Design and synthesis of a highly selective fluorescent turn-on probe for thiol bioimaging in living cells†

Xin Li,[a] Shijing Qian,[b] Qiaojun He,[b] Bo Yang,[b] Jia Li[c] and Yongzhou Hu*[a]

Received 17th March 2010, Accepted 18th June 2010
First published as an Advance Article on the web 30th June 2010
DOI: 10.1039/c004344c

A new fluorescent turn-on probe for the rapid optical sensing of thiols has been designed and synthesized. The probe displays high on/off signal ratios and high selectivity towards thiols. The potential of the probe as a biosensor for thiols was demonstrated by imaging of thiols in living cells.

The optical sensing of thiols in biological systems is of significant interest due to their crucial roles, for example, in maintaining the higher-order structures of proteins, and in regulation of the intracellular redox state.[1] Thiols are also active in the catalytic sites of many enzymes, and play important roles in the regulation of protein function.[2] The levels of certain thiols, such as homocysteine, have been linked to a number of diseases including cardiovascular and Alzheimer's diseases.[3] Thiol levels may also be affected in response to the oxidative stress that has been associated with numerous cellular processes linked to aging and the development of age-related diseases.[4]

The detection of important biological thiols has been the focus of numerous research efforts and a wide variety of fluorescent probes for thiols are commercially available,[5] with the majority based on redox chemistry or development of fluorophores bearing electrophilic groups. However, these often suffer from low selectivity and strong background signal, necessitating washing and isolation steps, therefore precluding rapid quantification. Thus more effective probes with good sensitivity, selectivity, and practicability in living cells still need to be exploited and the focus of study has been shifted to the development of selective fluorescent turn-on probes based on different mechanisms.[6]

Recently, it was found in our lab that nucleophilic aromatic substitution of phenyl-2,4-dinitrobenzenesulfonate by thiophenol proceeds fast at room temperature to give (2,4-dinitrophenyl)(phenyl)sulfane and phenol in nearly quantitative yield. Since the arenesulfonate moiety has received much attention in the design of fluorescence quenched probes,[7] the above observation inspired us to see if we could design a fluorescent turn-on probe for the detection of thiols in biological systems. Due to its strong reduction potential, the 2,4-dinitrobenzenesulfonate moiety attached to a fluorophore may incur donor-excited photoinduced electron transfer (d-PeT) and accept an electron transferred from the excited fluorophore, resulting in the quenching of the fluorescence.[8] Nucleophilic aromatic substitution of the arenesulfonate ester with thiols will release the hydroxyl donor, and thus the fluorophore will resume its strong fluorescence due to the push-pull character (Fig. 1).

