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Fluorescein propiolate: a propiolate-decorated fluorescent probe with remarkable selectivity towards cysteine†

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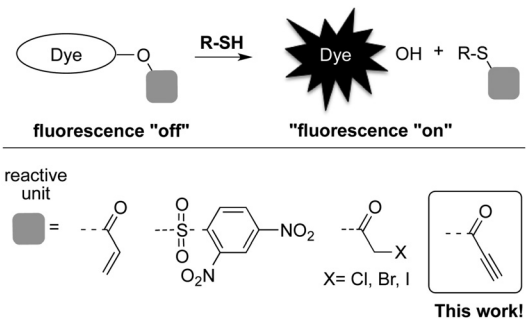
A fluorescent probe decorated with an alkynyl ester unit (*e.g.* propiolate) displayed a selective turn-on type fluorescent response towards cysteine. Following a sequential addition–cyclisation pathway mediated by the addition of cysteine, the pre-fluorescent dye rapidly transformed into a new structure and induced a fluorescent response clearly observable with the naked eye.

Scientists in diverse fields have long sought to understand the roles of intracellular species in biological processes. As members of intracellular species, biothiols such as cysteine (Cys), homocysteine (Hcy) and glutathione (GSH) play essential roles in the functioning of living organisms, including in physiological events ranging from protein synthesis and tissue growth to cellular redox homeostasis and metabolism.[1] At the same time, aberrant changes in biothiol levels are associated with various diseases, including cardiovascular disease, Alzheimer's disease and cancer.[2]

Visualising the production and distribution of biothiols in living systems is therefore vital to investigating cell function and the early diagnosis of some thiol-associated diseases. Research to those ends continues to focus on the development of analytical tools for probing biothiols, among which fluorescent-based assays have attracted particular attention.[3] Researchers have developed numerous types of thiol-responsive fluorescent probes that mobilise various detection strategies, most of which exploit the reactive nature of sulfhydryl (–SH) and operate *via* specific chemical reactions such as Michael addition,[4] cyclization reaction with aldehydes,[5] cleavage of sulfonate esters,[6] nucleophilic substitution,[7] addition to haloacetates[8] and others.[9] In general, designing thiol-specific probes relies on masking a fluorescent dye with a reactive unit, which upon interacting with –SH splits from the dye to deliver the emissive form of the fluorophore (Scheme 1). The analytical performance of such probes depends heavily on the specificity of the recognition unit employed in the probe skeleton. Among the widely used recognition units, acrylates are currently the most popular due to their affinities to thiols, especially cysteine.[4a,10]



Scheme 1 Selected thiol-reactive masking groups.

Despite notable achievements in developing fluorescent probes for sensing thiols, differentiating biothiols with similar chemical structures remains a significant challenge. Given the demand for easily accessible recognition motifs with improved analyte selectivity, sensitivity, biocompatibility and response times, however, we have sought new thiol-specific reactive groups that can be easily integrated into the skeletons of fluorophores.

In this paper, we present the design, synthesis and spectral investigation of a novel probe that exploits a unique reaction scheme for recognising cysteine with remarkable selectivity compared to other species. In particular, we devised **FL-PRP**, a prefluorescent dye with a fluorescein-based fluorophore as a signal reporter and an alkynyl ester (*e.g.* propiolate) as the recognition scaffold, which marks a first in research on cysteine sensing. Similar to an acrylate, an alkynyl ester can function as a Michael acceptor that readily reacts with thiols to yield thioacrylates.[11] We hypothesised that amino acids with a dual reactive character—for example, cysteine—would interact with the probe's reactive site to mediate a sequential addition–cyclisation process that could induce the formation of the emissive form of the probe (Scheme 2b).

We prepared the probe (**FL-PRP**) according to the synthetic route outlined in Scheme 3. First, we integrated propiolate into

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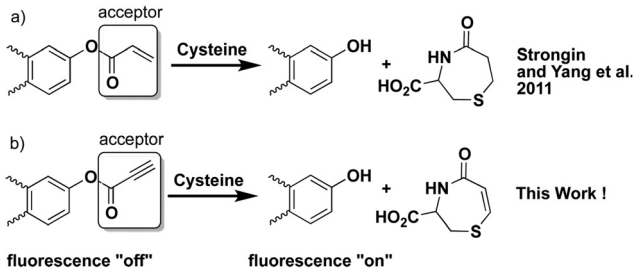
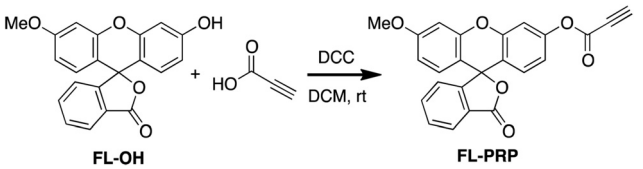
† Electronic supplementary information (ESI) available: Characterization of **FL-PRP** and all data for UV-vis and fluorescence measurements. See DOI: 10.1039/c9cc01774g



Scheme 2 Cysteine addition/cyclization reaction sequence for acrylate and propiolate-based fluorophores.



