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A Bioluminescent Probe for Imaging Endogenous Hydrogen Polysulfides in Live Cells and Murine Model of Bacterial Infection†

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In this work, we report the first bioluminescent probe BP-PS for detecting H_2S_n with high specificity and sensitivity. Owing to the bioluminescence imaging without requiring an excitation light source, tissue autofluorescence is eliminated and BP-PS shows a high signal-to-noise ratio. Moreover, BP-PS was successfully utilized to visualize endogenous H_2S_n in live cells and murine model of bacterial infection.

Hydrogen polysulfides (H_2S_n, n > 1) are known as reactive sulfur species (RSS), playing prominent roles in biological systems.[1] Reactions caused by cystathionine β-synthase (CBS) or cystarhionine-γ-lyase (CSE) could generate endogenous H_2S_n.[2, 3] As redox form of H_2S, H_2S_n work with H_2S collectively to maintain the balance of sulfur redox. Though H_2S has been widely studied as a gasotransmitter, the functions of H_2S_n are still poorly understood. In some physiological activities, it was thought to be mediated by H_2S, while it may be actually mediated by H_2S_n. For example, H_2S was originally thought to react with cysteine residues to yield persulfides. Actually, this reaction is mainly mediated by H_2S_n.[4] Abnormally high concentrations of H_2S_n are found in inflammation, in which CSE is overexpressed.[5] Thus, H_2S_n are closely associated with diverse physiological and pathophysiological conditions. And, in order to better understand its role in biological processes, it is significant to develop accurate and sensitive tools for sensing and bioimaging of H_2S_n *in vivo*.

Conventional H_2S_n analytical methods, such as UV-vis spectroscopy,[6] are not sensitive and suitable for detection of H_2S_n in live cells and *in vivo*. To date, the use of fluorescent probe as a detection and imaging tool has numerous advantages, including simplicity, fast analysis, and real-time observation.[7-10] Although various fluorescent H_2S_n probes have been reported and applied to image H_2S_n in live cells or tissues,[11-20] they suffer from autofluorescence interference, limited exciting light penetration depth, phototoxicity or low photostability of photobleaching, limiting the *in vivo* applications.

Bioluminescence imaging is a powerful technique for visualizing biological events (such as the change of reactive species levels, the activity of an enzyme, the expression of a gene, or the tumor growth and metastasis) occurring in live cells or *in vivo*.[21-23] For firefly luciferase-luciferin system, the firefly luciferase (fLuc) catalyzes substrate D-luciferin into oxyluciferin to emit a visible light in the presence of ATP, O_2, and Mg^{2+}.[24] Compared with fluorescence imaging, bioluminescence imaging does not require external excitation which features its minimal phototoxicity, low autofluorescence and deep tissue penetration *in vivo* imaging.[25] To date, this tool has been developed for bioluminescence imaging of various analytes. The analytic objects have included metal ions,[26] small molecules,[27] reactive oxygen/nitrogen species,[28-30] and enzyme,[31] etc. With advantages of high signal-to-noise contrast and excellent biocompatibility, bioluminescence imaging holds great promise to realize highly sensitive detection of H_2S_n *in vivo*. However, bioluminescent H_2S_n probes are still unavailable, to our best knowledge.

Herein, we presented the first bioluminescent probe **BP-PS** for detecting H_2S_n *in vitro* and *in vivo*. We constructed **BP-PS** by installing a H_2S_n-responded moiety onto a fLuc substrate.[13] **BP-PS** can be converted to D-luciferin through reaction with H_2S_n, generating bioluminescence (Scheme 1a). **BP-PS** showed high specificity and sensitivity to H_2S_n. With excellent biocompatibility, we successfully applied **BP-PS** to visualize the change of H_2S_n in live cells. More importantly, **BP-PS** was used to visualize endogenous H_2S_n in bacterial infection of live mice.