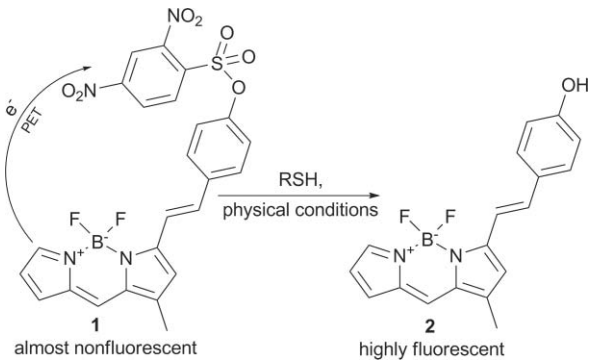


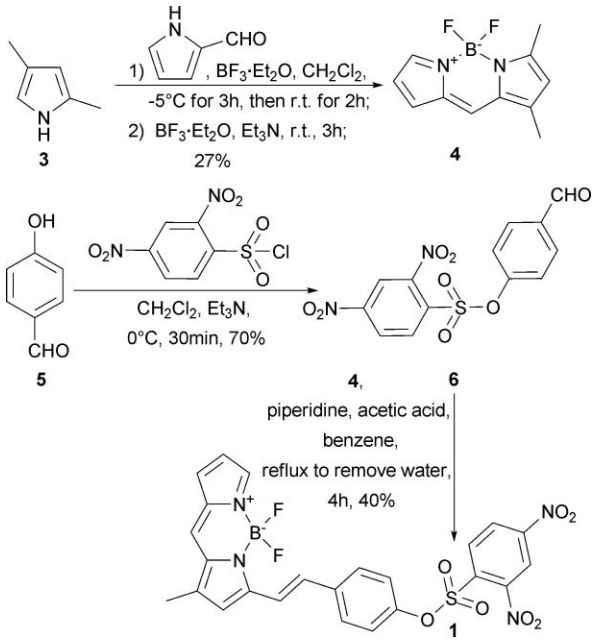
Fig. 1 Design of fluorescent turn-on probe **1**. Probe **1** is hypothesized to be nonfluorescent due to donor-excited photoinduced electron transfer, while thiol-specific reaction will release the hydroxyl donor, and in turn makes the BODIPY fluorophore **2** resume its strong fluorescence ascribed to its push-pull character.

4,4-Difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY) has outstanding photophysical properties as a fluorescent scaffold, such as high photostability, high quantum yield and pH-insensitivity.[9] Sparked by the new tactics in the functionalization of the BODIPY core, a veritable BODIPY renaissance has come into being within the last five years, and its chemical modification has been well studied.[10] It is reported that the 3- and 5-methyls in the BODIPY scaffold readily undergo Knoevenagel reaction, resulting in a styryl-substituted BODIPY dye displaying bathochromic shift fluorescent emissions due to the extended π-conjugation.[11] With this knowledge in hand, we envisioned to incorporate the 2,4-dinitrobenzenesulfonate moiety into the BODIPY core *via* Knoevenagel reaction, giving designed probe **1** (Fig. 1).

Probe **1** is prepared in three steps straightforwardly as outlined in Scheme 1. Condensation of 2,4-dimethylpyrrole **3** and pyrrole-2-carboxylaldehyde at low temperature, followed by the addition of $BF_3{\cdot}OEt_2$ and Et_3N, constructed the BODIPY core **4** in **27%** yield.[12] Arenesulfonate ester moiety **6**, prepared by sulfonating the hydroxyl group of **5** with 2,4-dinitrobenzene-1-sulfonyl chloride, was then introduced into **4** under Knoevenagel reaction conditions to furnish probe **1** conveniently.[13]

With probe **1** in hand, we first examined its fluorescence properties in the absence and presence of a thiol. Working solution of **1** was prepared by 100-fold dilution of its ethanolic solution with aqueous phosphate buffer (PBS, 10 mM, pH 7.3). As shown

[a]*ZJU-ENS Joint Laboratory of Medicinal Chemistry, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou 310058, China. E-mail: huyz@zju.edu.cn; Fax: 086-571-88208460; Tel: 086-571-88208460*
[b]*Institute of Pharmacology and Toxicology, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou 310058, China*
[c]*State Key Laboratory of Drug Research, Shanghai Institute of Material Medica, Chinese Academy of Science, Shanghai 201203, China*
† Electronic supplementary information (ESI) available: Synthetic methods; supplementary figure (Fig. S1); and spectra. See DOI: 10.1039/c004344c



Scheme 1 Synthesis of probe **1**.

