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A Highly Selective Ratiometric Fluorescent Probe for Peroxynitrite Detection in Aqueous Media

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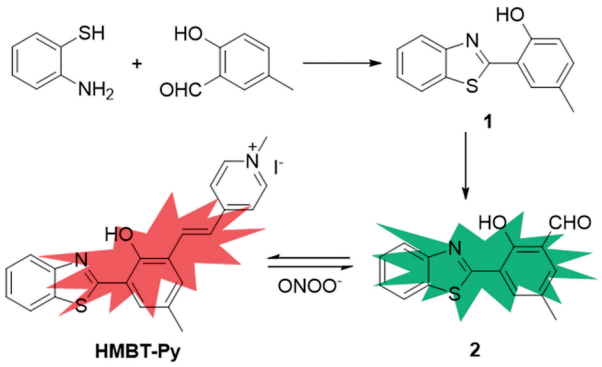
Herein we developed a fast-responsive fluorescent probe 2-(benzo[*d*]thiazol-2-yl)-4-methylphenol (**HMBT**)**-Py** for $ONOO^-$ detection. **HMBT-Py** can react with $ONOO^-$ by an activated C=C oxidation cleavage reaction, causing a ratiometric change in the fluorescence spectra. In addition, **HMBT-Py** showed high selectivity to $ONOO^-$ over other reactive species.

Keywords: Fluorescence probe | Peroxynitrite | C–C double bond cleavage

Highly reactive oxygen species (hROS), including $^1O_2$, •OH, OOH, $ONOO^-$, HOCl, and HOBr,[1] can easily cause oxidative stress (OS), which is an established risk factor because they can cause huge damage to cells or tissues when they are overproduced and mismanaged.[2] As the pK_a of peroxynitrite anion ($ONOO^-$) is 6.8, $ONOO^-$ is the major form under most biological conditions, rather than the unstable protonated state (ONOOH) at pH 7.40 for instance.[3] Endogenous $ONOO^-$ is generated by a combination reaction of nitric oxide (•NO) with superoxide radical ($O_2^{\bullet-}$)[4] with a short half-life (ca. 1 s at pH 7.40).[5] Due to strong oxidative and nitrative abilities, $ONOO^-$ can easily react with a variety of biomolecules (e.g. lipid, protein, and nuclear acid), leading to various diseases like inflammatory and cardiovascular diseases, neurodegeneration, and cancer.[6] On the other hand, $ONOO^-$ served as a redox signaling molecule, modulating the cell signal transduction process.[7] Considering the complex life process in vivo, it is a wonderful choice to conduct in vitro simulation to investigate the role of $ONOO^-$ in physiology and pharmacology.

Till now, several detection methods of $ONOO^-$ monitoring has been developed, including optical sensing,[8] electrochemical analysis,[9] ^{19}F magnetic resonance, and electron spin resonance (ESR).[10,11] Among these methods, fluorescence sensing has some advantages, such as simplicity, high temporal and spatial resolutions, and high sensitivity, and it is widely applied in $ONOO^-$ detection.[4,12–18] Most of the fluorescent probes for $ONOO^-$ are based on the specific oxidation reactions, such as aryl ether group oxidation,[13] aryl boronate oxidation,[4,14] phenol group oxidation,[15] heteroatom (Se, Te for instance) oxidation,[16] activated carbon–carbon (C=C) double bond oxidative cleavage,[17] and others.[18] Although many probes have been reported for $ONOO^-$,[19] most of them have a long response time (>5 min) or suffer interference from other reactive species. As endogenous $ONOO^-$ exists in low concentration and has a short lifetime, more sensitive and extremely fast responsive fluorescent probes should be developed to meet the demand of detecting $ONOO^-$. Herein, we reported a ratiometric fluorescent probe for peroxynitrite with fast response time (within 5 min).