BP-PS was successfully synthesized following the procedure showing in Scheme 1b and the structures of the compounds were verified by MS and NMR. The spectroscopic bioluminescence emission and absorption characteristics of the probe **BP-PS** for H_2S_n were carried out in PBS. As we expected, **BP-PS** itself only exhibited very weak bioluminescence signal. However a significant enhancement of bioluminescence intensity was observed after adding 100 μM Na_2S_4 to **BP-PS** solution (Fig. 1a). And the absorption wavelength of **BP-PS** red-shifted to 345 nm for blending with Na_2S_4 in the UV-vis spectrum as shown in Fig. 1b. The

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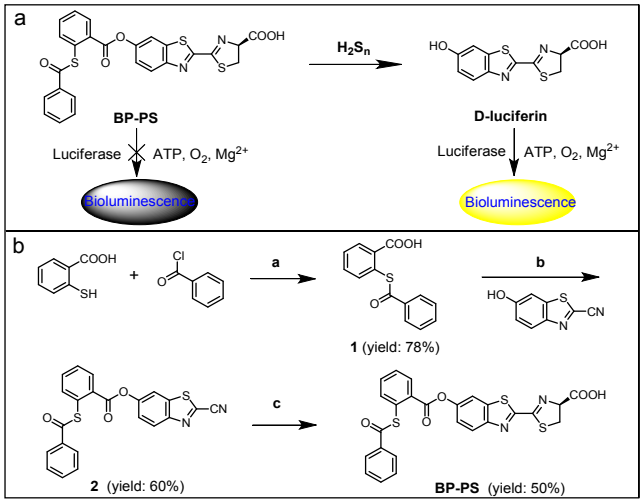

Scheme 1 (a) Strategy for the design of **BP-PS** for detecting H_2S_n. (b) Synthesis procedure of **BP-PS**. Reagent and reaction conditions: a) $NaHCO_3$, H_2O, 0 °C, 15 min, RT, 1 h; b) EDCI, DMAP, CH_2Cl_2, RT, 6 h; c) D-cysteine hydrochloride, K_2CO_3, CH_2Cl_2/MeOH (1:1, v/v), RT, 15 min.

increased bioluminescence intensity and red-shift in absorption spectra were presumably attributed to the release of D-luciferin caused by Na_2S_4-triggered reaction. We further confirmed our conjecture by HPLC analysis (Fig. S1, ESI†). The data showed that **BP-PS** (HPLC retention time, T_R =13.2 min) was converted to D-luciferin (T_R = 4.0 min). And ^{1}H NMR spectrometry analysis of the purified compound in mixed solution of **BP-PS** and Na_2S_4 corresponded to D-luciferin (Fig. S2, ESI†). These data verified the response mechanism of **BP-PS** to H_2S_n.

To study the sensitivity of **BP-PS**, we treated **BP-PS** with different concentrations of Na_2S_4 (0-200 μM) in black 96-well plate, then added fLuc solution into every set of solution. As shown in Fig. 2, with addition of 100 μM Na_2S_4, the bioluminescence intensity (total flux) was 7.3-fold higher than that without Na_2S_4 addition, although high concentration of Na_2S_4 may slightly affect the activity of fLuc (Fig. S3, ESI†). Notably, obvious bioluminescence intensity was still observed with Na_2S_4 concentration as low as 0.1 μM, indicating the high sensitivity of **BP-PS** for bioluminescence detection of Na_2S_4. The bioluminescence intensity showed a good linear relationship with the Na_2S_4 concentration in the range of 0.1 to 2 μM (Fig. 2b). The detection limit was calculated to be 30 nM (based on 3σ rule). The concentration of endogenous H_2S_n has been reported to be in the nanomolar levels.[32] Therefore, **BP-PS** is promising to visualize endogenous H_2S_n in living systems.

