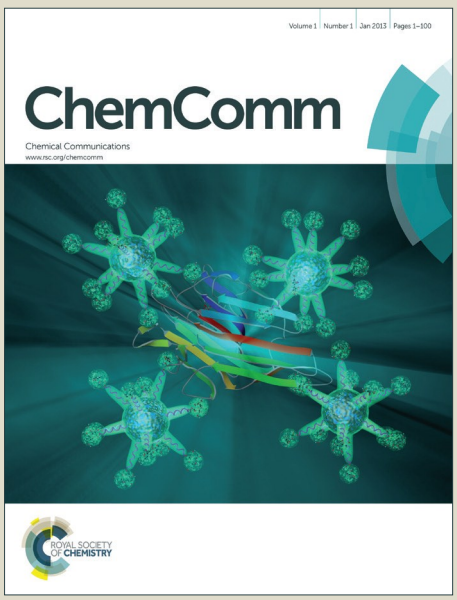
ChemComm

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

ChemComm

COMMUNICATION

Naphthalimide-based fluorescent probe for selectively and specifically detecting glutathione in lysosome of living cells

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Meijiao Cao,[ad] Haiyan Chen,[bd]* Dan Chen,[b] Zhiqiang Xu,[a] Sheng Hua Liu,[a] Xiaoqiang Chen,[c]* and Jun Yin[a]*

A novel naphthalimide-based fluorescent probe by employing a sulfonamide unit as a thiol-responsive group was reported. It is capable of efficiently distinguishing GSH from cysteine and homocysteine. Bioimaging shows that it has highly selectivity in living cells and can visualize the level of GSH in lysosome. It is worth mentioning that the different group on the imide unit can affect the selectivity and reaction dynamics of the probe towards thiols.

Biothiols such as Glutathione (GSH), cysteine (Cys), and homocysteine (Hcy), with a wide range of cellular biological functions, plays an important role in the body's biochemical defense system such as redox homeostasis which maintain the equilibrium of reduced free thiol and oxidized disulfide forms.[1] However, the level of biothiols too high or too low could affect the normal physiological functions and pathological functions, resulting in a number of diseases, such as cancer, AIDS, liver damage, Alzhei-mer's disease, osteoporosis, heart, inflammatory bowel and cardi-ovascular disease.[2] Therefore, to maintain the stability of biothiols in body is very important.

GSH, the most abundant biothiol with a concentration in the millimolar range in living system, undergoes many cellular func-tions, containing maintenance of intracellular redox activities, xenobiotic metabolism, intracellular signal transduction, gene regulation, and liver damage.[3] In these significant functions, GSH as an important antioxidant protects the thiol molecules in proteins and enzymes from oxidative damage caused by free radicals and reactive oxygen species (ROS). Thus, exactly assessing the level of intracellular GSH prospectively provides some crucial evidence for clinical medicine to diagnose diseases that are related to GSH.[3a, 4]

In recent years, numerous efforts have paid to the fluorescence-responsive chemosensors to find the highly selective and specific fluorescent probes of GSH due to its simplicity, high sensitivity, high selectivity and operability.[5] Owing to the fact that Cys, Hcy possess similar molecular backbone and reactivity (such as SH group) as well as GSH, it is considerably difficult to effectively distinguish GSH from Cys and Hcy. Despite many probes have been confirmed to be highly selective or specific, it still suffers the challenge from few examples having high selectivity and specificity at the same time. For GSH-responsive fluorescent probes, the mechanism of nucleophilic substitution is one of the most widely utilized design principles.[6] For example, the sulfonyl group with 2,4-dinitrobenzene unit is considered as one of the most available systems owing to its high efficiency and definite mechanism.[6b, 6f] Accordingly, plenty of GSH-responsive fluorescent probes were reported by employing 2,4-dinitrobenzene-1-sulfonyl moiety as a reactive group with thiol group.[6b, 6f] Besides, the dansyl also possesses a similar sulfonyl moiety. However, its derivatives were often used to investigate the interaction with metal ions.[7] Recently, Yoon and we firstly employed a dansyl moiety as a GSH-response group to construct a near-infrared cyanine-based fluorescent probes, which can monitor the level of GSH in living cells and in vivo.[6a] For two types of sulfonyl-based probes, they present a similar strategy that the GSH in products was installed on the 2,4-dinitrobenzene and 5-(dimethylamino)naphthalene units, as shown in Fig. 1a. If the GSH is located on the fluorophore after reaction, it would be significant to provide a strategy for labeling the GSH and its analogues with GSH backbone. Based on the consideration described above, we developed a novel sulfonyl-based naphthalimide fluorescent probe based on the mechanism described in Fig. 1b. Investigation on the response of thiols shows that it can selectively detect the GSH not only in vitro but also in living cells. Furthermore, it can specifically visualize the level of GSH in lysosome of living cells.

