Organic LETTERS

Letter

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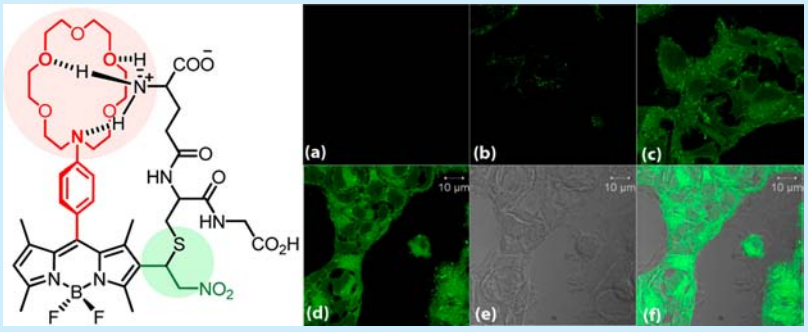
Designing an Intracellular Fluorescent Probe for Glutathione: Two Modulation Sites for Selective Signal Transduction

Murat Işık,[†,‡] Ruslan Guliyev,[§] Safacan Kolemen,[†] Yigit Altay,[§] Berna Senturk,[†] Turgay Tekinay,[†] and Engin U. Akkaya*[,†,§]

[†]UNAM-National Nanotechnology Research Centerand [§]Department of Chemistry, Bilkent University, Ankara 06800, Turkey

[‡]Department of Metallurgical and Materials Engineering, Bingol University, Bingol, 12400, Turkey

Supporting Information



ABSTRACT: A selective probe for glutathione was designed and synthesized. The design incorporates spatial and photophysical constraints for the maximal emission signal. Thus, pHs, as well as the intracellular thiol concentrations, determine the emission signal intensity through a tight control of charge-transfer and PeT processes. The probe works satisfactorily inside the human breast adenocarcinoma cells, highlighting GSH distribution in the cytosol.

Chemosensor development has evolved into an attractive field of study, with a large number of promising examples emerging at a steady pace with worldwide participation in this endeavor.[1] Most successful probes or chemosensors emerge through modulation of photophysical processes operational for the selected chromophore. The modulation can be the result of a reversible association (e.g., ligand−metal ion) or a chemical conversion of the probe in the presence of a targeted analyte. Rational design of selectivity, on the other hand, would benefit tremendously by the judicious incorporation of multiple structural and electronic handles on the signal transduction process.

Biological thiols such as cysteine (Cys), homocysteine (Hcy), and glutathione (GSH) are known to be important parameters in health and disease states.[2] High cysteine concentration is clearly associated with myocardial and cerebral infarctions,[3] whereas elevated plasma Hcy level is an indicator of arterial and venous thrombosis.[4] GSH, on the other hand, is the major endogenous antioxidant with a number of biological roles.[5] Consequently, a great deal of recent effort has been placed on developing sensors and probes for each and every one of these three species. The substantial progress made in the field has been reviewed recently by Yoon and co-workers.[6] It is an interesting side note that a probe selective for GSH remained elusive until recently.[7] Commercially available probes have acknowledged deficiencies such as the need for UV excitation (*o*-phthaldialdehyde, naphthalene-2,3-dicarboxaldehyde, ThiolTracker Violet), high background signal, nuclear accumulation of the adduct (monochlorobimane), and nonselective reactions in all cases.[8]

In our work focusing on advancing new strategies and/or tactics in sensing and signaling,[9] we found Bodipy dyes to be particularly useful and amenable to modification in accordance with any design requirements.[10] Their impressive spectroscopic properties (quantum yield, extinction coefficient, tunability) coupled with the ease of modulation of these properties through, among other photophysical processes, photoinduced electron transfer[9b,11] (PeT) and internal charge transfer[9b,12] (ICT), made them a favorite among similar fluorophores. In designing a selective GSH sensor, we also noted that sensing of thiols is mostly based on their strong nucleophilic character, apparent even in aqueous solutions.[13] Conjugate addition reactions, altering the spectroscopic properties of the probe is a very common theme encountered in a large number of molecular sensor designs.[14] Our goal was to target GSH specifically, and we thought that could be achieved by incorporating an additional recognition site for the N-terminal ammonium group found in GSH. The structure of our target molecule is shown in Figure 1. We also thought the emission signal of the probe could be further modulated by the protonation state of the azacrown amine moiety which would alter the rate of PeT. The quenching in *meso*-dialkylaminophenyl substituted Bodipys is most likely a

