RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

A Ratiometric Fluorescent Probe for Rapid and Sensitive Visualizing Hypochlorite in Living Cells

Jiayu Zha,[a] Boqiao Fu,[b] Caiqin Qin,[b] Lintao Zeng*[a,b] and Xichao Hu*[b]

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

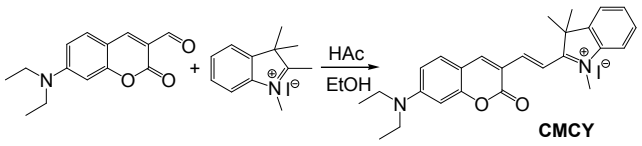
DOI: 10.1039/b000000x

A ratiometric fluorescent probe for ClO^- has been developed based on coumarin–hemicyanine, which displayed colorimetric and fluorescence response to ClO^- with high selectivity, fast response (within 2 min) and extremely low detection limit (0.08 μM). This probe was successfully used to visualize hypochlorite in living cells.

Hypochlorite anion (ClO^-)/hypochlorous acid (HOCl), a biologically important reactive oxygen species (ROS), is generally produced in living organisms from the myeloperoxidase (MPO)-mediated peroxidation of chloride ions and hydrogen peroxide. [1] Hypochlorite anion (ClO^-) plays a key role in human immune defense system and inflammation by destruction of the invading bacteria and pathogens.[2] Whereas, excessive or misplaced production of ClO^- can lead to tissue damage and diseases, such as atherosclerosis[3], arthritis,[4] cancer,[1b] and neurodegeneration.[5] Therefore, it is of great importance to investigate the complex contributions of HOCl/ClO^- to our health and study the mechanism of action and specific functions of HOCl in living organisms. Scientists have conducted extensive research to elucidate the mechanism by which HOCl kills bacteria and destroys human tissue.[6] However, the detailed understanding of HOCl formation during pathogenic biological events still remains a challenge due to the lack of methods for monitoring HOCl in living organisms.[7]

Fluorescence-based assays are useful tools for real-time sensing and visualizing some biologically important species in the living organisms because of their high sensitivity, high temporal and spatial resolution.[8] Several fluorescent probes for the detection and visualization of HOCl in living cells have been developed on the basis of HOCl-mediated oxidation reaction of various functional groups such as *p*-methoxyphenol,[9] ether,[10] thioether,[11] oxime,[12] and hydroxamic acid.[13] However, most of these probes just show changes in emission intensity, which is usually interfered by environmental condition, probe distribution, and instrumental efficiency.[14] By contrast, a ratiometric probe which utilizes the ratio of two emissions at different wavelengths as the detecting signal,[15] can provide a built-in correction for the above mentioned factors and thus allow more accurate analysis. Fluorescence resonance energy transfer (FRET) mechanism is an effective approach for construction of a ratiometric fluorescent probe. This strategy has been successfully employed to design a few of ratiometric fluorescent probes for ClO^- and achieved good performance.[16,17] Nevertheless, it is difficult to construct such a system because of the complicated synthetic routes as well as the requirement of strong spectral overlap between emission of donor and absorption of acceptor. Thus, it is highly desired to develop a ratiometric probe for ClO^- based on a simple and efficient approach.

Diethylamino-coumarin dye possesses several favourable fluorescence properties, such as excitation and emission wavelengths in the visible region as well as a high fluorescence quantum yield, which are desirable for the intended biological applications of probe. In this work, we chose diethylamino-coumarin aldehyde as one of a building block, and then modified it by 1, 2, 3, 3-tetramethyl-3*H*-indolium iodide to produce a water soluble **CMCY** with long emission wavelength (shown in Scheme 1). The structure of **CMCY** was confirmed by ^{1}H NMR, ^{13}C NMR and HR-MS. It is well known that hypochlorite anion (ClO^-)/hypochlorous acid (HOCl) have strong reactivity to double bond. We expect that ClO^- will react with double bond and destroy the large π-conjugation of probe, giving rise to a colorimetric and ratiometric fluorescence response to ClO^-. This hybrid coumarin–hemicyanine probe exhibited an emission maximum at 632 nm. Upon addition of ClO^-, the colour of the resultant solution changed from purple to colourless and the fluorescence of solution changed from red to blue.