[a] Key Laboratory of Pesticide and Chemical Biology, Ministry of Education, College of Chemistry, Central China Normal University, Wuhan 430079, P. R. China. E-mail: yinj@mail.ccnu.edu.cn
[b] Department of Biomedical Engineering, School of Engineering, China Pharmaceutical University, 24 Tongjia Lane, Gulou District, Nanjing 210009, P. R. China. E-mail: chenhaiyan@cpu.edu.cn
[c] State Key Laboratory of Materials-Oriented Chemical Engineering, College of Chemistry and Chemical Engineering, Nan-jing Tech University, Nanjing 210009, P. R. China. E-mail: chenxq@njtech.edu.cn
[d] They contributed equally to this work.
†Electronic Supplementary Information (ESI) available: experiment section, UV-Vis absorption, fluorescence emission, theoretical calculation, 1H NMR, 13C NMR and MS spectra of all the new compounds were available in Supporting Information. See DOI: 10.1039/x0xx00000x

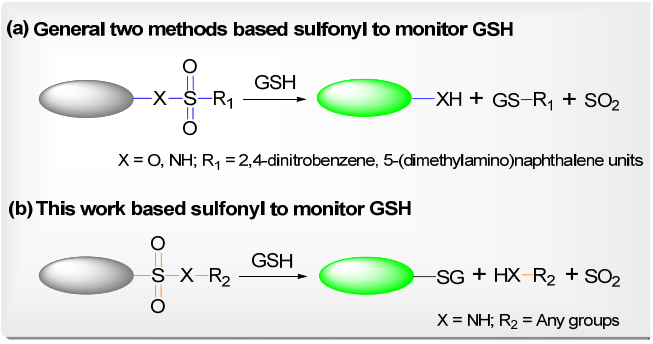
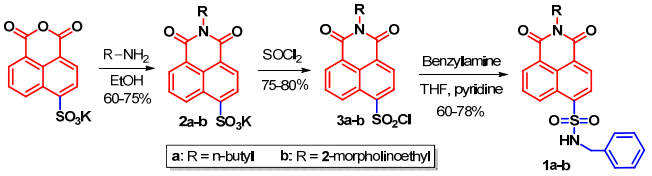


Fig. 1 Two types of sulfonyl-based fluorescence probe for GSH detection.



Scheme 1 Synthesis of probes **1a** and **1b**.

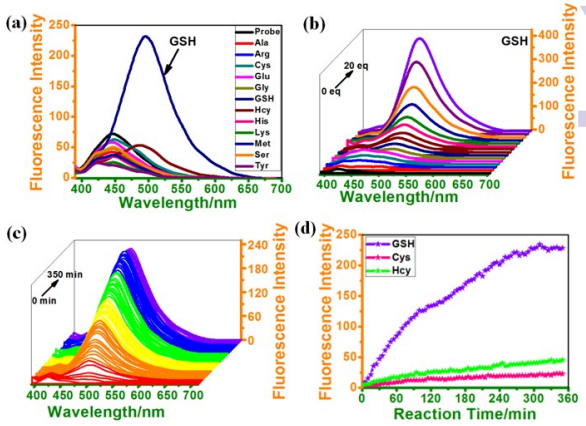


Fig. 2 (a) The fluorescence spectra of **1b** (10 μM) with various amino acids (100 μM) in HEPES buffer (0.02 M, pH = 7.4) containing 10% DMSO. (b) The titration fluorescence spectra of **1b** (10 μM) with GSH (0 - 200 μM). (c) Time-dependent fluorescent spectrum of **1b** (10 μM) with 10 equiv of GSH. (d) Time dependence of fluorescence intensity of **1b** (10 μM) at 495 nm with 10 equiv of GSH, Cys or Hcy, respectively. λ_{ex} = 370 nm.

