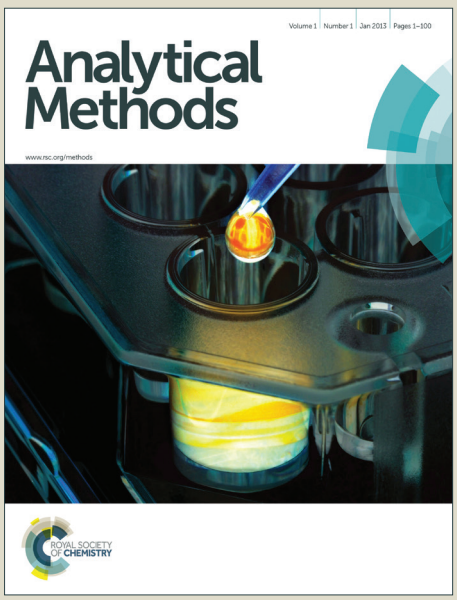
Analytical Methods

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/methods

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

A long-wavelength fluorescent turn-on probe for video detection of biological thiols in living cells

Wenqing Jiang,[a] Hua Chen,[a] Yue Pan[a], and Weiying Lin*[, a, b]

Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX

DOI: 10.1039/b000000x

We have designed and synthesized the compound FR-thiol based on a new far-red platform as a turn-on fluorescent probe for biological thiols. The turn-on probe FR-thiol displayed a high selectivity and sensitivity to thiols. Finally, we have demonstrated that FR-thiol is suitable for video observation of biological thiols in living cells.

Introduction

Small-molecular-weight biological thiols, including cysteine (Cys), homocysteine (Hcy), and glutathione (GSH), play a critical role in many biological processes[1]. However, abnormal levels of Cys, Hcy, and GSH are implicated in a variety of diseases, such as liver damage, skin lesions, slowed growth, edema,[2] Therefore, it is of intense interest for the detection of biological thiols with high sensitivity and selectivity.

So far, several analytical techniques, including high-performance liquid chromatography (HPLC),[3] capillary electrophoresis (CE),[4] electrochemical assay,[5] UV/Vis spectroscopy,[6] Fourier transform infrared (FTIR) spectroscopy,[7] mass spectrometry,[8] and fluorescence spectroscopy,[9-12] have been devoted to the detection of thiols. Although these methods provide sensitive analysis, they require complicated sample preparation, sophisticated instrumentation, or destruction of tissues or cells. By contrast, fluorescence sensing via microscopy is noninvasive.

Thus, the development of fluorescent probes for biological thiols is of high interest. However, fluorescent thiols probes with both maximal absorption and emission wavelength in the far-red or near-infrared region are relatively few[13], although they are highly desirable for biological imaging of thiols in living cells. Thus, it is necessary to construct long-wavelength fluorescent probes with favorable properties for monitoring thiols in living cells.

Herein, we rationally designed probe **FR-thiol** as a novel fluorescence turn-on probe for thiols. The probe exhibits large turn–on signal upon treatment with biological thiols. Importantly, we have demonstrated that the probe could detect thiol in biological samples.

Results and Discussion

Design and synthesis of probe FR-thiol

The protection/deprotection approach has been extended to cage fluorescent dyes to construct the fluorescent probes for the particular analyte[14]. In this work, we designed a fluorescent turn-on probe based on the protection/deprotection approach. As is well known, an ideal fluorescent probe consists of two parts, a recognition moiety with high selectivity for one kind of analyte only (responsible for the selective reaction with the target analyte) and a fluorescent dyes moiety that is highly sensitive to the environment (the analyte of interest and leading to the release of the free fluorescent molecule). As shown in Fig. 1, probe **FR-thiol** is composed of a new long-wavelength fluorescent dye and a 2,4-dinitrobenzenesulfonate group. The 2,4-dinitrobenzenesulfonate group was chosen for reaction site based on several considerations. Firstly, the 2,4-dinitrobenzenesulfonate group is a specific reaction site for thiol. Secondly, it can act as a protection group for this new dye. We thus anticipated that probe **FR-thiol** may be essentially non-fluorescent. However, upon reaction with thiol to release the 2,4-dinitrobenzenesulfonate unit, the fluorescence should be recovered.