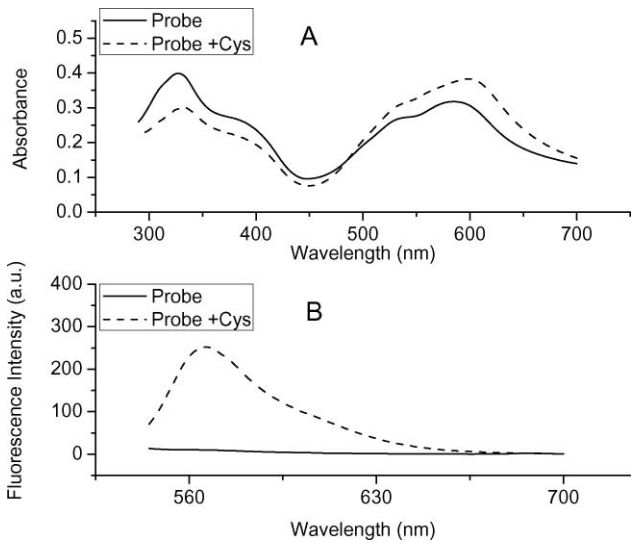
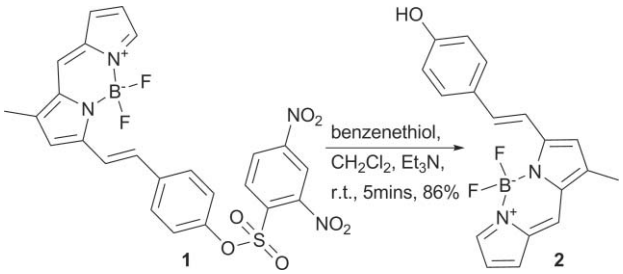


Fig. 2 (A) Absorption and (B) emission spectra (λ_{ex} = 527 nm) of probe **1** before (—) and after (---) the addition of cysteine. Final concentrations of **1** and cysteine are 2.0×10^{-5} M and 20×10^{-5} M, respectively, in aqueous PBS buffer (10 mM, pH 7.3) containing 1% EtOH. Data were collected before or 1 h after the addition of cysteine.

in Fig. 2, probe **1** has an absorption maximum at 325 nm (ε = 19 500 mol L^{-1} cm^{-1}) and is weakly fluorescent with an emission centered at 500 nm (Φ_F 0.03, λ_{ex} 360 nm). Upon addition of cysteine, the absorption maximum is red-shifted to 600 nm (ε = 20 200 mol L^{-1} cm^{-1}) and a significant increase in fluorescence intensity (λ_{em} 570 nm, Φ_F 0.28) is observed, due to the *in situ* generation of the highly fluorescent probe **2** (Φ_F 0.69). These observations indicate that attachment of the 2,4-dinitrobenzenesulfonate moiety to the styryl BODIPY has induced d-PeT and quenched effectively the fluorophore's fluorescence.

The validity of the proposed sensing mechanism was confirmed by the preparative-scale synthesis of the free dye **2** from **1** and benzenethiol (Scheme 2).



Scheme 2 Cleavage of probe **1** with thiophenol.

We then tested the selectivity of probe **1** for thiols by screening its response to biologically relevant analytes under simulated physical conditions (37 °C, 10 mM PBS buffer, pH 7.3). As shown in Fig. 3, probe **1** was highly selective to thiols with remarkable fluorescence intensity enhancement for thiophenol, glutathione, cysteine and thioglycol, while no significant enhancement for CH_3NH_2, *n*-$BuNH_2$, glycine, alanine and cystine was observed, indicating the high selectivity of the probe toward thiols.

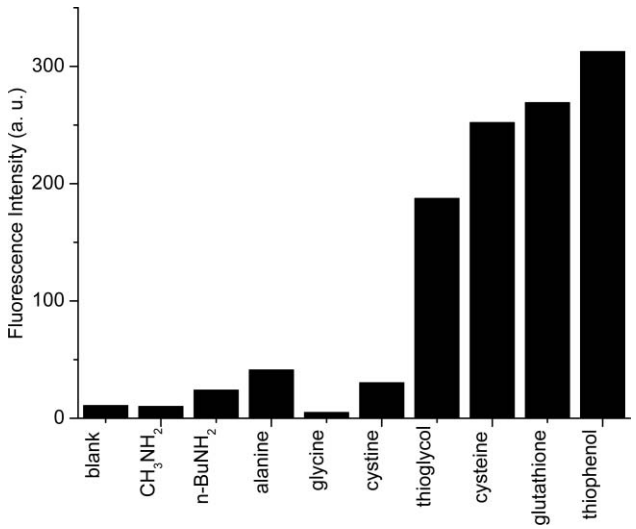


Fig. 3 Selectivity of probe **1** toward different analytes. Relative fluorescence intensity of probe **1** (20 μM in 10 mM of PBS buffer containing 1% EtOH, pH 7.3) was measured at 570 nm (λ_{ex} = 527 nm) after incubation at 37 °C for 1 h in the presence of 200 μM (final concentrations) of analytes.