Scheme 1 Synthesis of probe

To examine the response of **CMCY** to ClO^-, UV-vis absorption spectra titrations were performed in PBS/MeOH (pH 7.4, v/v = 9:1) at ambient temperature. As shown in Fig. 1, free probe displayed a strong absorption band centred at 570 nm. Upon addition of ClO^-, the absorption band centred at 570 nm was gradually decreased and a new absorption band at 392 nm emerged with a well-defined isosbestic point at 455 nm. As a result, an obvious colour change from purple to colourless was observed, allowing colorimetric detection of ClO^- by the naked eyes (Fig. 1a inset). When the amount of ClO^- reached 20 equivalents with respect to the probe, these changes were found to reach a plateau. A linear calibration of the UV-vis absorption response to ClO^- concentrations from 0 to 73 μM was obtained (Fig. 2a), indicating that **CMCY** could be potentially used to

quantitatively detect ClO^- concentrations. We further examined the time course of the ratiometric response of **CMCY** to ClO^- (Fig. 3a). **CMCY** was highly stable under the assay conditions. However, upon addition of 200 μM ClO^-, a dramatic colour change from purple to colourless and a large increase in the ratio were observed within 2 min. This finding suggested that **CMCY** was a fast-response probe for ClO^- and might be suitable for real-time sensing of ClO^- in living cells.

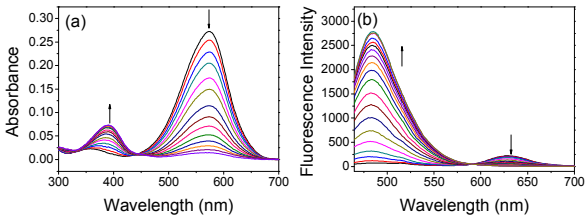


Fig. 1 (a) UV−vis absorption responses of **CMCY** (10 μM) to ClO^- (0 to 200 μM) in 50 mM PBS solution (pH 7.4). Inset: The colour of **CMCY** solution (10 μM) changed from purple to colourless; (b) Fluorescence responses of **CMCY** (10 μM) to ClO^- (0 to 200 μM) in 50 mM PBS solution (pH 7.4), λ_{ex} = 460 nm. Inset: The fluorescence of **CMCY** (10 μM) changed from red to blue.

As shown in Fig. 1b, **CMCY** displayed a characteristic emission band centred at 631 nm. Upon addition of increasing amount of ClO^-, the fluorescence intensity at 631 nm gradually decreased and accompanied with a new emission band centred at 480 nm. Consequently, the fluorescence of **CMCY** solution changed from red to blue. In the presence of 20 equiv. ClO^-, the ratio of emission intensity (I_{480}/I_{631}) showed a drastic enhancement (ca. 400-fold enhancement), establishing that **CMCY** could serve as a ratiometric fluorescent probe for ClO^-. It is worthy to note that such a huge change of signal ratios at two wavelengths is highly desirable for ratiometric fluorescent probes, as the sensitivity and the dynamic range of ratiometric probes are controlled by the ratios. By plotting the ratio of emission intensity (I_{480}/I_{631}) versus the concentration of ClO^-, a good linear relationship (R^2 = 0.9932) was obtained with the ClO^- concentration ranging from 0 to 73 μM (shown in Fig. 2b). The detection limit of probe **CMCY** for ClO^- was determined to be 0.08 μM according to the signal-to-noise ratio (S/N = 3), which was comparable to the previously reported ratiometric fluorescent probes for ClO^-.[16,18]

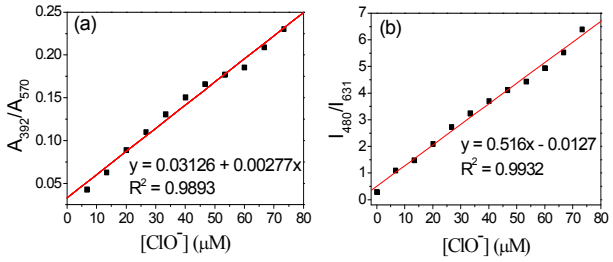


Fig. 2 (a) Absorbance changes and (b) fluorescence intensity changes of **CMCY** (10 μM) versus concentrations of ClO^- in PBS solution (pH 7.4). The data were collected after ClO^- was added into the **CMCY** (10 μM) solution for 3 minute.

The ratiometric fluorescence responses of **CMCY** to ClO^- at different pH values were also investigated, as were shown in Fig. 3b. In the absence of ClO^-, **CMCY** did not display any fluorescence changes over a wide range of pH values from 4.0 to 7.8. However, in the presence of 200 μM ClO^-, a very large ratiometric fluorescence enhancement was observed from pH 6.5 to 7.8, which indicated that **CMCY** would be suitable for bio-applications at the physiological condition.