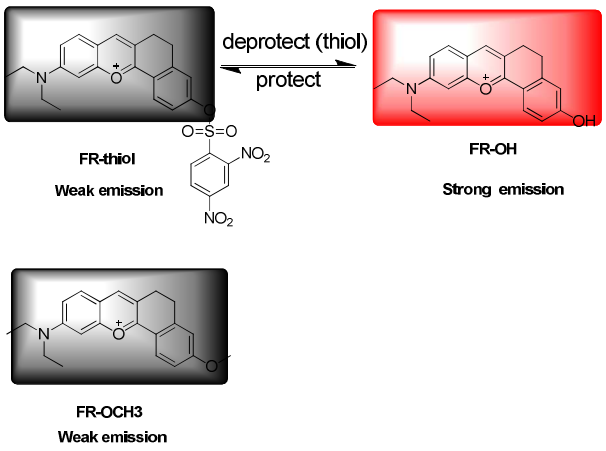


Fig. 1 The design concept of the fluorescent turn-on probe **FR-thiol,** and the molecular structure of reference compound **FR-OCH3**.

Probe **FR-thiol** was readily prepared in three steps (Scheme S1). Reference compound **FR-OCH3** was synthesized by sacid-catalyzed condensation reaction between benzocyclohexanone derivatives **1** and salicylaldehyde derivatives **2**. The staring materials **1** and **2** were commercially available. All new compounds were carefully characterized by ^{1}H NMR, ^{13}C NMR,

MS (ESI), and HRMS (ESI).

Optical properties

To further support our design concept of the fluorescent turn-on probe **FR-thiol**, we then proceeded to investigate their optical properties. The absorption and emission profiles of compounds **FR-OCH3** and **FR-OH** in distinct solvents (CH_3CH_2OH, PBS containing 10% CH_3CH_2OH) are shown in Fig. 2. Firstly, we investigated the spectral properties of **FR-OCH3** and **FR-OH** dyes in protic solvent CH_3CH_2OH. **FR-OCH3** and **FR-OH** dyes have a maximum absorption peak at around 561 nm and 594 nm, respectively. Fluorescence spectra of **FR-OH** exhibits strong fluorescence with a maximum emission peak at around 624 nm in CH_3CH_2OH. Reference compound **FR-OCH3**, which bears no electron-donating group, displays weak fluorescence. To get more insight into the photophysical properties of this **FR-OCH3** and **FR-OH** dyes, we continue to test the spectral properties of these dyes in PBS containing 10% CH_3CH_2OH. Upon excitation at 550 nm, compound **FR-OH** displays a strong emission bands at around 624 nm and **FR-OCH3** shows a weak emission (Fig. 2).

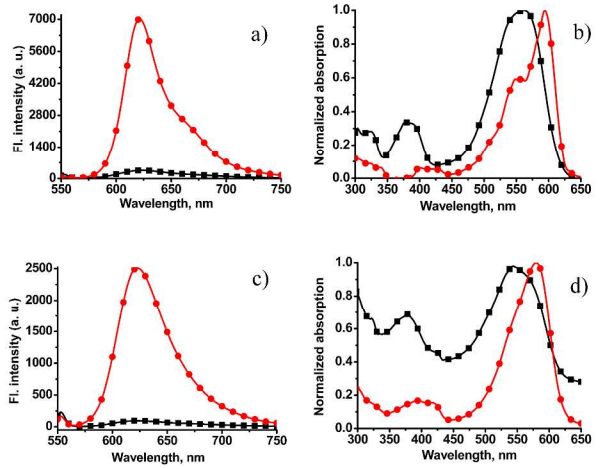


Fig. 2 (a) The fluorescence emission spectra of compounds **FR-OH** (●), and **FR-OCH3** (■) in CH_3CH_2OH; (b) The normalized absorption spectra of compounds **FR-OH** (●), and **FR-OCH3** (■) in CH_3CH_2OH; (c) The fluorescence emission spectra of compounds **FR-OH** (●), and **FR-OCH3** (■) in PBS; (d) The normalized absorption spectra of compounds **FR-OH** (●), and **FR-OCH3** (■) in PBS.