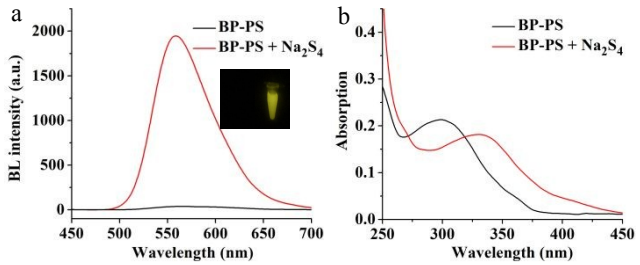

Fig. 1 (a) Bioluminescence spectra of 20 μM **BP-PS** in PBS with 10 mM $MgCl_2$, 2 mM ATP and fLuc before (black) and after (red) Na_2S_4 (100 μM) treatment. (b) Absorbance of 20 μM **BP-PS** in PBS before (black) and after (red) Na_2S_4 (100 μM) treatment for 30 min. Inset of (a): A photo of **BP-PS** (left) and **BP-PS** after Na_2S_4 treatment (right) after addition of fLuc solution.

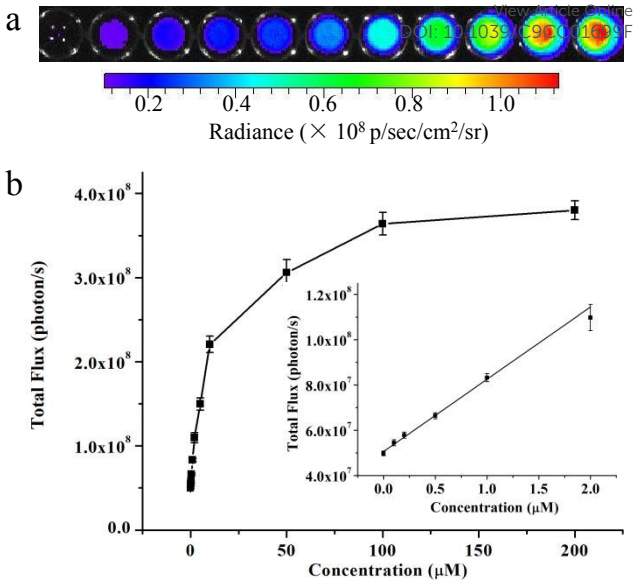

Fig. 2 (a) Bioluminescence images of 20 μM **BP-PS** to different Na_2S_4 concentrations (0, 0.1, 0.2, 0.5, 1, 2, 5, 10, 50, 100, 200 μM). (b) Quantification of bioluminescence signal intensity with different Na_2S_4 concentrations. Inset of (b): The fitted calibration line between the total flux and Na_2S_4 concentration.

As we know, selectively recognizing its target analyte is an essential feature for a probe. To further evaluate whether **BP-PS** had a much higher selectivity for H_2S_n relative to other biologically relevant analytes, we incubated 20 μM **BP-PS** with H_2S_n and other potentially interfering species in 96-well plate. As shown in Fig. 3, we observed high turn-on bioluminescence intensity in the well containing of H_2S_n and no obvious increase in the bioluminescence intensity in other wells. It indicated that our probe **BP-PS** had an excellent selectivity for H_2S_n and possibility of detecting H_2S_n in real biological samples.