The synthetic scheme for the preparation of probes **1a** and **1b** was shown in Scheme 1. The intermediates **2** and **3** were synthesized according to reported synthetic procedures.[8] The condensation of **3** and benzylamine afforded the probes **1a** and **1b** in 60-78% yields, respectively. Their structures were characterized by ^{1}H NMR, ^{13}C NMR and ESI-MS.

Studies were performed to evaluate the application of fluorescent probes 1a and 1b to detect amino acids containing a thiol group. We firstly explored the selectivity of probes **1a** and **1b** to different amino acids by UV-Vis absorption and fluorescence spectra in HEPES buffer (0.02 M, pH = 7.4) containing 10% DMSO. Analysis of UV-Vis absorption spectroscopic changes of **1a** showed that there were no obvious changes observing from the UV-Vis spectra (Fig. S1a, ESI†). Meanwhile, upon excitation of 356 nm, only a little enhancement of fluorescence intensity at 450 nm was observed in fluorescent spectra when 10 equivalents of GSH were added (Fig. S1b, ESI†), compared with **1a**, probe **1b** replaced n-butyl with 2-morpholinoethyl obtained high selectivity. Despite probe **1b** did not exhibit obvious changes in UV-Vis absorption spectra under the same condition (Fig. S2b, ESI†), the addition of GSH (10.0 equiv.) induced an obvious green emission (Fig. S2a, ESI†) with a dramatically increasing of fluorescence intensity at 495 nm due to the formation of product of nucleophilic substitution (Fig. 1b), as shown in Fig. 2a. However, no obvious changes took place when other amino acids including thiols Hcy and Cys were added. The attachment of partially protonized moieties such as 2-morpholinoethyl in physiological conditions made probe **1b** possess good water-soluble and excellent selectively detection for GSH due to the sequestration effect of positive charges in physiological pH value.[9] Subsequently, we exploited competitive fluorescence assay of **1b** toward GSH in the presence of variety kinds of amino acids. The result suggested that the interference from other amino acids was negligible (Fig. S3a, ESI†). All these data demonstrated that **1b** had high selectivity for GSH and could be utilized to discriminate GSH from other amino acids, especially for Cys and Hcy.

Next, we evaluated the absorption and fluorescence titration performance of **1b** to different concentration of GSH. Upon the addition of GSH (0 - 200 μM), the weak fluorescence emission at 425 nm gradually emerged red-shift along with an increasing of fluorescent intensity in Fig. 2b while no obvious color changes were observed in UV-Vis absorption spectra (Fig. S3b, ESI†). Next, we investigated the kinetics of probe **1b** toward three biothiols such as GSH, Cys and Hcy. The results of an experiment probing the time-dependent fluorescence response of probe **1b** (10 μM) to GSH (100 μM) in HEPES buffer (0.02 M, pH= 7.4) containing 10% DMSO showed that the intensity increased with time, reaching a maximum in 5 min. In comparison to GSH, the other thiol-based amino acids, Cys and Hcy, did not promote as rapid a fluorescence response of probe **1b** (Fig. 2c and 2d). Compared with those response-fast probes mainly in 10~30 min,[6a, 6g] probe **1b** has enough time to enter the detection position of living system, which was assumed to reach and react with GSH completely. Nevertheless, some efficient approach such as using the interior hydrophobicity of supramolecular micelles can also be used to advance the reactivity.[10]

To assess the biological application of probe **1b**, studies were carried out to investigate the utility in fluorescence imaging of cellular thiols. To achieve this purpose, HepG2 cells were grown in DMEM medium supplemented with 10% (v/v) calf serum, penicillin (100 U/mL) and streptomycin (100 mg/mL) and were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. All the cells were washed by ice-