The molar extinction coefficients of the new **FR-OCH3** and **FR-OH** dyes are 39480 $M^{-1}cm^{-1}$ and 61920 $M^{-1}cm^{-1}$, respectively. Importantly, compound **FR-OH** has a fluorescence quantum yield of 0.57 in CH_3CH_2OH, which is relatively large for classical Rhodamine dyes. Furthermore, the fluorescence quantum yield of compound **FR-OCH3** is only 0.016, much less than that of compound **FR-OH**. This is attributed to the alkyloxy substituent of **FR-OCH3** which reduces the electron-donating ability of the oxygen atom and thus forbids the formation of the zwitterionic resonance form. Thus, these data suggest that the optical properties of new far-red dye **FR-OH** can be regulated by alkylation on the hydroxyl group.

To further understand the role of the hydroxyl group playing on the photophysical properties, we then examined the fluorescence profiles of the new **FR-OH** dye at different pH values. As shown in Figure S1, with the enhancement of pH from 3.0-10.0, the absorption band at around 550 nm undergoes a red-shift to a peak at around 584 nm. As shown in Fig. 3, The emission profiles of compound **FR-OH** is pH-dependent. Upon excitation at 550 nm, enhancement of pH from 3.0-10.0 induces a fluorescence turn-on signal in the far-red region (Fig. 3). The pK_a of compound **FR-OH** was calculated to be 6.45 based on the Henderson–Hasselbach-type mass action equation.[15]. These results implies the protection/deprotection approach can be extended to cage fluorescent dyes to construct the fluorescent probes.

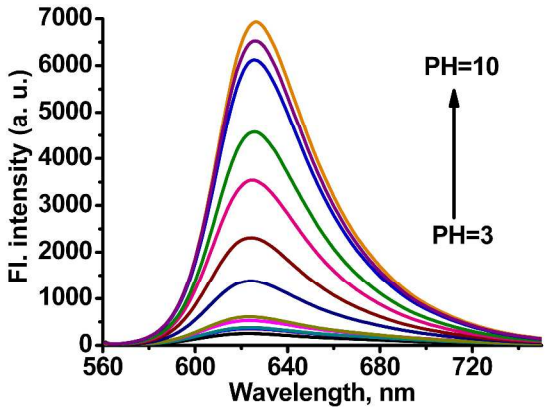


Fig. 3 pH-dependence of the emission spectra of compound **FR-OH** (5 μM) with the arrows indicating the change of the emission intensities with pH enhancement from 3 to 10.

Sensing of the probe to thiol

With the probe **FR-thiol** in hand, we firstly evaluated the capability of probe **FR-thiol** to detect cysteine in PBS. As designed, the free probe is almost non-fluorescent in PBS (Figure 4a). However, addition of cysteine induces a dramatic change in the fluorescence spectra. A significant fluorescence turn-on response at 624 nm (up to 20-fold) was observed. Consistently, a marked red-shift from 544 to 580 nm was noted in the absorption spectra upon treatment of the probe with cysteine (Figure S2). Both the mass spectrometry and NMR analyses confirmed that the fluorescence turn-on is indeed due to the thiol-mediated removal of the 2,4-dinitrobenzenesulfonate moiety (Figure S3 and Figure S4).[16] The detection limit for **FR-thiol** was calculated to be 1.87×10^{-6} M (Figure S5), indicating that the probe is highly sensitive to cysteine.

We also evaluated the capability of **FR-thiol** to detect other biological thiols (GSH) in PBS buffer.The titrations of the novel probe **FR-thiol** (5 μM) with GSH were conducted in PBS buffer (pH 7.4, containing 10% EtOH as a cosolvent). Fig 4b shows the fluorescence emission spectra (excitated at 550nm) of compounds **FR-thiol** treated with different concentrations of GSH. When the GSH concentration was increased, the intensity of the maximum emission peak at 624 nm progressively increased. A maximal fluorescence enhancement (about 11-fold) was obtained when the concentration of GSH reached 5.0 equiv. These data

demonstrate that the probe is capable of monitoring biological thiol in PBS.