The C=C double bond can be oxidized by strong oxidative species to generate various double-bond cleavage products.[20] Activated C=C was already applied to the recognition of $ONOO^-$ using the oxidative double-bond cleavage reaction.[17] On the other hand, pyridine cation can act as the accepted part in the "donor–acceptor" (D–A) system, which can easily cause bathochromic shift of the emission wavelength,[21] and the new D–A system might result in a ratiometric phenomenon when it is interrupted.[22] Herein, we introduced a C=C bond linkage, which is connected with a well-known fluorophore 2-(benzo[*d*]thiazol-2-yl)-4-methylphenol (**HMBT**, compound **1**) and *N*-methylpyridine cation to obtain a target probe **HMBT-Py**. We expected that the probe can act as an excellent probe for $ONOO^-$ monitoring using the activated C=C to react with $ONOO^-$ and interrupt the D–A system (Scheme 1). As expected, when **HMBT-Py** reacts with $ONOO^-$ in aqueous solution, a remarkable ratiometric change is observed (Figure 1).



Scheme 1. Synthetic routes of target probe and sensing mechanism.

With **HMBT-Py** in hand, basic spectral properties of **HMBT-Py** were investigated. First of all, fluorescence spectra of **HMBT-Py** in different solvents were recorded (Figure S1). As can be seen, in the investigated solvents (DMSO, DMF, MeOH, EtOH, THF, MeCN, and PBS buffer (pH 7.40, 10 mM)), the emission maximum wavelengths were almost similar (around 660 nm). Although the intensity in PBS buffer was weak, it is enough to conduct the following experiments in PBS buffer. Two major peaks (312 and 492 nm) and a side peak (400 nm) were found in the absorbance spectra (Figure S2). As shown in Figure 1, after reacting **HMBT-Py** with $ONOO^-$, the emission peak (645 nm) disappeared and a new peak centered at 545 nm was observed. We supposed this hypsochromic shift should be induced by a disruption of the D–A system in **HMBT-Py** molecule after the oxidation cleavage of C=C bond by $ONOO^-$, which was reported to generate aldehyde product in the oxidation reaction.[17] Evidences were also found in high-resolution mass spectrum (HRMS) and TLC results of the reaction solution between **HMBT-Py** and $ONOO^-$. As shown in the ES^- result in Figure S3, a peak of m/z 268.0432 was found,

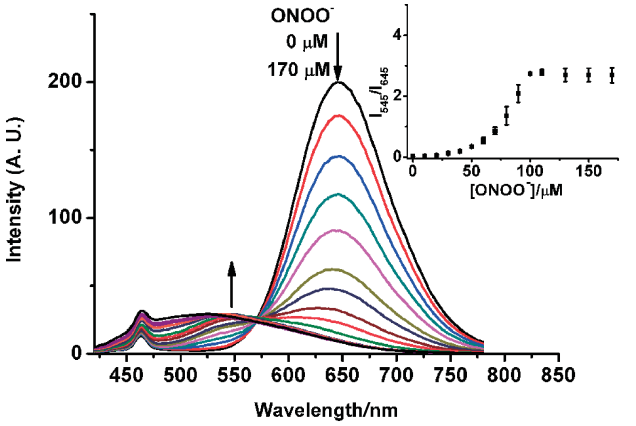


Figure 1. Fluorescent responses of probe **HMBT-Py** (10 μM) reacted with different concentrations (including: 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 130, 150, and 170 μM) of $ONOO^-$ in PBS buffer after 5 min at room temperature. $\lambda_{ex} = 400$ nm, slits: 5/5 nm.

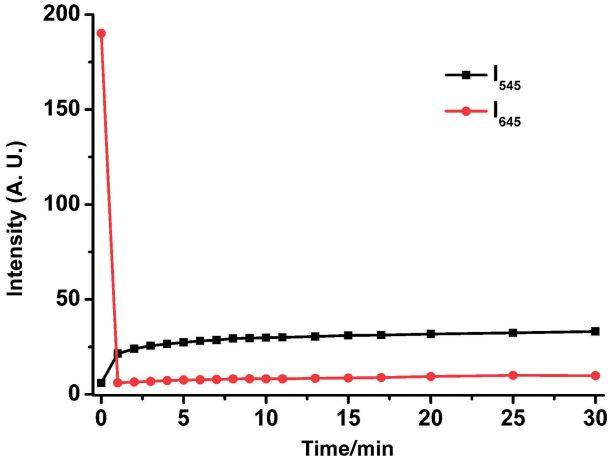


Figure 2. Time-dependent fluorescence intensity at 545 and 645 nm of **HMBT-Py** (10 μM) in the present of $ONOO^-$ (100 μM) in PBS buffer at room temperature. Different time points including: 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 13, 15, 17, 20, 25, and 30 min.