cold PBS thrice and then incubated with FBS free fresh media for subsequent cell imaging. As presented by viewing the confocal fluorescence microscope, bright field and merged images displayed in Fig. 3a, a significant green image was generated when HepG2 cells were incubated with probe **1b** (20 μM) at 37°C for 1.5 h. The result indicated that probe **1b** was capable of permeating into cells and reacting with endogenous thiols to generate the green fluorescence images. As described above, probe **1b** possessed high selectivity for GSH in vitro. Subsequent control experiments were performed to investigate the selectivity of probe **1b** in living cells. When HepG2 cells were pretreated with the thiol-blocking reagent N-ethyl maleimide (NEM) for 0.5 h and then incubated with **1b** (20 μM) for 1.5 h, the green fluorescence was completely quenched in Fig. 3b. Subsequently, addition of GSH (100 μM) to this NEM-pretreated HepG2 cells and then incubating another 1.5 h, gave rise to a markedly stronger green fluorescence while addition of Cys or Hcy (100 μM) did not result in obviously green fluorescence observing (Fig. 3c-e). This experiment further confirmed that probe **1b** is a GSH-selective fluorescent probe and can be used as a biomarker to monitor the intracellular GSH.

Numerous researches have shown that morpholine has a capability of targeting lysosome of living cells.[11] Next, we investigated its specificity of lysosome. To better gain the fluorescence images, especially in subcellular organelle lysosome, a commercially available lysosome probe LysoTracker Red was employed as a reference for lysosome staining. Probe **1b** was incubated with NEM-pretreated HepG2 cells at 37°C for 1.5 h, and then followed by further staining with LysoTracker Red (10 μM) for 0.5 h. Before addition of GSH, no obvious green fluorescence was observed. A remarkable green fluorescence image took place when GSH (100 μM) was added to the above system and incubated for 1.5 h. The merged fluorescence images as shown in Fig. 4 implied that probe **1b** can be efficiently localized to the lysosome of living HepG2 cells. Results arising from the data describe above suggested that probe **1b** not only had high selectivity for GSH and remarkable lysosome-specificity.

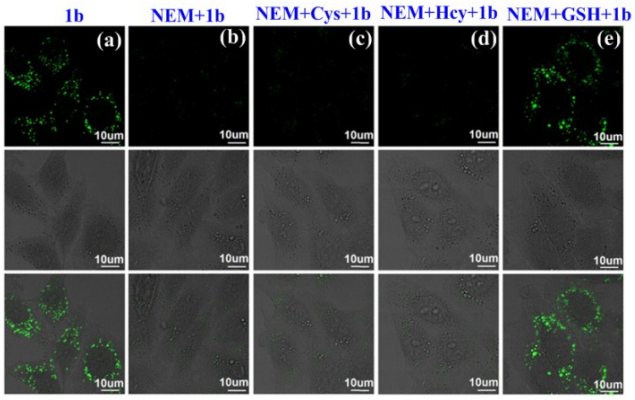


Fig. 3 Confocal microscope images of 1b in HepG2 cells (a) Fluorescence image of HepG2 cell incubated with 1b (20 μM) for 1.5 h. (b) Fluorescence image of HepG2 cell pretreated with NEM (1 mM) for 0.5 h, and incubated with 1b (20 μM) for 1.5 h. (c) Fluorescence image of HepG2 cell pretreated with NEM (1 mM) for 0.5 h, treated with 1b (20 μM) for 1.5 h and then incubated with Cys (100 μM) for another 1.5 h. (d) Fluorescence image of HepG2 cell pretreated with NEM (1 mM) for 0.5 h, treated with 1b (20 μM) for 1.5 h and then incubated with Hcy (100 μM) for another 1.5 h. (e) Fluorescence image of HepG2 cell pretreated with NEM (1 mM) for 0.5 h, treated with 1b (20 μM) for 1.5 h and then incubated with GSH (100 μM) for another 1.5 h.

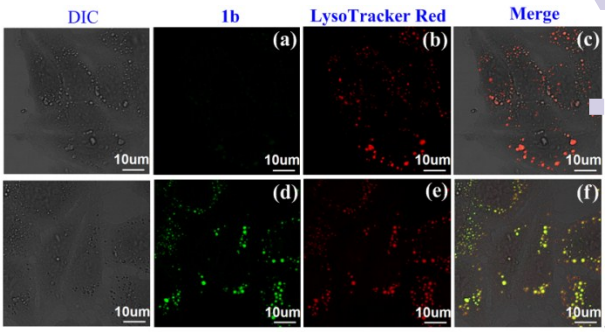


Fig. 4 The first line: confocal microscope images HepG2 cell pretreated with NEM (1 mM) for 0.5 h, and then incubated with **1b** (20 μM) for 1.5 h and LysoTracker Red (10 μM) for 0.5 h; (a) in green channel; (b) in red channel; (c) merged image of (a) and (b). The second line: confocal microscope images of HepG2 cell pretreated with NEM (1 mM) for 0.5 h and then incubated with **1b** (20 μM) for 1.5 h, GSH (100 μM) for 1.5 h and LysoTracker Red (10 μM) for 0.5 h; (d) in green channel; (e) in red channel; (f) merged image of (d) and (e).