Scheme 3 Synthesis of **FL-PRP**.

the skeleton of fluorescein by setting the individually prepared fluorescein derivative (**FL-OH**) in reaction with propiolic acid in the presence of dicyclohexylcarbodiimide (DCC). The reaction proceeded smoothly (1 h, 45% yield) and afforded the title compound in a reasonable yield. Following chromatographic purification, we confirmed the unambiguous identity of **FL-PRP** by using NMR spectroscopy and mass spectrometry.

We investigated the spectroscopic response of **FL-PRP** to a range of biothiols, including cysteine, homocysteine and glutathione, as well as other reactive species, by using ultraviolet-visible and fluorescence spectroscopy. As expected, in the absence of an analyte, the probe solution—10 μM **FL-PRP**, CH_3CN : PBS buffer (10 mM, pH 7.4, 6 : 4 v/v)—exhibited no features of absorption or emission in the visible region, indicating that the ring-closed probe structure was preserved. Upon adding cysteine (10 equiv., 100 μM), a new emission band appeared at 515 nm (Fig. 1), and its intensity became saturated within 30 min (Fig. S4, ESI†).

With the systematic addition of cysteine, the emission band at 515 nm increased linearly over a wide range of concentrations,

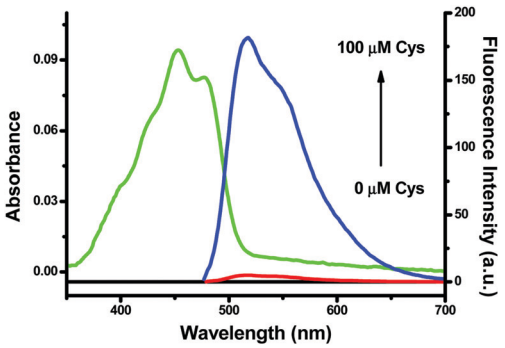


Fig. 1 Absorbance spectra of **FL-PRP** (10 μM) in the absence (black line) and presence (green line) of 10 equiv. of Cys and fluorescence spectra of **FL-PRP** (10 μM) in the absence (red line) and presence (blue line) of 10 equiv. of Cys in CH_3CN : PBS buffer (10 mM, pH 7.4, 6 : 4 v/v).

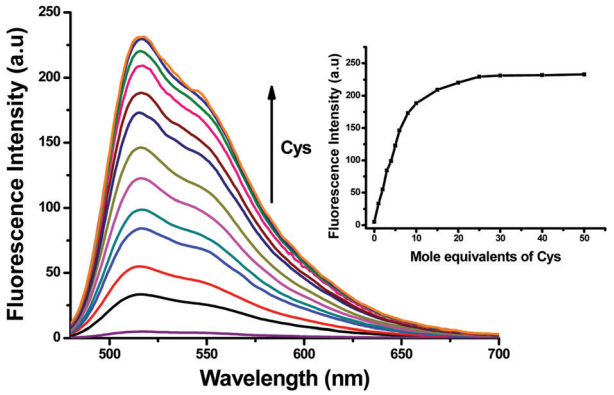


Fig. 2 Fluorescence spectra of **FL-PRP** (10 μM) in CH_3CN : PBS buffer (6 : 4 v/v) at pH 7.4 (λ_{ex} = 460 nm) in the presence of Cys (mole equivalents 1–50). Inset: Plot of fluorescence intensity depending on the number of equivalents of Cys.

and the band intensity stabilised with the addition of 30 equiv. of cysteine, with an enhancement factor of more than 35-fold (Fig. 2). In light of analytical data collected from fluorescence analysis, we estimated the limit of detection to be 182 nM, given that S/N = 3 (see the ESI† for more details).

Meanwhile, the probe solution became distinctly green, which we attributed to a structural modification (*e.g.* ring opening) of the probe structure. As measured in a time course study performed with different biothiols, the response of **FL-PRP** towards cysteine was distinctly faster and enhanced greater fluorescence emission than towards other biothiols (*e.g.* homocysteine) (Fig. 3a). The observed rate constants (k_{obs}) at pH 7.4 were 2.35×10^{-3} and 4.6×10^{-4} s^{-1} for cysteine and homocysteine, respectively (Fig. S5, ESI†).