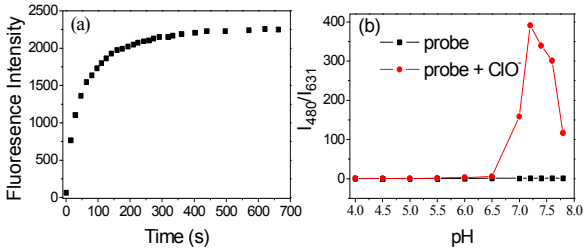


Fig. 3 (a) Time-dependent fluorescence responses of **CMCY** (10 μM) to ClO^- (200 μM) in PBS solution (50 mM, pH 7.4); (b) The ratiometric fluorescence responses of probe (10 μM) to ClO^- (200 μM) at different pH values.

Good selectivity is a crucial requirement for all kinds of detection methods. To test the selectivity, **CMCY** (10 μM) was incubated with 10 equiv. of representative species including ROS, anions and ions. As shown in Fig. 4, a large ratiometric signal with I_{480}/I_{631} = 15.7 was observed in the presence of 10 equiv. of ClO^-. By contrast, other ROS species (hydrogen peroxide, hydroxyl radical, superoxide, *t*-BuOOH and $ONOO^-$) induced a very weak ratiometric response with I_{480}/I_{631} < 0.5. Some common anions (CH_3COO^-, F^-, ClO_4^-, PO_4^{3-}, HCO_3^-, NO_3^-, SO_3^{2-}, NO_2^- and SO_4^{2-}) and heavy metal ions (Hg^{2+}, Cu^{2+}) caused only negligible changes in the emission ratio (I_{480}/I_{631} < 0.2). These results suggest that the probe has excellent selectivity for ClO^- over other anions and ROS species. Although ozone is a very strong oxidation reagent and can break alkene to give aldehyde and carboxylic acid, it also easily oxidize many biological species including GSH, protein and antibody. As we known, the content of GSH, protein and antibody are very rich in living cells. As a result, ozone has been largely consumed by these biological species, and thus have litter interference on our probe. Therefore, the probe **CMCY** has potential applications for ClO^- detection in complex biological environments.

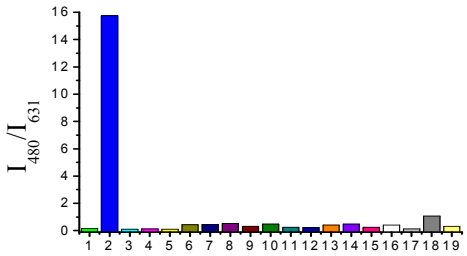
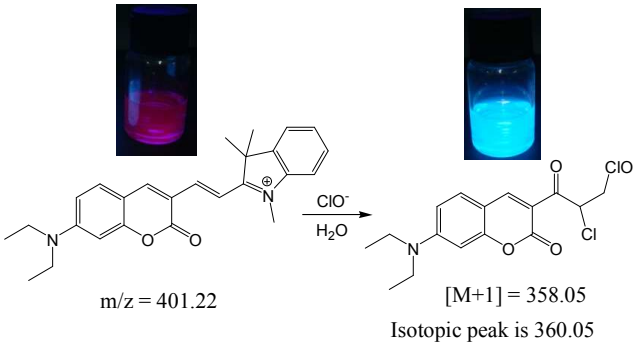


Fig. 4 Fluorescence intensity ratio (I_{480}/I_{631}) of **CMCY** (10 μM in PBS solution, pH 7.4) in the presence of various species (100 μM). 1, blank; 2, ClO^-; 3, HCO_3^-; 4, NO_3^-; 5, CH_3COO^-; 6, SO_3^{2-}; 7, F^-; 8, ClO_4^-; 9, PO_4^{3-}; 10, NO_2^-; 11, Cu^{2+}; 12, Hg^{2+}; 13, GSH; 14, Cys; 15, *t*-BuOOH; 16, hydroxyl radicals; 17, $ONOO^-$; 18, H_2O_2; 19, O_2^-; λ_{ex} = 460 nm.

To get insight into the sensing mechanism, **CMCY** was treated with 20 equiv. ClO^- in PBS for 30 min, and then the major fluorescent product was separated by silica gel column for

MS analysis. The ESI-MS (+ mode) showed the presence of a dominant peak at m/z = 358.0976 and a noticeable isotopic peak at 360.0947, which was corresponding to a coumarin derivative shown in Scheme 2 and Fig. S2†. Hence, spectral changes in the presence of ClO^- were originated from the oxidative cleavage of **CMCY** by ClO^-.