To further investigate the recognition mechanism, time-dependent density functional theory (TD-DFT) calculations at the B3LYP/6-31G* level using the Gaussian 09 program were carried out. The absorption spectra and the molecular orbital containing the main electronic transitions with the largest oscillator strength of **1b** and **1a** were listed in Fig. 5 and Table S1 (ESI†). An intense transition for **1a** was predicted at about 339 nm with larger oscillator strength of 0.2659 (HOMO-2→LUMO). The HOMO-2 was mainly assigned to naphthalimide moiety and benzyl group whereas in the electron density of LUMO orbital was predominantly located over naphthalimide moiety, implying that probe **1a** had a weak fluorescence owing to an electron transfer. The result was well in agreement with an experimental data (Fig. S4a, ESI†). For **1b**, an intense transition was predicted at 340 nm with larger oscillator strength of 0.2319 (HOMO-3→LUMO). The HOMO-3 was chiefly delocalized over the entire molecule while LUMO orbital was on the naphthalimide moiety. Accordingly, probe **1b** involved in a process of stronger proton electron transfer (PET), resulting in fluorescence quenching compared with probe **1b**, which kept in step with experimental result (Fig. S4b, ESI†). After treatment with GSH, probe **1b** displayed an intense transition at 362 nm with larger oscillator strength of 0.2307 (HOMO-1→LUMO). The frontier molecular orbital profiles of HOMO-1 and LUMO were mainly localized at the naphthalimide moiety, suggesting that there was no obvious

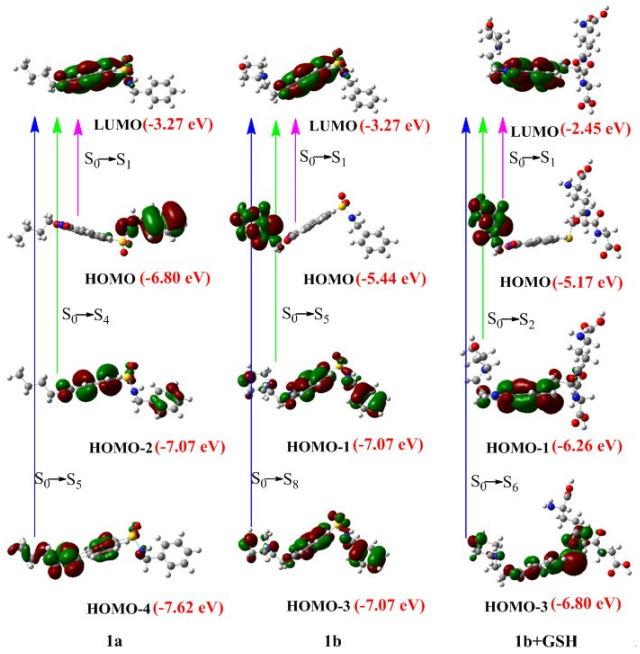


Fig. 5 The main contribution frontier molecular orbital of **1a**, **1b** and **1b**+GSH involved in the absorption processes.

electron transfer as a result of new turn-on fluorescence at 495 nm. Results of theoretical calculation further confirmed the experimental data. To gain further support for the reactive product, the ESI mass spectrometry was employed to follow the reaction of the probe **1b** with GSH, Cys and Hcy. The peak at m/z = 615.1 (GSH), 430.1 (Cys) and 444.1 (Hcy) can be assigned to the products of nucleophilic substitution, respectively, which strongly confirmed the mechanism of probe described in Fig. 1b (Fig. S5, ESI†). We can refer that GSH, Cys and Hcy can react with the sulfonamide unit of naphthalimide, but Cys and Hcy can't induce the turn-on fluorescence.