The selectivity test for **FL-PRP** with other amino acid derivatives, including alanine, arginine, aspartic acid, glycine, glutamine, lysine, methionine and proline, resulted in no obvious spectral changes, which indicated **FL-PRP**'s clear selectivity towards cysteine. Fig. 3 shows the spectral changes that **FL-PRP** underwent with the addition of various analytes.

To assess possible interference from other species, we also examined the fluorescence response of **FL-PRP** to cysteine in the presence of other reactive species. Remarkably, the spectral response induced by cysteine remained almost unaffected in the presence of each of those species. Such results indicate that **FL-PRP** could properly detect cysteine in mixtures with other related species (Fig. S8, ESI†). To rule out any discrepancies arising from acidity changes, we investigated the effect of pH fluctuations on the fluorescence behavior of the sensing system, and found that the response of **FL-PRP** to the addition of cysteine remained unaffected by changing the pH of the sensing medium. **FL-PRP** thus seemed to operate efficiently over a wide range of pH (pH 2–12), especially at physiological pH (*e.g.* pH 7.4), which fulfils a basic requirement for cell-imaging applications (Fig. S9, ESI†).

Adding cysteine to the probe solution instantaneously induced a change in colour and fluorescence, which was easily detectable by the naked eye under light and UV irradiation.

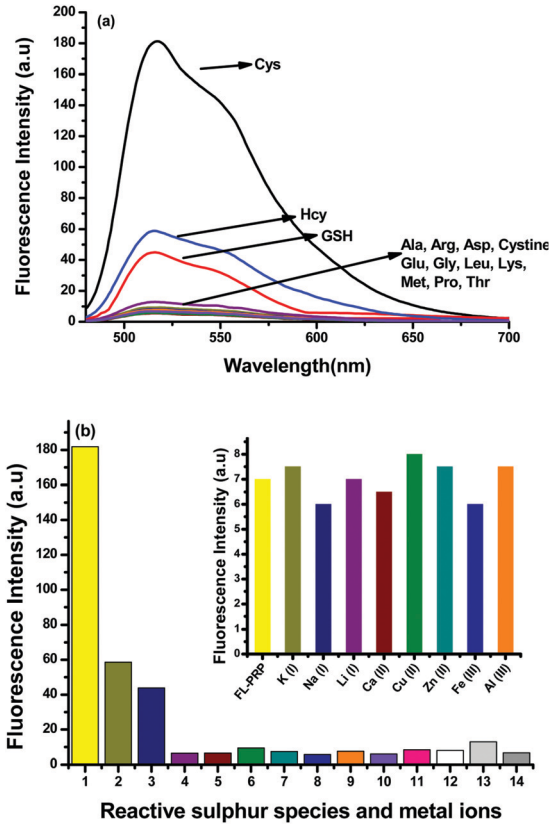
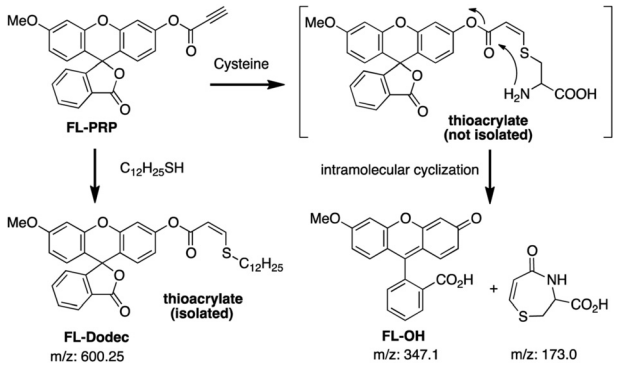


Fig. 3 Fluorescence intensities of **FL-PRP** (10 μM) in CH_3CN : PBS buffer (10 mM, pH 7.4, 6 : 4 v/v) in the presence of reactive sulphur species and other amino acids (10 equiv.), (λ_{ex} = 460 nm). 1, Cys; 2, Hcy; 3, GSH; 4, Ala; 5, Arg; 6, Asp; 7, cystine; 8, Glu; 9, Gly; 10, Leu; 11, Lys; 12, Met; 13, Pro; 14, H_2S (source: Na_2S). Inset: Fluorescence intensities of **FL-PRP** (10 μM) in the presence of metal ions (10 equiv.).



Scheme 4 Proposed mechanism for the detection of cysteine.