Scheme 2 The proposed mechanism for sensing of ClO^-

Furthermore, we explored the potential application of **CMCY** for sensing ClO^- in living cells. HeLa cells incubated with **CMCY** (5 μM) for 30 min exhibited clear cell profile with red colour (Fig. 5b), which indicated that **CMCY** has penetrated into the cells and retained. When the HeLa cells were further treated with 100 μM ClO^- for 30 minutes, blue fluorescence from HeLa cells was observed (shown in Fig. 5e). Meanwhile, the original red fluorescence from **CMCY** was not detected, implying that **CMCY** has been consumed by ClO^- and produced some products with blue fluorescence. Cell staining results indicated that probe **CMCY** was cell membrane permeable and capable of imaging of ClO^- in the living cells.

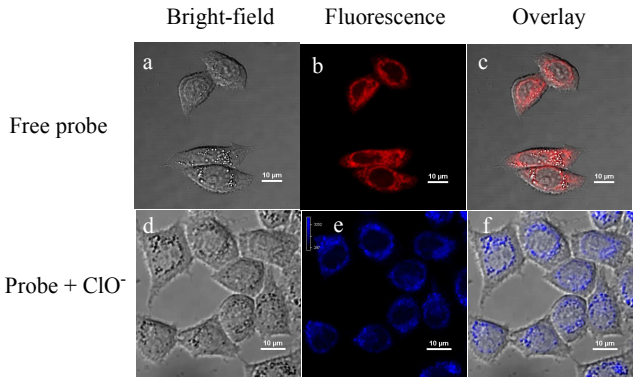


Fig. 5 Confocal fluorescence images of HeLa cells. Cells were incubated with **CMCY** (5 μM) for 30 min (a), (b), (c). Image of cells after treatment with **CMCY** (5 μM) for 30 min and subsequent treatment of the cells with 100 μM ClO^- for 30 min (d), (e), (f). Scale bar: 10 μm.

In summary, we have designed a water-soluble red-emitting probe based on coumarin–hemicyanine, which displayed a noticeable colorimetric and fluorometric dual response to ClO^- on the basis of a simple alkene cleavage reaction. **CMCY** exhibited fast response to ClO^- in physiological condition with high sensitivity and selectivity, and extremely low detection limit (0.08 μM). By plotting the ratio of emission intensity (I_{480}/I_{631}) versus the concentration of ClO^-, a good linear relationship (R^2 = 0.9932) was obtained with the ClO^- concentration ranging from 0 to 73 μM. Cell staining results indicated **CMCY** was cell membrane permeable and could be used for visualizing exogenous and endogenous hypochlorite in living cells.

This work was financially supported by NSFC (No.21203138, 31371750), the Natural Science Foundation of Tianjin (13JCQNJC05300), Hubei Provincial Educational Department (D20132702), and Hubei Co-Innovation Center for Utilization of Biomass Waste.

Notes and references

[a] *School of Chemistry & Chemical Engineering, Tianjin University of Technology, Tianjin 300384, PR China. Fax: +86 22 60214252; E-mail: zlt1981@126.com*

[b] *Department of Chemistry and Material Sciences, Hubei Engineering University, Hubei Xiaogan 432000, PR China. Fax: +86 712 2345265.*

† Electronic Supplementary Information (ESI) available: [Synthetic details and charicterization data]. See DOI: 10.1039/b000000x/

‡ Footnotes should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

1 (a) Y. W. Yap, M. Whiteman and N. S. Cheung, *Cell. Signalling*, 2007, **19**, 219. (b) D. I. Pattison and M. J. Davies, *Biochemistry*, 2006, **45**, 8152.

2 (a) D. Roos and C. C. Winterbourn, *Science*, 2002, **296**, 669; (b) F. C. Fang, *Nat. Rev. Microbiol.,* 2004, **2**, 820.

3 S. Sugiyama, K. Kugiyama, M. Aikawa, S. Nakamura, H. Ogawa and P. Libby, *Arterioscler., Thromb., Vasc. Biol.*, 2004, **24**, 1309.

4 M. J. Steinbeck, L. J. Nesti, P. F. Sharkey and J. J. Parvizi, *Orthop. Res.*, 2007, **25**, 1128.

5 D. I. Pattison and M. J. Davies, *Chem. Res. Toxicol.*, 2001, **14**, 1453.

6 (a) M. D. Rees, C. L. Hawkins and M. J. Davies, *J. Am. Chem. Soc.*, 2003, **125**, 13719; (b) L. J. Marnett, *J. Org. Chem.*, 2012, **77**, 5224. (c) J. Park, H. Kim, Y. Choi and Y. Kim, *Analyst*, 2013, **138**, 3368.