In summary, we have developed an efficient probe containing a naphthalimide fluorophore linked to sulfonamide functional groups which is capable of selectively monitoring GSH and efficiently distinguishing GSH from Cys and Hcy in vitro and in vivo. Further, confocal images shows that it has highly selectivity in living cells and can visualize the level of GSH in lysosome. It is worth mentioning that the different group on the imide unit can affect the selectivity and reaction dynamics of probe towards thiols.

We acknowledge financial support from National Natural Science Foundation of China (21272088, 21472059, 21402057 and 81371684) and the Program for Academic Leader in Wuhan Municipality (201271130441).

Notes and references

1 Z. A. Wood, E. Schroder, J. R. Harris and L. B. Poole, *Trends Biochem. Sci*., 2003, **28**, 32.

2 (a) D. M. Townsend, K. D. Tew and H. Tapiero, *Biomed. Pharmacother*., 2003, **57**, 145; (b) T. J. Meade and S. Aime, *Acc. Chem. Res*., 2009, **42**, 821; (c) T. Ueno and T. Nagano, *Nat. Methods,* 2011, **8**, 642; (d) P. V. Chang and C. R. Bertozzi, *Chem. Commun.,* 2012, **48**, 8864; (e) D. Lee, G. Kim, J. Y[illegible] and J. Yoon, *Chem. Commun.,* 2015, **51**, 6518; (f) Y. Zhang, H. Chen, D. Chen, D. Wu, X. Chen, S. H. Liu and J. Yin, *Org. Biomol. Chem.,* 2015, **13**, 9760.

3 (a) P. K. Pullela, T. Chiku, M. J. Carvan and D. S. Sem, *Anal. Biochem*., 2006, **352**, 265; (b) S. M. Kanzok, R. H. Schirmer, Iozef, R. Türbachova and K. J. Becker, *Biol. Chem.,* 2000, **275**, 40180; (c) C. Saito, C. Zwingmann and H. Jaeschke, *Hepatology,* 2010, **51**, 246.

4 (a) X. Chen, X. Tian, I. Shin and J. Yoon, *Chem. Soc. Rev.*, 2011, **40**, 4783; (b) L. M. Hyman and K. J. Fanz, *Coord. Chem. Rev*., 2012, **256**, 2333; (c) Y. Feng, J. Cheng, L. Zhou, X. Zhou and H. Xiang, *Analyst,* 2012, **137**, 4885; (d) K. Xu, M. Qiang, W. Gao, R. Su, N. Li, Y. Gao, Y. Xie, F. Kong and B. Tang, *Chem. Sci.,* 2013, **4**, 1079; (e) X. Xiong, F. Song, G. Chen, W. Sun, J. Wang, P. Gao, Y. Zhang, B. Qiao, W. Li, S. Sun, J. Fan and X. Peng, *Chem. Eur. J.,* 2013, **19**, 6538; (f) W. X. Ren, J. Han, T. Pradhan, J.-Y. Lim, J. H. Lee, J. Lee, J. H. Kim and J. S. Kim, *Biomaterials,* 2014, **35**, 4157.

5 (a) L.-L. Yin, Z.-Z. Chen, L.-L. Tong, K.-H. Xu and B. Tang, *Chin. J. Anal. Chem.,* 2009, **37**, 1073; (b) X. Chen, Y. Zhou, X. Peng and J. Yoon, *Chem. Soc. Rev.,* 2010, **39**, 2120; (c) Y. Zhou and Y. Yoon, *Chem. Soc. Rev.,* 2012, **41**, 52; (d) H. S. Jung, X. Chen, J. S. Kim and Y. Yoon, *Chem. Soc. Rev.,* 2013, **42**, 6019; (e) C. Yin, F. Huo, J. Zhang, R. Martínez-Máñez, Y. Yang, H. Lv and S. Li, *Chem. Soc. Rev.,* 2013, **42**, 6032; (f) J.-G. Yu, X.-H. Zhao, Y. Y. Yu, H. Yang, X.-Q. Chen and J.-H. Jiang, *Curr. Org. Synth.,* 2014, **11**, 377; (g) K. Wang, H. Peng and B. J. Wang, *Cell Biol.* 2014, **115**, 1007; (h) S. Wang, S. Shen, Y. Zhang, X. Dai and X. Zhao, *Chin. J. Org. Chem.,* 2014, **34**, 1717; (i) L.-Y. Niu, Y.-Z. Chen, H.-R. Zheng, L.-Z. Wu, C.-H. Tung and Q.-Z. Yang, *Chem. Soc. Rev.,* 2015, **44**, 6143.

