cells within a few minutes after addition of the probe to the medium. Intracellular localization of the fluorescence signal was in accordance with our previous finding that the fluorescent product, HMRG, tends to accumulate in lysosomes.[14] To evaluate whether this rapid activation of DNs-HMRG in living cells reflects the intracellular GSH level, N-ethylmaleimide (NEM: a thiol-blocking reagent) was added before application of DNs-HMRG. The fluorescence increase was decreased in a dose-dependent manner by the pretreatment with NEM (Fig. 2). These results clearly indicate that DNs-HMRG is cell-permeable and is efficiently activated by biothiols in living cells. Considering that GSH is most abundant component of intracellular biothiols, it is reasonable to conclude that activation of DNs-HMRG in living cells is mainly caused by intracellular GSH.

Next, we applied DNs-HMRG to two cell lines having different GSH levels: SHIN3 (ovarian cancer cells; GSH-high) and HUVEC (normal epithelial cells; GSH-low). There was a clear difference