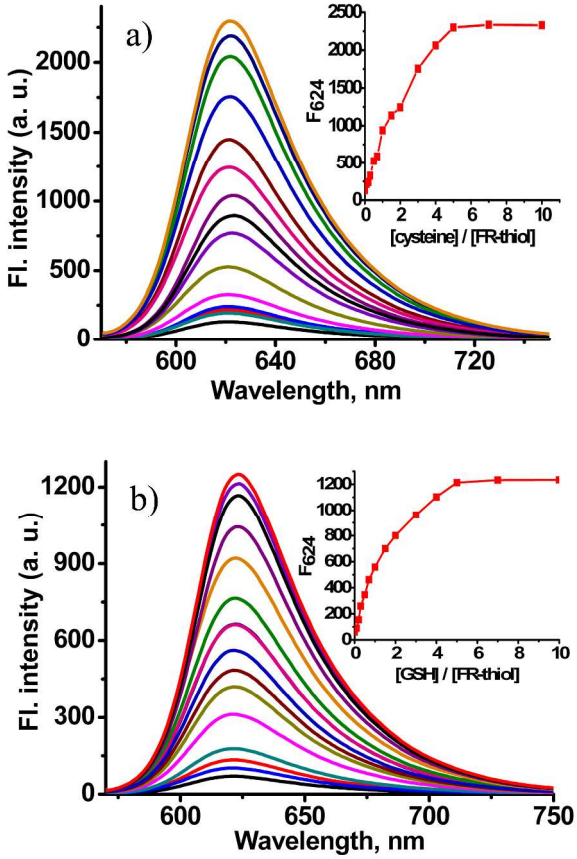


Fig. 4 (a) Emission spectra (excitated at 550 nm) of the novel probe **FR-thiol** (5 μM) in the presence of various amounts of cysteine (0-50μM) in PBS (pH 7.4, containing 10% EtOH as a cosolvent). Inset: fluorescence intensity changes at 624 nm of the probe (5 μM) with the amount of cysteine; (b) Emission spectra (excitatied at 550 nm) of the novel probe **FR-thiol** (5 μM) in the presence of various amounts of GSH (0-50μM) in PBS (pH 7.4, containing 10% EtOH as a cosolvent). Inset: fluorescence intensity changes at 624 nm of the probe (5 μM) with the amount of GSH.

Selectivity studies

To verify the specific response of probe **FR-thiol** toward biological thiols, we then examined the selectivity of the probe **FR-thiol** toward other disturb species. (such as representative amino acids, glucose, metal ions, reactive oxygen species, reducing agents, nucleosides, and small-molecule thiols) in buffer solution (25 mm phosphate buffer, pH 7.4) and monitored by emission spectroscopy. As exhibited in Fig. 5, introduction of Cys/Hcy/GSH to a solution of probe **FR-thiol** resulted in a visible increase of fluorescence intensity. However, no noticeable changes were observed upon addition of amino acids (Gly, Arg, Glu, Val, Ser, Leu, and Lys), metal ions (K^+, Ca^{2+}, Na^+, Mg^{2+}, and Zn^{2+}), a reactive oxygen species (hydrogen peroxide), a reducing agent (nicotinamide adenine dinucleotide (NADH)), or glucose, which indicates that the probe **FR-thiol** showed high selectivity to thiols over other interference ions examined.

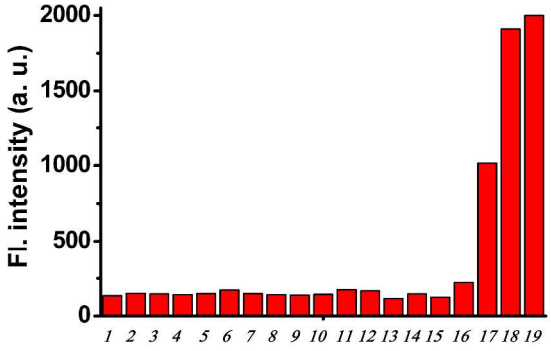


Fig. 5 Fluorescence probe **FR-thiol** (5 μM) in the presence of various analytes (25 μM) in PBS buffer (pH 7.4, containing 10% CH_3CH_2OH as a cosolvent), excitated at 550nm. including representative amino acids, metal ions, small-molecule thiols, reactive oxygen species, reducing agents, nucleosides, and glucose in buffer solution. 1. blank, 2. K^+, 3. Ca^{2+}, 4. Na^+, 5. Zn^{2+}, 6. Mg^{2+}, 7. H_2O_2, 8. Glucose. 9. NADH, 10. Gly, 11. Arg, 12. Glu, 13. Lys, 14 Leu, 15. Ser, 16. Val, 17 GSH, 18. Hcy, 19. Cys. Excitation at 550 nm. Emission at 624 nm.