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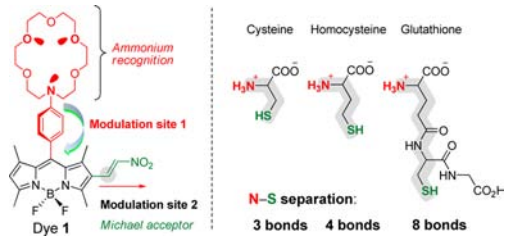

Figure 1. Structure and the signal modulation sites of the target probe. The distance between the terminal amine and the thiol suggests a much better match for GSH and the probe than the other two biological thiols.

combination of ICT and PeT processes, the former being more apparent in less polar organic solvents (Supporting Information, Figures S31–S34). For use in polar or aqueous solvents, rationally designed probes based on PeT modulation in Bodipy dyes abound, and the Nagano group has reported many with significant potential.[11e,15]

The target molecule was synthesized in a few straightforward steps (Figure 2) starting from the corresponding aza-crown

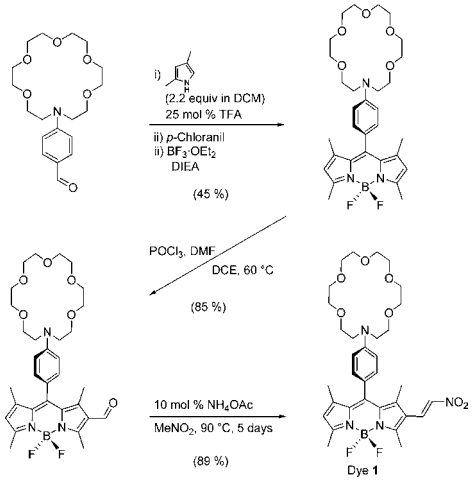

Figure 2. Synthetic route for the synthesis of GSH probe dye **1**.

substituted benzaldehyde. Formylation[16] of the 2-position of the Bodipy core, followed by nitromethane condensation, yielded the desired product. It is very well-known that electron-donating and -withdrawing substituents alter both the ground-state and excited-state properties and result in larger changes in dipole moment on excitation. Thus, dye **1** shows a red-shifted absorbance with maximum at 518 nm (Supporting Information, Figure S20) and it is essentially nonfluorescent ($\phi_{fl} < 0.01$). A control reaction with mercaptoethanol causes an enhancement of the emission intensity with a concomitant blue-shift (−20 nm) in the absorption spectrum. Biological thiols (Cys, Hcy, and GSH) also react similarly. Selected photophysical data are presented in Table S2 and in Figure S22 in the Supporting Information.

Thus, we felt confident that the first criterion for the putative GSH sensor, namely reactivity toward thiols and thus transforming the probe so that the typical green emission of an unaltered Bodipy core could be enhanced, was satisfied. This is a result of diminished conjugation and charge withdrawal, as initially the nitroethenyl substituent is in conjugation with the Bodipy core, and on reaction with a thiol, the nitro group becomes isolated from the Bodipy π-system due to the formation

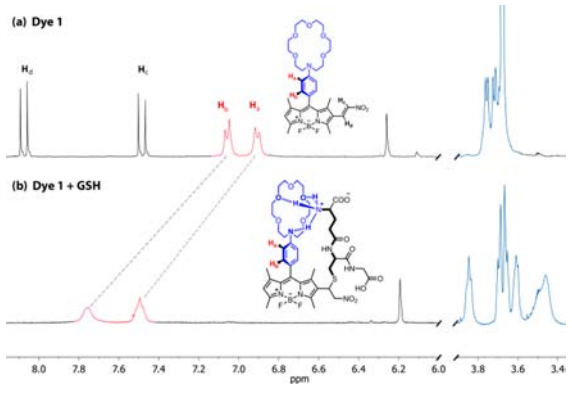

Figure 3. Partial ^{1}H NMR (in CD_3OD, 298 K) spectra depicting the changes on GSH conjugate addition to the dye **1**: The adduct was isolated by preparative HPLC following a room temperature reaction of the probe and GSH in aqueous acetonitrile. *Trans*-coupled protons disappear in the product, and the azacrown peaks show a more spread out cluster of peaks, suggesting an emergence of noncovalent, nonsymmetric interaction.

of the thioether adduct. ^{1}H NMR data (Figure 3) strongly corroborate with the emission intensity changes, providing clear evidence for the conjugate addition.

It is also evident that if there is any difference between the biological thiols in terms of the intensity of the emission signal it should be most likely due to the relative rate of the reactions, perhaps in competition with any side reactions. Thus, unless there are other built-in structural selection criteria, cysteine and homocysteine should react faster than the larger/bulkier nucleophile GSH, and for shorter reaction (incubation) periods they should generate larger emission responses.