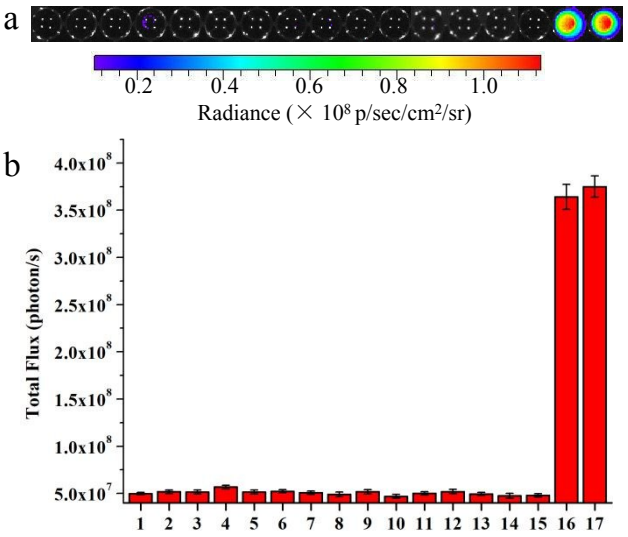

Fig. 3 (a) Bioluminescence images of 20 μM **BP-PS** with different species (2-14: 200 μM): (1) **BP-PS** only, (2) NaClO, (3) H_2O_2, (4) H_2S, (5) HNO, (6) NO, (7) SO_3^{2-}, (8) $ONOO^-$, (9) $O_2^{\bullet-}$, (10) ascorbic acid, (11) Cys, (12) Hcy, (13) GSSG, (14) $S_2O_3^{2-}$, (15) GSH (5 mM), (16) Na_2S_2 (100 μM) and (17) Na_2S_4 (100 μM). (b) Quantification of bioluminescence signal intensity of **BP-PS** with different species.

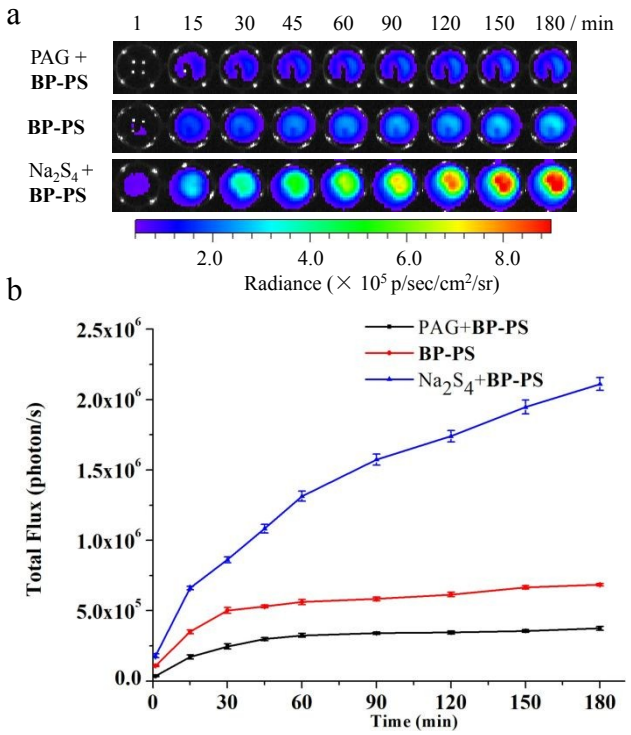

Fig. 4 (a) Bioluminescence images of MDA-MB-231-luc cells preincubated with PAG (100 μM) for 30 min and then with 20 μM **BP-PS** (first row), incubated with 20 μM **BP-PS** (second row), preincubated with Na_2S_4 (100 μM) and then with 20 μM **BP-PS** (third row). Bioluminescence images were captured at 1, 15, 30, 45, 60, 90, 120, 150, and 180 min. (b) bioluminescence signal intensity quantification of the bioluminescence images.