Notably, probe **1** showed a quick response toward cysteine based on the study of reaction time (Fig. 4). A pronounced fluorescent intensity increase up to 25-fold was obtained even 1 min after the addition of cysteine. Limited enhancement was observed within the next hour, while almost no change appeared when longer reaction time was examined.

The sensitivity of probe **1** was then studied by fluorescence response of the probe toward different concentrations of cysteine. As shown in Fig. 5, the fluorescence enhancement was displayed in a concentration dependent manner with the increase being linear with a coefficient of 0.9938 when low concentration of cysteine (1–100 μM) was added (Fig. 5, inner panel), indicating that probe **1** could detect thiol quantitatively.

The stability of probe **1** in PBS buffer solution was then examined by detecting the change of its fluorescent intensity. First of all, probe **1** is essentially stable over a range of pH 6–8, with a low level of its hydrolysis being detected at pH 8.0, indicated by

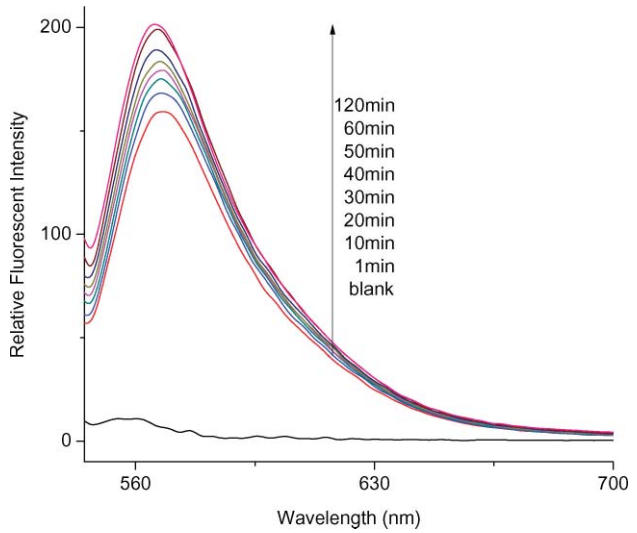


Fig. 4 Reaction-time profile of probe **1** and cysteine. The fluorescence intensity of probe **1** (20 μM in 10 mM of PBS buffer containing 1% EtOH, pH 7.3) was studied at room temperature in the absence and presence of cysteine (5.0 equiv.) after the specified time periods (1, 10, 20, 30, 40, 50, 60, 120 min) with λ_{ex} 527 nm.

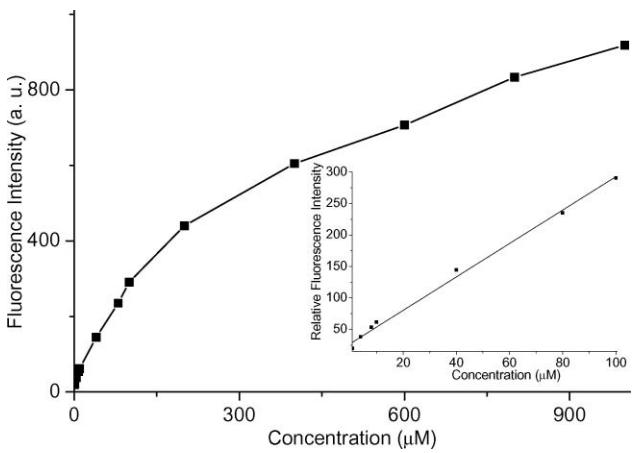


Fig. 5 Fluorescence response of probe **1** (20 μM) upon reaction with cysteine in 1, 4, 8, 10, 40, 80, 100, 200, 400, 600, 800, 1000 μM. The fluorescence intensity at λ_{em} = 570 nm was plotted after 1 h of incubation at room temperature. The excitation wavelength was 527 nm. The inner panel displays the fluorescence enhancement of probe **1** toward cysteine in 1, 4, 8, 10 μM.

