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Bio-specific and bio-orthogonal chemistries to switch-off the quencher of a FRET-based fluorescent probe: application to living-cell biothiol imaging.

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Abstract: We report the first molecular system that is responsive to both a bio-specific and a bio-orthogonal stimulus. This dual activation process was applied for the design of a biothiol-specific FRET-based fluorescent probe that could be turned-on *via* an original concept of quencher bleaching.

The field of chemistry in living organisms has long been focused on fine tuning of structural motifs' reactivity in order to achieve chemo-selective reaction with biological substrates such as an endogenous reactive metabolite, an amino acid, a DNA sequence, a ROS or acidic pH. This type of biospecific chemistry led to important developments in various domains such as imaging probes,[1] pathology specific prod-drugs[2] or advanced biomaterials.[3] The idea of using exogenous reactants to trigger a chemical act in a living organism (bio-orthogonal reaction) appeared more recently and has nevertheless gained much interest. Among landmark works, Meggers[4] and later Bradley[5] showed that organometallic catalysts were able to mediate intracellular bond breaking and bond forming processes. Likewise, Bertozzi,[6] Weissleder,[7] and others pioneered the *in vivo* use of bio-orthogonal Cu-free click reactions.

An attractive goal to extend the scope of such bio-functional chemistries would now be to combine into a single molecular system both the capabilities to react with an endogenous metabolite (bio-specific reaction) and an exogenous reagent (bio-orthogonal reaction). Such endeavour relies on chemist's ability to tweak the reactivity of functional groups and reagents in order to make them functioning under the particular constrains of complex biological media. For instance, such molecular system should demonstrate water solubility, water stability, fast reaction kinetics at low concentration, bio-specificity and low toxicity. These stringent operating constrains as well as the precise bio-selectivity window required for such dual mode of activation make their development an attractive challenge for organic chemists.

In this paper, we report our study on *N*-(dicyanomethylene)aniline oxide that we found to be bio-specifically reactive with endogenous biothiols and bio-orthogonally reactive with sodium dithionite, a bio-compatible exogenous reagent.[8] We also found that reaction with any of the above stimuli resulted in a strong hypsochromic shift of *N*-(dicyanomethylene)aniline absorption. Based on this combination of chemo and bio-functionalities, we report a novel concept of FRET probe which can be activated by either a bio-specific or a bio-orthogonal bleaching of its quencher. (Fig. 1)

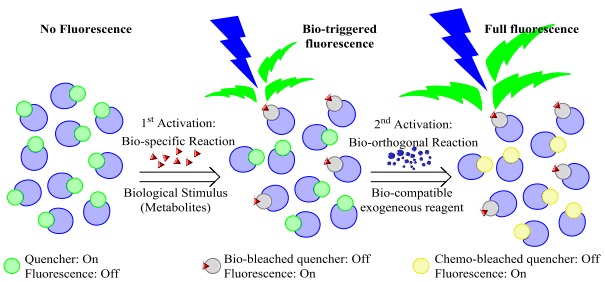

Fig. 1 Principle of FRET-based probe with a biologically and chemically bleachable quencher. Metabolite-specific reaction shifts the quencher's absorption towards lower wavelengths and thus switches fluorescence on. Further treatment with a bio-compatible exogenous reagent results in complete bleaching of the quencher and consequently reveals the inactivated probes.

Pro-fluorescent probes in complex biological media are activated *via* bio-specific reactions involving biothiols such as Michael additions,[9] aromatic nucleophilic additions,[10] thiazolidine ring formation,[11] sulfonamide or sulfonate esters cleavage,[12] disulfide cleavage and addition-elimination that activates FRET-based probes.[13]

It is noteworthy that even though the reported activation mechanisms have allowed some degree of biospecific activation to be obtained, none of them is compatible with a subsequent activation with an exogenous bio-orthogonal reagent. Following a reactivity screening, we have found that *N*-(dicyanomethylene)aniline oxide **1** (See Fig.

2), synthesized from nitroso aniline and tetracyanoethylene, exhibits suitable features to become an effective chemo-bio-bleachable quencher.[14]

Firstly, nitrone **1** showed a maximum absorption at λ_{max} = 552 nm (Fig. S1). Such high maximum absorption wavelength overlaps the emission spectrum of the tetramethylrhodamine (TAMRA), a widely used fluorophore that absorbs at λ_{max} = 550 nm and emits at λ_{max} = 580 nm. Secondly, we found that upon treatment of nitrone **1** (50 μM) with Cys (1 mM) in phosphate buffer (0.1 M, pH 7.4) the absorption band at 552 nm almost instantaneously disappeared with concomitant and transient appearance of a new absorption band at 442 nm corresponding to the formation of a Cys-adduct (Fig. 2). This shift of maximum absorption peak would represent a good fluorescence turn-on process of a Nitrone-TAMRA FRET probe. Along with this change of absorption, a solution of nitrone **1** turned from dark purple to colourless (Fig. S4). Thirdly, absorbance of compound **1** was instantaneously neutralized upon treatment with sodium dithionite in phosphate buffer (0.1 M, pH 7.4). This would be a smooth chemo-bleaching process of the corresponding Nitrone-TAMRA FRET probe. Finally, MTT assays showed no inherent toxicity of nitrone **1** in HaCaT cells under physiological conditions (Fig. S5).