It is known that the biocompatibility is a precondition for probe **BP-PS** to monitor H_2S_n in living systems. MTT assays were conducted to assess its potential cytotoxicity. The data in Fig. S4 (ESI†) showed that the cells viability had no marked change even incubating the cells with high concentration of **BP-PS** (200 μM) for 24 and 48 h. These results indicated that at the tested concentrations, **BP-PS** possesses no significant cytotoxicity. Then, we used **BP-PS** to monitor the changes of H_2S_n in the cells. Hydrogen polysulfide-derivatives are known to be generated from L-cystine by CSE.[2, 3] In addition, it is observed that CSE was expressed in MDA-MB-231 cells.[33, 34] We treated the cells with PBS, Na_2S_4 or PAG (CSE inhibitor),[35] respectively. Then we added the same doses of **BP-PS** into the wells. The plates were imaged for 180 min. As shown in Fig. 4a, we found that the cells incubated with **BP-PS** exhibited obvious light production. And the bioluminescence signal increased with the incubation time increasing from 1 to 180 min. The results indicated that **BP-PS** was potential to detect of endogenous H_2S_n in live cells. For further confirmation, cells were preincubated with Na_2S_4 and PAG, respectively. With addition of Na_2S_4, a further increase in the bioluminescence intensity was measured compared to that of adding **BP-PS** only. It can be explained by the increased level of H_2S_n resulted in increased bioluminescence intensity. With addition of PAG, reduction of H_2S_n generation leads to decreased bioluminescence intensity (Fig. 4b). Again, when cells were treated with the same doses of PAG and D-luciferin, bioluminescence signal exhibits no differences (Fig. S5a, b, ESI†). All these demonstrated that **BP-PS** had the ability to monitor H_2S_n levels in live cells.

We further applied **BP-PS** for bioluminescence imaging H_2S_n *in vivo*. As an inflammatory mediator, LPS can induce the CSE overexpression and H_2S_n could be generated enzymatically by CSE. After administration of LPS for 12h, fLuc and **BP-PS** were locally injected (Fig. 5a). Then we measured the bioluminescence signal and observed an obvious enhancement in LPS-stimulated inflammation tissues in contrast with the normal tissues (Fig. 5b). Previous studies suggested that bacterial infection can cause severe inflammation.[36] Furthermore, a murine model of bacterial infection was established by subcutaneous injection of *E. coli* into right legs of mice (Fig. 5d). And after 12 h, we found that the tissues infected with *E. coli* displayed an obviously enhanced bioluminescence signal in contrast with the normal tissues (Fig. 5e). Moreover, when the inflammation tissues were first treated with NEM (a RSS scavenger), the bioluminescence signal was significantly weakened (Fig. S6, ESI†). Taken together, these results proved that probe **BP-PS** could be used to visualize endogenous H_2S_n in live inflamed and bacterial infected mice.

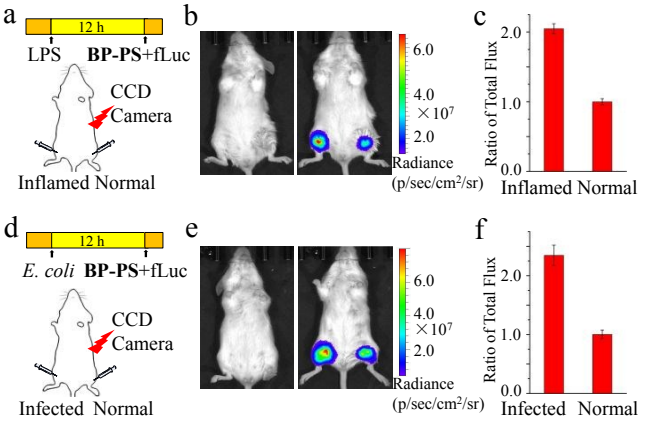

Fig. 5 (a) LPS was injected into right hind leg of each mouse. 12 h later, the mice were locally injected with fLuc and **BP-PS**. (b) Bioluminescence images of mice pre- (left) and post-injection (right) of fLuc and **BP-PS**. (c) Ratio of bioluminescence emission intensity at normal and inflamed areas in b (right). (d) 100 μL of *E. coli* (5×10^6 CFU mL^{-1}) was injected into right hind leg of each mouse. 12 h later, the mice were locally injected with fLuc and **BP-PS**. (e) Bioluminescence images of mice pre- (left) and post-injection (right) of fLuc and **BP-PS**. (f) Ratio of bioluminescence emission intensity at normal and bacterial infected areas in e (right).