the slight enhancement of its fluorescent intensity (ESI, Fig. S1†). Furthermore, probe **1** is basically stable in PBS buffer solution at pH 7.3 with no obvious enhancement of fluorescent intensity being observed over 12 h at room temperature. Dependence of the present fluorometric assay on pH was studied next by using cysteine as an analyte with the pH ranging from 6 to 8. The results showed that probe **1** could act as a probe only at pH up to 7.0, this is mainly due to the fact that nucleophilic aromatic substitution is generally dependent on the deprotonated thiolate anion while the pK_a values of aliphatic thiols are generally 8–9 (ESI, Fig. S1†).

To test the capability of our new fluorescence probe to image thiols in living cells, we next applied **1** to monkey renal fibroblast COS-7 cell line. After being incubated with a solution of **1** (5 μM in 1 : 1000 DMSO–PBS v/v) for 10 min at 37 °C, cells were observed by fluorescence microscopy. As shown in Fig. 6, probe **1** was found to be cell-permeable and to react with intracellular thiols, resulting in strong fluorescence emission. When *N*-methylmaleimides, which selectively react with thiols by covalent Michael addition, were supplemented to cells before they were stained with probe **1**, a distinct decrease of fluorescence intensity was observed. This confirms the specificity of **1** for thiols over other analytes in living cells and clearly demonstrates its feasibility as a biosensor for thiols.

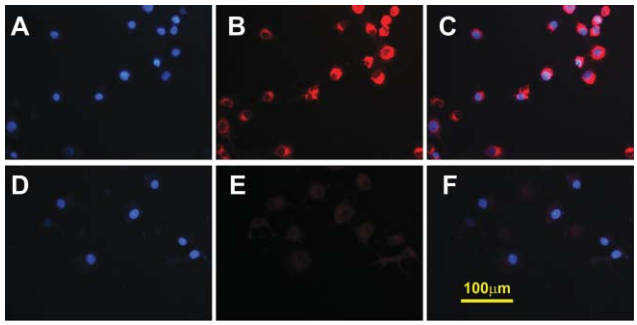


Fig. 6 Fluorescence microscopic images of live COS cells. Upper: cells were incubated with probe **1** (5 μM) for 10 min at 37 °C, and stained with a nucleus-staining dye, DAPI. (A) Fluorescence image collected with a DAPI dye filter set; (B) fluorescence image collected with a Cy3 dye filter set; and (C) overlay image of B and C. Bottom: corresponding control images of cells pretreated with *N*-methylmaleimide (0.1 mM) for 60 min at 37 °C and then incubated with **1** as above. Images were detected by LERCA DMI4000B. Scale bar represents 100 μm.

In conclusion, we have developed a new fluorescent turn-on probe, in which the fluorescence is quenched by d-PeT. The probe can recover its strong fluorescence only by thiol-specific nucleophilic aromatic substitution to release the BODIPY fluorophore. The probe has several advantages. Firstly, it is highly selective toward thiols and the detection is rapid. Secondly, it can be excited with visible light and demonstrates high off/on signal ratios. Thirdly, it can be used for the quantitative analysis of low concentrations of thiol. Fourthly, it can be applied to physical conditions with pH ranging from 7 to 8 that is compatible with most biological applications. The probe was confirmed to be applicable for detecting thiols in living cells.

Acknowledgements

This work was supported by National Natural Science Foundation of China (30901858), National Key Tech Project for Major Creation of New Drugs (2009ZX09501-003), China Postdoctoral Science Foundation (20090461390) and the State Key Laboratory of Drug Research (SIMM1004KF-13).