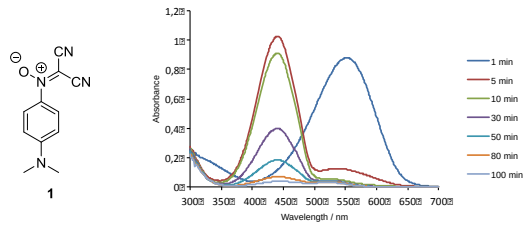

Fig. 2 Structure of nitrone **1** and absorption spectra of **1** (50 μM) upon addition of Cys (1 mM) in phosphate buffer (0.1 M, pH 7.4) at 1, 5, 10, 30, 50, 80 and 100 min.

Synthesis of the Nitrone-TAMRA FRET probe **7** started with the preparation of the intermediate **2** (Fig. 3). Amine alkylation between *N*-methylaniline and methyl-4-bromobutyrate gave compound **2**. Nitrosation with sodium nitrite led to nitroso derivative **3**. After saponification, **4** reacted with tetracyanoethylene (TCN) to give **5**. Finally, coupling of **5** with TAMRA amine **6** gave the turn-on fluorescent probe **7** after purification by semi-preparative HPLC.

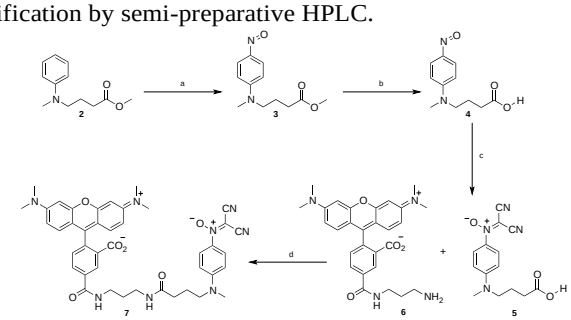

Fig. 3 Synthetic pathway for the formation of probe **7**. Reagents and conditions: a) $NaNO_2$, HCl, CH_3OH, 0 °C, 30 min → 30 °C, 4 h (38%); b) NaOH, CH_3OH, RT, overnight (87%); c) TCN, DMF, RT, overnight (15%); d) EDC, HOBT, Et_3N, DMF, RT, overnight (27%).

To assess the chemoselectivity of *N*-(dicyanomethylene)aniline oxide moiety activation, probe **7** (1 μM) was treated with various biological analytes (5 mM) including amino acids, oxidizing and reducing agents, metal ions, glucose, nucleosides, ascorbic acid and thiols (Cys, Hcy, GSH) in phosphate buffer (0.1 M, pH 7.4) (Fig. S10). The results demonstrated a significant fluorescence increase at 580 nm only upon reaction with thiol derivatives. With all the other analytes, no real fluorescence emission enhancement was observed. This thiol-specific reactivity was further confirmed by inhibiting the reaction with iodoacetate, a thiol-alkylating agent (Fig. S11). Next, our probe **7** was reacted with increasing equivalents of cysteine and a concentration-dependent increase in fluorescence response was recorded. We calculated a detection limit for thiols of 50 nM (Fig. S8). We also measured a quantum yield of fluorescence of 15% upon activation with cysteine (Fig. S12). Bio-specific and bio-orthogonal activation of probe **7** was then studied in living cells. Probe **7** (40 μM) was incubated with normal embryonic fibroblast 3T3 cells for 1 h and with 3T3 cells, pre-treated with *α*–lipoic acid (500 μM) for 48 h. The purpose of *α*–lipoic acid (ALA) is to increase the intracellular level of reduced GSH.[15] Fluorescence emission was measured with a microplate reader (Fig. 4).