In conclusion, we describe the first bioluminescent probe **BP-PS** for detecting H_2S_n. Upon incorporating a H_2S_n sensitive moiety onto firefly luciferin, **BP-PS** is converted to free D-luciferin after reaction with H_2S_n, resulting in a bioluminescence response in the presence of fLuc. Owing to the bioluminescence without excitation light, autofluorescence is eliminated and **BP-PS** was effectively applied to the detection and imaging of H_2S_n at nanomolar concentration with high signal-to-noise ratio. Additionally, with excellent biocompatibility, **BP-PS** allowed for bioluminescence imaging of endogenous H_2S_n in live cells and murine model of inflammation induced by LPS and bacterial infection. With these results, we believe that **BP-PS** will be a powerful and promising tool for finding

the biological functions of H_2S_n in living systems.

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Conflicts of interest

There are no conflicts to declare.

Notes and references

1. T. V. Mishanina, M. Libiad and R. Banerjee, *Nat. Chem. Biol.*, 2015, **11**, 457-464.
2. P. K. Yadav, M. Martinov, V. Vitvitsky, J. Seravalli, R. Wedmann, M. R. Filipovic and R. Banerjee, *J. Am. Chem. Soc.*, 2015, **138**, 289-299.
3. T. Ida, T. Sawa, H. Ihara, Y. Tsuchiya, Y. Watanabe, Y. Kumagai, M. Suematsu, H. Motohashi, S. Fujii, T. Matsunaga, M. Yamamoto, K. Ono, N. O. Devarie-Baez, M. Xian, J. M. Fukuto and T. Akaike, *Proc. Natl. Acad. Sci. U. S. A.*, 2014, **111**, 7606-7611.
4. Y. Kimura, Y. Mikami, K. Osumi, M. Tsugane, J.-i. Oka and H. Kimura, *FASEB J.*, 2013, **27**, 2451-2457.
5. X.-Y. Zhu, S.-J. Liu, Y.-J. Liu, S. Wang and X. Ni, *Cell. Mol. Life Sci.*, 2010, **67**, 1119-1132.
6. R. Greiner, Z. Pálinkás, K. Bäsell, D. Becher, H. Antelmann, P. Nagy and T. P. Dick, *Antioxid. Redox Signal.*, 2013, **19**, 1749-1765.
7. D. Wu, A. C. Sedgwick, T. Gunnlaugsson, E. U. Akkaya, J. Yoon and T. D. James, *Chem. Soc. Rev.*, 2017, **46**, 7105-7123.
8. J. Qian and B. Z. Tang, *Chem*, 2017, **3**, 56-91.
9. W. Sun, S. Guo, C. Hu, J. Fan and X. Peng, *Chem. Rev.*, 2016, **116**, 7768-7817.
10. Z. Mao, M. Ye, W. Hu, X. Ye, Y. Wang, H. Zhang, C. Li and Z. Liu, *Chem. Sci.*, 2018, **9**, 6035-6040.
11. W. Chen, E. W. Rosser, D. Zhang, W. Shi, Y. Li, W.-J. Dong, H. Ma, D. Hu and M. Xian, *Org. Lett.*, 2015, **17**, 2776-2779.
12. N. Gupta, S. I. Reja, V. Bhalla and M. Kumar, *Org. Biomol. Chem.*, 2017, **15**, 6692-6701.
13. W. Chen, E. W. Rosser, T. Matsunaga, A. Pacheco, T. Akaike and M. Xian, *Angew. Chem. Int. Ed.*, 2015, **54**, 13961-13965.