Next, we tested the response of the probe to three biological thiols in aqueous solutions. We carried out the first set of experiments in pH 7.4 buffered aqueous solutions. When the reactions were complete, we observed a turn on of fluorescence emission (Figure 4, right), together with a blue shift, just as it was

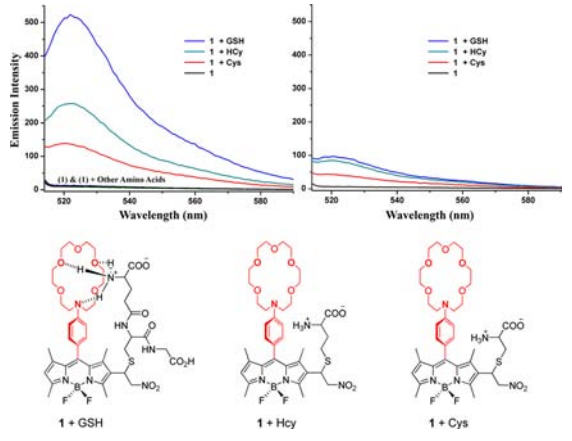

Figure 4. Emission response to biological thiols at two different pH values, 7.4 (right, 60:40, 30 mM MES buffer/acetonitrile) and 6.0 (left, 60:40, 30 mM MOPS buffer/acetonitrile). A small change in pH causes more than 5-fold increases in emission intensity for the GSH-1 adduct. For Cys and Hcy, the change is approximately 2.5-fold. The concentrations of the thiols and other amino acids (Gly, His, Ile, Leu, Met, Phe, Pro, Ser, Thr, Tyr, Val) were 1.6 mM and the dye 1 concentration was 8.0 μM. Excitation was at 500 nm, with 5 nm slit-widths.

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in the case of simpler thiol, mercaptoethanol. However, when the reactions were repeated in slightly acidic solutions mimicking the typical pH values for tumor tissues (pH 6.0), in accordance with our design, the results showed a very clear-cut preference for GSH for the strongest emission signal (Figure 4, left). Selectivity at pH 6.0 was further established in a series of measurements including the number of amino acids and the three biological thiols (Figure 5). Nonthiol amino acids showed no response, and

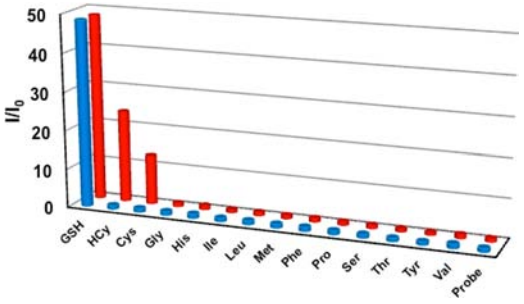

Figure 5. Selective emission response of the dye **1**. Blue bars correspond to the emission enhancement ratios (I_0 being the emission intensity of the probe) when biological thiols are introduced at their respective intracellular concentrations[17−19] in aqueous medium (60:40, pH 6.0, 30 mM MOPS buffer/acetonitrile) with 8.0 μM dye **1** concentration. Red bars show the emission enhancement when all analytes are introduced at 1.6 mM, in the same solvent system and probe concentration. Excitation was at 500 nm, and emission data at 522 nm (I and I_0) were used in calculations.

selectivity at actual intracellular concentrations of three biological thiols was spectacular. Two conclusions can be drawn from these results, (1) the steric fit of the protonated ammonium group at the N-terminal is optimal in GSH, and (2) since the other two thiols, Cys and Hcy, are shorter they cannot provide an ammonium group reaching the *N*-phenylazacrown receptor as the second recognition site (Figure 1) once the thiol adduct is formed. The second more important result is the PeT modulation: the *N*-phenylazacrown is a strong PeT donor. Its protonation would stop or slow down PeT leading to strong enhancement of the emission (off-on type). However, our expectation based on the pK_a data of the aromatic amines was such that the aromatic amine moiety should not be protonated to a significant extent at even pH 6.0 (Figure 6, left), so no clear enhancement should be seen in the case of Cys or Hcy.