7 K. Setsukinai, Y. Urano, K. Kakinuma, H. J. Majima and T. Nagano, *J. Biol. Chem.*, 2003, **278**, 3170.

8 (a) J. Chan, S. C. Dodani and C. J. Chang, *Nat. Chem.*, 2012, **4**, 973; (b) Y. Yang, Q. Zhao, W. Feng and F. Li, *Chem. Rev.*, 2013, **113**, 192; (c) L. Zeng, N. Fan, J. Zha, X. Hu, B. Fu, C. Qin and L. Wang, *Analyst*, 2013, **138**, 7083.

9 Z.-N. Sun, F.-Q. Liu, Y. Chen, P. K. H. Tam and D. Yang, *Org. Lett.*, 2008, **10**, 2171.

10 J. Shepherd, S. A. Hilderbrand, P. Waternan, J. W. Heinecke, R. Weissleder and P. Libby, *Chem. Biol.*, 2007, **14**, 1221.

11 (a) S. Kenmoku, Y. Urano, H. Kojima and T. Nagano, *J. Am. Chem. Soc.*, 2007, **129**, 7313; (b) Y. Koide, Y. Urano, K. Hanaoka, T. Terai, T. Nagano, *J. Am. Chem. Soc.*, 2011, **133**, 5680.

12 (a) W. Lin, L. Long, B. Chen and W. Tan, *Chem. Eur. J.*, 2009, **15**, 2305; (b) X. Cheng, H. Jia, T. Long, J. Feng, J. Qin and Z. Li, *Chem. Commun.*, 2011, **47**, 11978; (c) G. Cheng, J. Fan, W. Sun, K. Sui, X. Jin, J. Wang and X. Peng, *Analyst*, 2013, **138**, 6091.

13 Y.-K. Yang, H. J. Cho, J. Lee, I. Shin, J. Tae. *Org. Lett.* 2009, **11**, 859.

14 (a) K. Komatsu, Y. Urano, H. Kojima and T. Nagano, *J. Am. Chem. Soc.*, 2007, **129**, 13447; (b) D. Srikun, E.W. Miller, D.W. Domaille and C. J. Chang, *J. Am. Chem. Soc.*, 2008, **130**, 4596; (c) L. Long, D. Zhang, X. Li, J. Zhang, C. Zhang and L. Zhou, *Analytica Chimica Acta*, 2013, **775**, 100; (d) Y. Zhou, J. Y. Li, K. H. Chu, K. Liu and C. Yao, *Chem. Comm.*, 2012, **48**, 4677.

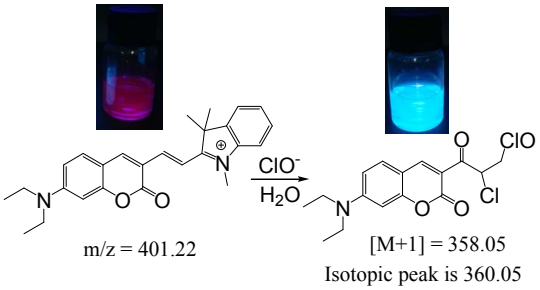
15 J.V. Mello and N.S. Finney, *Angew. Chem. Int. Ed.*, 2001, **40**, 1536.

16 (a) L. Yuan, W. Lin, J. Song and Y. Yang, *Chem. Comm.*, 2011, **47**, 12691; (b) L. Yuan, W. Lin, Y. Xie, B. Chen and J. Song, *Chem. Eur. J.*, 2012, **18**, 2700.

17 (a) X. Wu, Z. Li, L. Yang, J. Han and S. Han, *Chem. Sci.*, 2013, **4**, 460. (b) G. Chen, F. Song, J. Wang, Z. Yang, S. Sun, J. Fan, X. Qiang, X. Wang, B. Dou and X. Peng, *Chem. Commun.*, 2012, **48**, 2949.

18 S. Chen, J. Lu, C. Sun and H. Ma, *Analyst*, 2010, **135**, 577.

A Ratiometric Fluorescent Probe for Rapid and Sensitive Visualizing Hypochlorite in Living Cells



A novel fluorescent probe displayed colorimetric response to ClO^- with high selectivity, fast response and low detection limit (0.08 μM).