To further examine the effective applications of the probe, the fluorescence responses of **FR-thiol** to cysteine in the presence of typical competing ions were studied. As shown in Fig. 6, most of competing ions only exhibited minimum interference in the detection of cysteine. The result showed that probe **FR-thiol** can selectively respond to cysteine even under competition from other related interference ions.

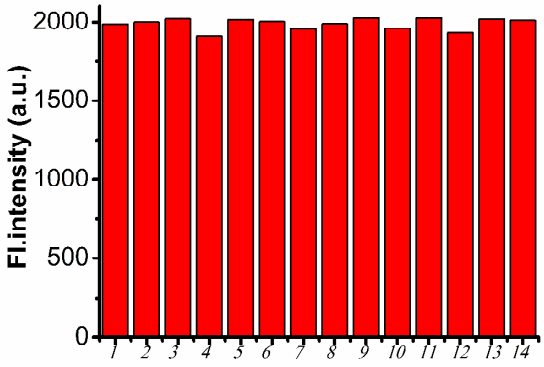


Fig. 6 Fluorescence response of probe **FR-thiol** (5 μM) to 5 equiv of cysteine in the presence of 5 equiv of different competing metal ions, Excitation at 550 nm. Emission at 624 nm. 1. K^+, 2. Ca^{2+}, 3. Na^+, 4. Zn^{2+}, 5. Mg^{2+}, 6. Glucose. 7. NADH, 8. Gly, 9. Arg, 10. Glu, 11. Lys, 12 Leu, 13. Ser, 14. Val.

Response time of probe FR-thiol

The time course of probe **FR-thiol** in the absence or presence of thiol was displayed in Figure. S5. Upon addition of thiol (100 μM) at room temperature, a dramatic enhancement in the fluorescence intensity at 624 nm was observed within 15 min, denoting the rapid reaction of probe **FR-thiol** with thiol. Probe **FR-thiol** responds rapidly to thiol (Figure S6), and the *pseudo*-first-order rate constant was calculated to be k = -0.2044 min^{-1} for cysteine and k = -3525 min^{-1} for GSH, respectively. (Figure S6),

These data indicated that the probe is highly rapid and may be suitable for studies of thiol in the living systems.

Detection of thiol in living cells

For the preliminary fluorescence imaging applications, the probe **FR-thiol** was incubated with the living HeLa cells pretreated with or without N-ethylmaleimide (as a thiol blocking agent). As shown in Fig. 7, the cells treated without N-ethylmaleimide (as a thiol blocking agent) exhibit strong fluorescence in the red channel. By contrast, when the cells pretreated with N-ethylmaleimide, then incubated with **FR-thiol** much weak fluorescence in the red channel was observed (Fig. 7). These data established that the probe **FR-thiol** is cell membrane permeable and can report biological thiols in the living cells. Furthermore, the results of the nuclear staining with Hoechst 33258 (Fig. 7) implied that the cells were alive during the imaging experiments.

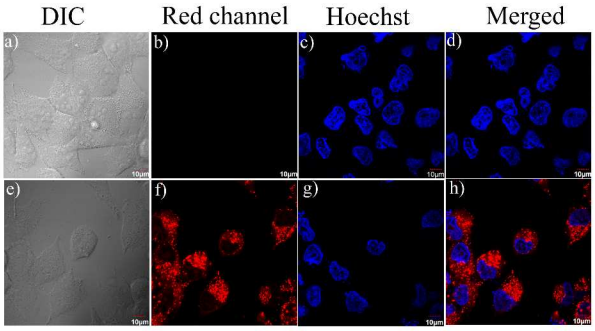


Fig. 7. (a-d) HeLa cells pre-incubated with N-ethylmaleimide (1 mM) for 30 min, and then co-incubated with **FR-thiol** (5 μM) and Hoechst 33258 (4.5 μM) for 30 min. (a) Brightfield image; (b) Emission from the red channel; (c) emission from the blue channel (nuclear staining); and (d) overlay of the blue and red channels. (e-h) HeLa cells only co-incubated with **FR-thiol** (5 μM) and Hoechst 33258 (4.5 μM) for 30 min. (e) Brightfield image; (f) Emission from the red channel; (g) emission from the blue channel (nuclear staining); and (h) overlay of the blue and red channels.