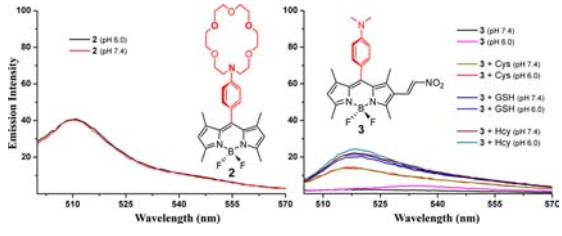

Figure 6. Structures of control Bodipy dyes 2 and 3. Dye 2 has a binding site for ammonium moiety, but no reactive group for thiols. (Left) Dye 3 is thiol reactive, but it does not have a site for ammonium recognition. (Right) Dye concentrations were 8.0 μM, and the biological thiols were introduced at 1.6 mM. Aqueous buffer solutions were used as solvents for pH 7.4 (60:40, 30 mM MES buffer/acetonitrile) and for pH 6.0 (60:40, 30 mM MOPS buffer/acetonitrile). Excitation was at 500 nm, with 5 nm slit-widths.

In addition, the protonation state would not be in any way linked to the thiol reaction. In GSH, however, N-terminal ammonium is in the right place for an effective ion−dipole and H-bonding interactions, which would change in the pK_a of the azacrown amine, and thus it will be protonated to larger extent at pH 6.0

We did not want to leave this issue as simple conjecture and synthesized two control compounds to check this experimentally. Dye **2** is a simple phenylazacrown substituted Bodipy and dye **3** has nitroethenyl Michael acceptor and an amine function (Supporting Information). These two compounds are to serve as negative controls of our design. To our delight, control dye **2** showed essentially no response to either moderate change in pH (7.4 to 6.0) or to the thiols (Figure 6, left). Control dye **3** also, as expected, showed only a small enhancement in emission on reaction with thiols, with small discrimination in terms of signal intensity, but PeT from the dimethylaminophenyl substitituent showed no signs of change within the pH range mentioned (Figure 6, right).

The control experiments prove that the designed dye **1** has a strong steric differentiation between the GSH and the other two biologically relevant thiols, more so at pH 6.0. pH 6.0 versus pH 7.4 responses are important as pH difference in this range are one of the characteristics separating tumors from healthy tissues. GSH is known to be present at highly elevated concentrations in tumor cells compared to healthy tissues.[17]

Finally, we wanted to demonstrate the feasibility of the designed probe for GSH imaging in intracellular medium using cell cultures. Figure 7 shows time-lapse images of dye **1** incubated cells. Within 2 h, strong green emission of the GSH adduct is clearly visible in the cytosol of the cells. Considering intracellular concentrations of all biological thiols, the green emission is clearly resulting from the reaction with intracellular GSH. As a control to eliminate the possibility of the contribution of nonspecific reactions (with other biological thiols, including proteins) to the generated emission signal, we performed an inhibition experiment (Figure 7). Buthionine sulfoximine (BSO) is a known selective inhibitor[20] of γ-glutamylcysteine synthetase (γ-GCS). HUVEC cells pretreated with the inhibitor showed

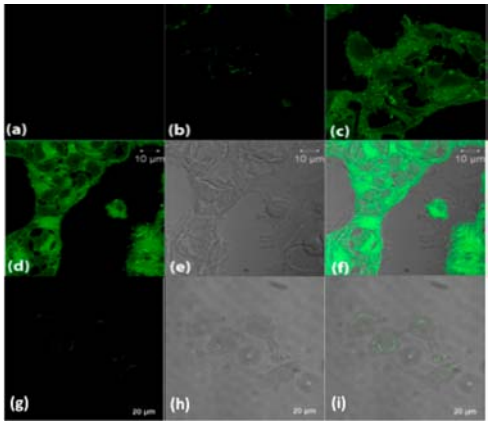

Figure 7. Time-lapse confocal microscopy pictures of human breast adenocarcinoma cells (MCF-7) cells incubated with dye **1** at 0.5 μM ((a) 0 min, (b) 1 min, (c) 120 min); (d, e) 120 min, fluorescence, optical images and (f) merged image. Human umbilical vein endothelial cells (HUVEC) cells pretreated with 5 mM BSO (g), DIC image (h), merge (i). The selective inhibition of GSH synthesis reduces fluorescence emission from the cells to a very low level, attesting the selectivity of the designed probe.

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very low residual emission compared to untreated cells on staining with dye **1**.

In conclusion, we have shown that selectivity for reaction-based probes can be improved by applying additional photophysical constraints. In this work, this was made possible by the simultaneous modulation at two different sites, as the adduct was generated. While the probe discussed here required a cosolvent (acetonitrile) for better solubility, the idea described in this work is perfectly transferable to other fluorophores and even to other reactions to be used in sensory systems. Work along these lines toward other rationally designed selective probes is in progress.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic procedures, ^{1}H, ^{13}C NMR, mass spectra, photophysical characterization/control experiments, details of cell culture studies, fluorescence kinetic experiments and pH titration studies. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: eua@fen.bilkent.edu.tr.

Notes

The authors declare no competing financial interest.

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