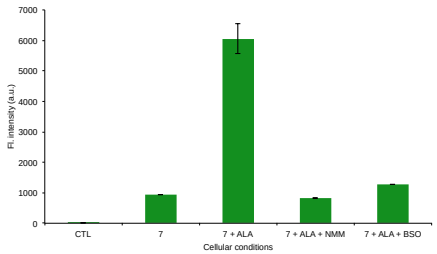

Fig. 4 Fluorescence of probe **7** (1 μM) incubated for 1 h with 3T3 cells, treated in various conditions: CTL = untreated 3T3 cells; **7** = probe **7** with non-treated cells; **7** + ALA = probe **7** with cells pre-treated for 48 h with ALA (500 μM); **7** + ALA + NMM = probe **7** with cells pre-treated for 48 h with ALA (500 μM), then treated for 30 min with NMM (500 μM); **7** + ALA + BSO = probe **7** with cells pre-treated for 48 h with ALA (500 μM) then treated for 30 min with BSO (100 μM). Data were measured with a microplate reader (excitation filter 530/25 nm; emission filter 590/35 nm).

A sevenfold increase of intracellular fluorescence intensity was observed in ALA treated cells compared to non-treated cells. Then, ALA-3T3 cells were further treated with *N*-methylmaleimide (NMM) to decrease the level of free thiols within the cells by electrophilic alkylation and with buthionine sulfoximine (BSO) a specific inhibitor of γ-glutamylcysteine synthetase and therefore a GSH synthesis inhibitor.[16] Under both conditions fluorescence intensity was found to be equivalent to that of untreated cells, thus indicating that activation of probe **7** was indeed descriptive of the intracellular level of free biothiols.

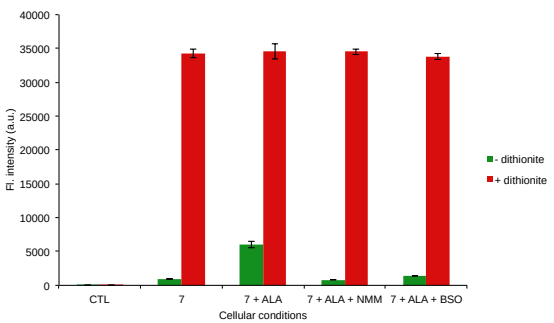

Fig. 5 Fluorescence of **7** in 3T3 cells in various conditions before (green) and after (red) addition of dithionite (10 mM). CTL = untreated 3T3 cells; **7** = probe **7** with untreated cells; **7** + ALA = probe **7** with cells pre-treated for 48 h with ALA (500 μM); **7** + ALA + NMM = probe **7** with cells pre-treated for 48 h with ALA (500 μM) then treated for 30 min with NMM (500 μM); **7** + ALA + BSO = probe **7** with cells pre-treated for 48 h with ALA (500 μM) then treated for 30 min with BSO (100 μM).

To validate the second mode of activation of probe **7**, namely bio-orthogonal chemical bleaching, sodium dithionite (10 mM) was added to each one of the above-described assays. (Fig. 5)
Finally, cells treated with probe **7** were imaged using confocal laser scanning microscopy. Cells were treated with blue Hoechst-33258 dye to localize their nuclei. An intense intracellular fluorescence was present in the cytosol. The recorded images reveal that the probe can penetrate the cell membrane and is present throughout the cytosol (Fig. 6a). As a control, cells were treated with NMM prior to the addition of probe **7**. As expected in that case, only very weak fluorescence could be observed (Fig. 6b). This shows that, in the absence of free biothiol, none of the other components of biological media activates the probe, thus illustrating the good bio-stability and bio-selectivity of the *N*-(dicyanomethylene)aniline oxide motif.

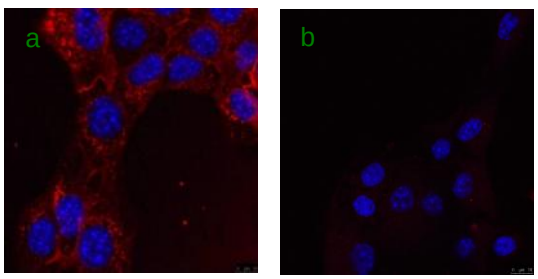

Fig. 6 Merged blue and red fluorescence images of living BNL CL.2 cells. (a) BNL CL.2 cells were incubated with 1 μM of **7** for 1 h, then with Hoechst 33258 (5 μg/mL) for 30 min. Fluorescence image of BNL CL.2 cells from the blue and the red channels. (b) BNL CL.2 were pretreated with ALA (500 μM) for 48 h, then with NMM (500 μM) for 20 min and finally incubated with 1 μM of **7** for 1 h and with Hoechst 33258 for 30 min.

In summary, we have reported the first molecular system that is responsive to both a bio-specific and a bio-orthogonal stimuli. This was visualized *via* activation of a FRET probe through an original mechanism of quencher bleaching. Such combination of bio-functional reaction and quencher-based fluorescence activation expands the repertoire of turn-on/off probes mechanism. It also illustrates one of the many-to-come possibilities of using biofunctional chemistry to investigate biological systems with compact and multi-functional molecular tools.

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Notes and references

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/c000000x/

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