References

1 S. M. Deneke, *Curr. Top. Cell. Regul.*, 2001, **36**, 151.
2 (*a*) T. Kooistra, P. C. Millard and J. B. Lloyd, *Biochem. J.*, 1982, **204**, 471; (*b*) S. A. Lipton, Y. B. Choi, H. Takahashi, D. Zhang, W. Li, A. Godzik and L. A. Bankston, *Trends Neurosci.*, 2002, **25**, 474.
3 (*a*) H. Refsum, P. M. Ueland, O. Nygard and S. E. Vollset, *Annu. Rev. Med.*, 1998, **49**, 31; (*b*) S. Seshadri, A. Beiser, J. Selhub, P. F. Jacques, I. H. Rosenberg, R. B. D'Agostino and P. W. F. N. Wilson, *N. Engl. J. Med.*, 2002, **346**, 476.

4 (*a*) T. Finkel, *Curr. Opin. Cell Biol.*, 2003, **15**, 247; (*b*) T. Finkel and N. J. Holbrook, *Nature*, 2000, **408**, 239.
5 R. P. Haugland, *Molecular Probes Handbook of Fluorescent Probes and Research Products*, Molecular Probes, Inc.: Eugene, OR, 9th edn 2002, 79.
6 (*a*) W. Wang, O. Rusin, X. Xu, K. K. Kim, J. O. Escobedo, S. O. Fakayode, K. A. Fletcher, M. Lowry, C. M. Schowalter, C. M. Lawrence, F. R. Fronczek, I. M. Warner and R. M. Strongin, *J. Am. Chem. Soc.*, 2005, **127**, 15949; (*b*) M. Zhang, M. Yu, M. Zhu, M. Li, Y. Gao, L. Li, F. Li, Z. Liu, J. Zhang, D. Zhang, T. Yi and C. Huang, *J. Am. Chem. Soc.*, 2007, **129**, 10322; (*c*) Y. H. Ahn, J. S. Lee and Y. T. Chang, *J. Am. Chem. Soc.*, 2007, **129**, 4510; (*d*) H. Chen, Q. Zhao, F. Li, Y. Wu, H. Yang, T. Yi and C. Huang, *Inorg. Chem.*, 2007, **46**, 11075; (*e*) X. Chen, S. K. Ko, M. J. Kim, I. Shin and J. Yoon, *Chem. Commun.*, 2010, **46**, 2751.
7 (*a*) H. Maeda, H. Matsuno, M. Ushida, K. Katayama, K. Saeki and N. Itoh, *Angew. Chem., Int. Ed.*, 2005, **44**, 2922; (*b*) W. Jiang, Q. Fu, H. Fan, J. Ho and W. Wang, *Angew. Chem., Int. Ed.*, 2007, **46**, 8445; (*c*) J. Bouffard, Y. Kim, T. M. Swager, R. Weissleder and S. A. Hilderbrand, *Org. Lett.*, 2008, **10**, 37.
8 H. Kobayashi, M. Ogawa, R. Alford, P. L. Choyke and Y. Urano, *Chem. Rev.*, 2010, **110**, 2620.
9 J. Karolin, L. B. A. Johansson, L. Strandberg and T. Ny, *J. Am. Chem. Soc.*, 1994, **116**, 7801.
10 (*a*) A. Loudet and K. Burgess, *Chem. Rev.*, 2007, **107**, 4891; (*b*) G. Ulrich, R. Ziessel and A. Harriman, *Angew. Chem., Int. Ed.*, 2008, **47**, 1184.
11 (*a*) Z. Dost, S. Atilgan and E. U. Akkaya, *Tetrahedron*, 2006, **62**, 8484; (*b*) S. Atilgan, Z. Ekmekci, A. L. Dogan, D. Guc and E. U. Akkaya, *Chem. Commun.*, 2006, 4398.
12 J. S. Lee, N. Y. Kang, Y. K. Kim, A. Samanta, S. Feng, H. K. Kim, M. Vendrell, J. H. Park and Y. T. Chang, *J. Am. Chem. Soc.*, 2009, **131**, 10077.
13 O. Buyukcakir, O. A. Bozdemir, S. Kolemen, S. Erbas and E. U. Akkaya, *Org. Lett.*, 2009, **11**, 4644.