6 (a) J. Yin, Y. Kwon, D. Kim, D. Lee, G. Kim, Y. Hu, J. H. Ryu and Y. Yoon, *J. Am. Chem. Soc.,* 2014, **136**, 5351; (b) M. Li, X. Wu, Y. Wang, Y. Li, W. Zhu and T. D. James, *Chem. Commun.,* 2014, **50**, 1751; (c) J. Liu, Y. Q. Sun, H. X. Zhang, Y. Y. Huo, Y. W. Shi and W. Guo, *Chem. Sci*., 2014, **5**, 3183; (d) L. Wang, H. Chen, H. Wang, F. Wang, S. Kambam, Y. Wang, W. Zhao and X. Chen, *Sens. Actuators, B,* 2014, **192**, 708; (e) F. Wang, L. Zhou, C. Zhao, R. Wang, Q. Fei, S. Luo, Z. Guo, H. Tian and W. H. Zhu, *Chem. Sci.,* 2015, **6**, 2584; (f) Q. Miao, Q. Li, Q. Yuan, L. Li, Z. Hai, S. Liu and G. Liang, *Anal. Chem*., 2015, **87**, 3460; (g) L. He, Q. Xu, Y. Liu, H. Wei, Y. Tang and W. Lin, *ACS Appl. Mater. Interfaces,* 2015, **7**, 12809; (i) J. Yin, Y. Kwon, D. Kim, D. Lee, G. Kim, Y. Hu, J. H. Ryu and Y. Yoon, *Nat. Protoc.*, 2015, **10**, 1742.

7 (a) J. Piao, J. Lv, X. Zhou, T. Zhao and X. Wu, *Spectrochim. Acta, Part A,* 2014, **128**, 475; (b) J. Huang, M. Liu, X. Ma, Q. Dong, B. Ye, W. Wang and W. Zeng, *RSC Adv*., 2014, **4**, 2296; (c) L. Y. Ma, J. Liu, L. Deng, M. Zhao, Z. Deng, X. Li, J. Tang and L. Yang, *Photochem. Photobiol. Sci.,* 2014, **13**, 1521; (d) M. Cao, L. Jiang, F. Hu, Y. Zhang, W. C. Yang, S. H. Liu and J. Yin, *RSC Adv.,* 2015, **5**, 23666; (e) X. Zhou, X. Wu and Y. Yoon, *Chem. Commun.,* 2015, **51**, 111; (f) Y. Yin, Y. Hu and Y. Yoon, *Chem. Soc. Rev.,* 2015, **44**, 4619.

8 (a) Z. Xu, Y. Xiao, X. Qian, J. Cui and D. Cui, *Org. Lett.,* 2005, **7**, 889; (b) Z. Xu, X. Qian and J. Cui, *Org. Lett.,* 2005, **7**, 3029; (c) Z. Li, C. Zhang, Y. Ren, J. Yin and S. H. Liu, *Org. Lett*., 2011, **13**, 6022.

9 Z. Wu and X. Tang, *Anal. Chem.,* 2015, **87**, 8613.

10 C. Zhao, X. Zhang, K. Li, S. Zhu, Z. Guo, L. Zhang, F. Wang, Q. Fei, S. Luo, P. Shi, H. Tian and W.-H. Zhu, *J. Am. Chem. Soc.*, 2015, **137**, 8490.

11 (a) H. Yu, Y. Xiao and L. Jin, *J. Am. Chem. Soc.,* 2012, **134**, 17486; (b) T. Liu, Z. Xu, D. R. Spring and J. Cui, *Org. Lett.*, 2013, **15**, 2310; (c) L. Yuan, L. Wang, B. K. Agrawalla, S. J. Park, H. Zhu, B. Sivaraman, J. Peng, Q. H. Xu and Y. T. Chang, *J. Am. Chem. Soc*., 2015, **137**, 5930.

Graphical Abstract