14. J. Zhang, X.-Y. Zhu, X.-X. Hu, H.-W. Liu, J. Li, L. L. Feng, X. Yin, X.-B. Zhang and W. Tan, *Anal. Chem.*, 2016, **88**, 11892-11899.
15. Y. Fang, W. Chen, W. Shi, H. Li, M. Xian and H. Ma, *Chem. Commun.*, 2017, **53**, 8759-8762.
16. J. Ma, J. Fan, H. Li, Q. Yao, F. Xu, J. Wang and X. Peng, *J. Mater. Chem. B*, 2017, **5**, 2574-2579.
17. W. Chen, A. Pacheco, Y. Takano, J. J. Day, K. Hanaoka and M. Xian, *Angew. Chem. Int. Ed.*, 2016, **55**, 9993-9996.
18. M. Gao, R. Wang, F. Yu and L. Chen, *Biomaterials*, 2018, **160**, 1-14.
19. K. Umezawa, M. Kamiya and Y. Urano, *Angew. Chem. Int. Ed.*, 2018, **57**, 9346-9350.
20. M. Gao, R. Wang, F. Yu, J. You and L. Chen, *J. Mater. Chem. B*, 2018, **6**, 2608-2619.
21. R. S. Negrin and C. H. Contag, *Nat. Rev. Immunol.*, 2006, **6**, 484-490.
22. S. Iwano, M. Sugiyama, H. Hama, A. Watakabe, N. Hasegawa, T. Kuchimaru, K. Z. Tanaka, M. Takahashi, Y. Ishida and J. Hata, *Science*, 2018, **359**, 935-939.
23. J. A. Prescher and C. H. Contag, *Curr. Opin. Chem. Biol.*, 2010, **14**, 80-89.
24. S. M. Marques and J. C. Esteves da Silva, *IUBMB life*, 2009, **61**, 6-17.
25. L. Mezzanotte, M. van't Root, H. Karatas, E. A. Goun and C. W. Löwik, *Trends Biotechnol.*, 2017, **35**, 640-652.
26. A. T. Aron, M. C. Heffern, Z. R. Lonergan, M. N. Vander Wal, B. R. Blank, B. Spangler, Y. Zhang, H. M. Park, A. Stahl and A. R. Renslo, *Proc. Natl. Acad. Sci. U. S. A.*, 2017, **114**, 12669-12674.
27. M. Zhang, L. Wang, Y. Zhao, F. Wang, J. Wu and G. Liang, *Anal. Chem.*, 2018, **90**, 4951-4954.
28. H. Takakura, R. Kojima, M. Kamiya, E. Kobayashi, T. Komatsu, T. Ueno, T. Terai, K. Hanaoka, T. Nagano and Y. Urano, *J. Am. Chem. Soc.*, 2015, **137**, 4010-4013.
29. Y. Nam, B. S. Kim and I. Shin, *Chem. Commun.*, 2016, **52**, 1128-1130.
30. J.-B. Li, L. Chen, Q. Wang, H.-W. Liu, X.-X. Hu, L. Yuan and X.-B. Zhang, *Anal. Chem.*, 2018, **90**, 4167-4173.
31. S. Li, R. Hu, C. Yang, X. Zhang, Y. Zeng, S. Wang, X. Guo, Y. Li, X. Cai and S. Li, *Biosens. Bioelectron.*, 2017, **98**, 325-329.
32. F. Yang, H. Gao, S.-S. Li, R.-B. An, X.-Y. Sun, B. Kang, J.-J. Xu and H.-Y. Chen, *Chem. Sci.*, 2018, **9**, 5556-5563.
33. N. Velusamy, A. Binoy, K. N. Bobba, D. Nedungadi, N. Mishra and S. Bhuniya, *Chem. Commun.*, 2017, **53**, 8802-8805.
34. S. Sen, B. Kawahara, D. Gupta, R. Tsai, M. Khachatryan, S. Roy-Chowdhuri, S. Bose, A. Yoon, K. Faull and R. Farias-Eisner, *Free Radical Biol. Med.*, 2015, **86**, 228-238.
35. V. S. Lin, A. R. Lippert and C. J. Chang, *Proc. Natl. Acad. Sci. U. S. A.*, 2013, **110**, 7131-7135.
36. Q. Chen, C. Liang, X. Sun, J. Chen, Z. Yang, H. Zhao, L. Feng and Z. Liu, *Proc. Natl. Acad. Sci. U. S. A.*, 2017, **114**, 5343-5348.