The **FR-OH** dye may be suitable for time-dependent video observation of biological thiols in living cells in light of their good optical properties. Toward this end, we successfully video image of **FR-thiol** responding to biological thiol in HeLa cells. As shown in Fig. 8, the fluorescence intensity in HeLa cells is time-dependent. Interestingly, this is consistent with the results of the time-dependent fluorescence changes of the probe upon incubation with biological thiols in the buffer (Figure S5). The HeLa cells were still alive after 30 min, indicating that the probe has minimum cytotoxicity (Fig. S7), in accordance with the results of the nuclear staining with Hoechst 33258 in living cells (Fig. 8).

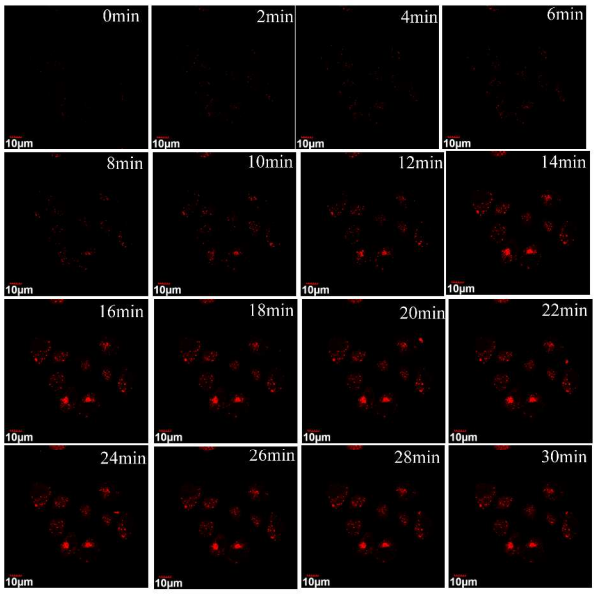


Fig. 8. Dynamic fluorescent pictures HeLa cells co-incubated with **FR-thiol**. Images were taken after only co-incubated with **FR-thiol** (5 μM).

Conclusions

In conclusion, a long-wavelength protection/deprotection dye platform was developed. Then, we have designed and synthesized probe **FR-thiol** as a new turn-on fluorescent probe for thiols based on this protection/deprotection dye platform. The turn-on probe **FR-thiol** displayed a high selectivity to thiols, and it is highly sensitive to thiols with a low detection limit. Furthermore, the turn-on probe can be employed to detect thiols in living cells. Finally, we have demonstrated that **FR-thiol** is suitable for time-dependent video detection of biological thiols in living cells.

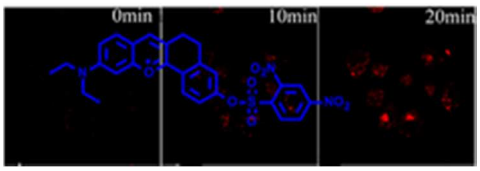
Notes and references

[a] *State Key Laboratory of Chemo/Biosensing and Chemometrics, College of Chemistry and Chemical Engineering, Hunan University, Changsha, Hunan 410082, China.*
[b] *Institute of Fluorescent Probes for Biological Imaging, School of Chemistry and Chemical Engineering, School of Biological Science and Technology, University of Jinan, Jinan, Shandong 250022, P.R. China.*
E-mail: Weiyinglin2013@163.com

† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

1. N. M. Giles, A. B. Watts, G. I. Giles, F. H. Fry, J. A. Littlechild and C. Jacob, *Chemistry & Biology*, 2003, **10**, 677.
2. (a) S. Shahrokhian, *Anal. Chem.*, 2001, **73**, 5972; (b) S. Seshadri, A. Beiser, J. Selhub, P. F. Jacques, I. H. Rosenberg, R. B. D' Agostino, P. W. F. Wilson and P. A. Wolf, *N. Engl. J. Med.*, 2002, **346**, 476; (c) A. Meister, *J. Biol. Chem.*, 1988, **263**, 17205.
3. (a) T. D. Nolin, M. E. Mc Menamin and J. Himmelfarb, *J. Chromatogr. B.*, 2007**, 852**, 554; (b) Y. Ogasawara, Y. Mukaib, T. Togawa, T. Suzuki, S. Tanabe and K. Ishii, *J. Chromatogr. B.*, 2007, **845**, 157.
4. (a) P. Ryant, E. Dolezelova, I. Fabrik, J. Baloun, V. Adam, P. Babula and R. Kizek, *Sensors.*, 2008, **8**, 3165; (b) G. Chen, L. Zhang and J. Wang, *Talanta.*, 2004**, 64**, 1018.

5. (a) S. D. Fei, J. H. Chen, S. Z. Yao, G. H. Deng, D. L. He and Y. F. Kuang, *Anal. Biochem.*, 2005**, 339**, 29; (b) P. R. Lima, W. J. R. Santos, R. d. C. S. Luz, F. S. Damos, A. B. Oliveira, M. O. F. Goulart and L. T. Kubota, *J. Electroanal. Chem.*, 2008, **612**, 87.
6. N. Shao, J. Y. Jin, S. M. Cheung, R. H. Yang, W. H. Chan and T. Mo, *Angew. Chem. Int. Ed.*, 2006, **45**, 4944.
7. Y. Sato, T. Iwata, S. Tokutomi and H. .Kando*ri, J. Am. Chem. Soc., 2005,* **127**, 1088.
8. J. M. Johnson, F. H. Strobel, M. Reed, J. Pohl and D. P. Jones, *Clin. Chim. Acta.*, 2008**, 396**, 43.
9. (a) X. Chen, Y. Zhou, X.Peng and J. Yoon, *Chem. Soc. Rev.*, 2010, **39**, 2120; (b) H. S. Jung, X. Chen, J. S. Kim and J. Yoon, *Chem. Soc. Rev.*, 2013, **42**, 6019; (c) C. Yin, F. Huo, J. Zhang, R. Martinez-Manez, Y. Yang, H. Lv and S. Li, *Chem. Soc. Rev.*,2013, **42**, 6032.
10. (a) L. Peng, Z. Zhou, R. Wei, K. Li, P. Song and A. Tong, *Dyes and Pigments*, 2014, **108**, 24; (b) H. Li, J. Fan, J. Wang, M. Tian, J. Du, S. Sun, P. Sun and X. Peng, *Chem. Commun.*, 2009, 5904. (c) X. Lou, Y. Hong, S. Chen, C. W. T. Leung, N. Zhao, B. Situ, J. W. Y. Lam and B. Z. Tang, *Scientific Reports*, 2014, **4**, 4272; (d) J. Liu, Y.-Q. Sun, Y. Huo, H. Zhang, L. Wang, P. Zhang, D. Song, Y. Shi and W. Guo, *J. Am. Chem. Soc.*, 2014, **136**, 574; (d) K. Cui, Z. Chen, Z. Wang, G. Zhang and D. Zhang, *Analyst*, 2011, **136**, 191.