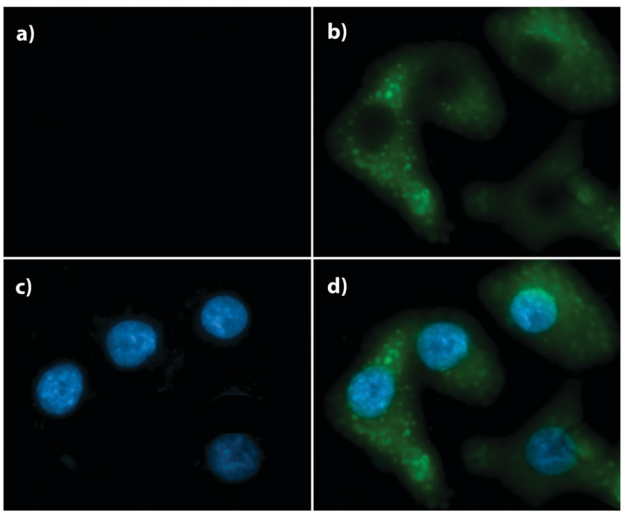


Fig. 4 Images of A-549 cells. (a) Cells pre-treated with 2 mM NMM for 30 min and then incubated with **FL-PRP** (5 μM) in the absence of Cys. (b) The fluorescence image of cells pre-treated with 2 mM NMM then incubated with **FL-PRP** (5 μM) with Cys (50 μM). (c) Cells are detected with DAPI nuclear counter-stain (control). (d) Merged images of (b) and (c).

A new green emissive compound, as monitored on a thin-layer chromatography plate, was additional clear evidence of the formation of an emissive fluorescein derivative (Fig. S10, ESI†). Given such experimental results, the recognition of cysteine likely proceeded by way of a sequential process initiated with the addition of the –SH unit of cysteine to form an adduct—namely, a thioacrylate—which subsequently cyclised over the primary amine to promote the cleavage of the ester and caused the formation of hydroxyl fluorescein along with a seven-membered lactam derivative (Scheme 4). We confirmed the unambiguous chemical identity of both products by using mass analysis (Page S13 in the ESI†).[12]

Control experiments performed with thiols lacking any primary amine functionality (*e.g.* *N*-acetyl cysteine and dodecanethiol) did not yield the emissive product, yet gave a thioacrylate (*e.g.* FL-Dodec) as the final product, which underscores the need for a primary amino group to mediate intramolecular cyclisation (Scheme 4). We suggest that reaction kinetics for intramolecular cyclisation thus determines analyte selectivity and that the formation of a seven-membered lactam thus occurs at a far greater rate than that of an eight-membered lactam, which accounts for the probe's selectivity towards cysteine.

The outstanding analytical performance of **FL-PRP** in solution encouraged us to further assess its feasibility to detect cysteine in living cells. First, the potential cytotoxicity of **FL-PRP** was evaluated using an MTT assay with human lung adenocarcinoma (A549) cells. The cellular viability was estimated to be 95% after 24 h, which showed that the probe (1–10 μM) has no cytotoxicity. We treated A549 cells with 2 mM of *N*-methylmaleimide (NMM), a thiol scavenger, to eliminate physiological thiol species from the cells. We next incubated the cells with **FL-PRP** (5 μM) for 20 min, after which we stained them with a nucleus staining dye, 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI) (1 mM), for another 20 min, and captured fluorescence images before and after the addition of cysteine (50 μM). As Fig. 4 shows, the cells incubated with **FL-PRP** did not display any fluorescence in the absence of cysteine yet began to emit strongly after their incubation with cysteine. The results of such preliminary cell imaging suggest that the probe can permeate cell membranes and be efficiently used to image cysteine in living cells.

In summary, we have reported the synthesis, spectral properties, and imaging application of **FL-PRP**, a new type of fluorescent probe, which showed a cysteine-specific turn-on fluorescence response.

We constructed the fluorescent probe on a fluorescein dye with an alkynyl ester (*e.g.* propiolate) as the recognition motif, which marks a first for cysteine sensing. In an addition–cyclisation reaction sequence mediated by cysteine, the non-emissive probe structure rapidly transformed into a new structure, which allowed the recognition of cysteine as an increase in emission intensity. Apart from the sensitive (182 nM), specific response in solution, the probe proved highly successful in imaging cysteine in living cells. Compared to acrylate-based probe systems reported earlier, the analytical performance of our system is highly competitive in terms of probe sensitivity, analyte selectivity and response time.[4,10b] We thus believe that integrating propiolates with other fluorescent tags can aid the efficient development of new probes with high specificities towards cysteine.

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Conflicts of interest

There are no conflicts to declare.

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12 See the ESI†.