11. (a) L. Wang, H. Chen, H. Wang, F. Wang, S. Kambam, Y. Wang, W. Zhao and X. Chen, *Sensors.*, 2014, **192**, 708; (b) X. Wang, J. Lv, X. Yao, Y. Li, F. Huang, M. Li, J. Yang, X. Ruan and B. Tang, *Chem. Commun.*, 2014, **50**, 15439; (c) F. Wang, J. An, L. Zhang and C. Zhao, *RSC Adv.*, 2014, **4**, 53437; (d) C. S. Lim, G. Masanta, H. J. Kim, J. H. Han, H. M. Kim and B. R.Cho, *J. Am. Chem. Soc.*, 2011, **133**, 11132; (e) J. Shao, H. Sun, H. Guo, S. Ji, J. Zhao, W. Wu, X. Yuan, C. Zhang and T. D. James, *Chem. Sci.*, 2012, **3**, 1049; (f) D. Lee, G. Kim, J. Yin and J. Yoon, *Chem. Commun.*, 2015, DOI: 10.1039/C5CC01071C; (g) L. Duan, Y. Xu, X. Qian, F. Wang, J. Liu and T. Cheng, *Tetrahedron Lett.*, 2008, **49**, 6624; (h) X. Wang, S.Wang and H. Ma, *Analyst*, 2008,**133**, 478.
12. (a) S. R. Malwal, A. Labade, A. S. Andhalkar, K. Sengupta and H. Chakrapani, *Chem. Commun.*, 2014, **50**, 11533; (b) B. Tang, Y. Xing, P. Li, N. Zhang, F. Yu and G. Yang, *J. Am. Chem. Soc.*, 2007, **129**, 11666; (c) F. Wang, L. Zhou, C. Zhao, R. Wang, Q. Fei, S. Luo, Z. Guo, H. Tian and W. Zhu, *Chem. Sci.*,2015, **6**, 2584; (d) J. Wu, R. Huang, C. Wang, W. Liu, J. Wang, X. Weng, T. Tian and X. Zhou, *Org. Biomol. Chem.*, 2013, **11**, 580; (e) X. F. Yang, Q. Huang, Y. Zhong, Z. Li, M. Lowry, J. O. Escobedo and R. M. Strongin, *Chem. Sci.*, 2014, **5**, 2177; (f) Z. Yang, N. Zhao, Y. Sun, F. Miao, Y. Liu, X. Liu, Y. Zhang, W. Ai, G. Song, X. Shen, X. Yu, J. Sun and W. Y. Wong, *Chem. Commun.*, 2012, **48**, 3442; (g) L. Y. Niu, Y. S. Guan, Y. Z. Chen, L. Z. Wu, C. H. Tung and Q. Z. Yang, *J. Am. Chem. Soc.*, 2012, **134**, 18928; (h) J. Lu, C. Sun, W. Chen, H. Ma, W. Shi and X. Li, *Talanta*, 2011, **83**, 1050; (i) S. Wu, Y. Song, Z. Li, Z. Wu, J. Han and S. Han, *Anal. Methods*, 2012, **4**, 1699; (j) Y. B. Ruan, A. F. Li, J. S. Zhao, J. S. Shen and Y. B. Jiang, *Chem. Commun.*, 2010, **46**, 4938.
13. (a) S. Xue, S. Ding, Q. Zhai, H. Zhang and G. Feng, *Biosensors and Bioelectronics*,2015, **68**, 316; (b) Y. S. Guan, L. Y. Niu, Y. Z. Chen, L. Z. Wu, C. H. Tung and Q. Z. Yang, *RSC Adv.*, 2014, **4**, 8360; (c) Z. Guo, S. W. Nam, S. Park and J. Yoon, *Chem. Sci.*, 2012, **3**, 2760; (d) S. Y. Lim, K. H. Hong, D. Kim, H. Kwon and H. J. Kim, *J. Am. Chem. Soc.*, 2014, **136**, 7018; (e) X. D. Jiang, J. Zhang, X. Shao and W. Zhao, *Org. Biomol. Chem.*, 2012, **10**, 1966; (f) F. Kong, R. Liu, R. Chu, X. Wang, K. Xu and B. Tang, *Chem. Commun.*, 2013, **49**, 9176; (g) D. Maity and T. Govindaraju, *Org. Biomol. Chem.*, 2013, **11**, 2098.
14. (a) N. Karton-Lifshin, L. Albertazzi, M. Bendikov, P. S. Baran and D. Shabat, *J. Am. Chem. Soc.*, 2012, **134**, 20412. (b) H. Chen, W. Lin, H. Cui and W. Jiang, *Chem. Eur. J*. 2015, **21**, 733.
15. The pKa was calculated according to the Henderson– Hasselbach-type mass action equation (log[(Fmax – F)/(F – Fmin)] = pKa – pH).
16. H. Maeda, H. Matsuno, M. Ushida, K. Katayama and K. Saeki, *Angew. Chem. Int. Ed.*, 2005, **44**, 2922.



19x6mm (300 x 300 DPI)