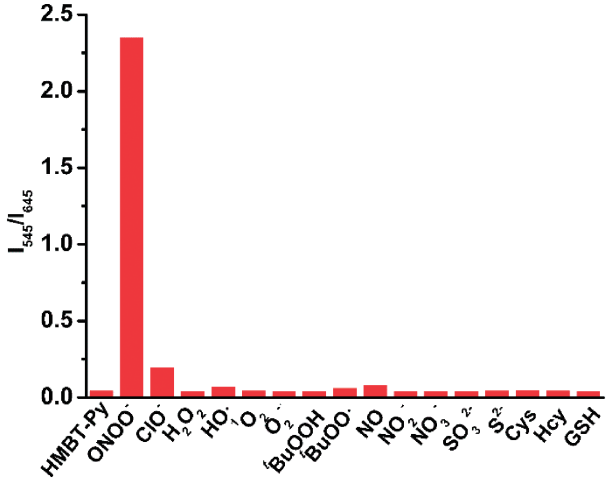


Figure 3. Selectivity of **HMBT-Py** (10 μM) to different reactive species (ROS, RNS, and RSS include: 100 μM for $ONOO^-$, ClO^-, NO, $^tBuOO•$, HO•, $^1O_2$, $O_2^{•-}$, SO_3^{2-}, and S^{2-}, 1 mM for H_2O_2, ^tBuOOH, NO_2^-, NO_3^-, Cys, Hcy, and GSH) in PBS (pH 7.40, 10 mM) at room temperature.

which towel matched with the result of compound **2** (calculated as m/z 268.0438). Moreover, a TLC image of the reaction solution of **HMBT-Py** and $ONOO^-$ was recorded (Figure S4), in which the R_f value of the new product in the reaction agreed to that of compound **2** ($R_f = 0.49$).

After the reaction conditions were confirmed, spectral responses of **HMBT-Py** in the presence of $ONOO^-$ were recorded. With increasing concentration of $ONOO^-$, absorbance decreased gradually in a broad range from 485 to 600 nm (Figure S2), and a ratiometric response was observed in the emission spectra with a weakened emission at 645 nm and an enhanced emission at 545 nm (Figure 1). As shown in the inset of Figure 1, the I_{545}/I_{645} ratio increased gradually to maximum till the $ONOO^-$ concentration reached 100 μM and finally to the equilibrium value. Consequently, 100 μM $ONOO^-$ was used in the following experiment to ensure a complete reaction. Next, the reaction kinetics of **HMBT-Py** (10 μM) with $ONOO^-$ (100 μM) in PBS buffer was investigated (Figure 2 and Figures S5, S6, and S7). After the addition of $ONOO^-$, ether absorbance or fluorescence emission spectra change immediately, and no obvious changes were observed after 5 min. Hence, 5 min was set as the final reaction time in the following experiment.

High selectivity is an important criterion for an excellent probe. Up to date, most $ONOO^-$ probes were still interfered by other ROS such as HClO and H_2O_2. Highly selective fluorescent probes for $ONOO^-$ recognition are in high demand. Hence, to demonstrate the selectivity of **HMBT-Py**, the reactions of different reactive species, including ROS and some typical reactive nitrogen species (RNS) or reactive sulfur species (RSS) (including ClO^-, H_2O_2, HO•, $^1O_2$, $O_2^{•-}$, ^{t}BuOOH, ^{t}BuOO•, NO, NO_2^-, NO_3^-, SO_3^{2-}, S^{2-}, Cys, Hcy, and GSH) to probe **HMBT-Py** were investigated in aqueous solution (Figure 3). As is shown, except $ONOO^-$, the other species could not cause any remarkable change demonstrating high selectivity of **HMBT-Py** to $ONOO^-$ over other reactive species in aqueous condition.

Finally, we investigated the response ability of **HMBT-Py** towards $ONOO^-$ in living HeLa and A549 cells (Figure 4). To our disappointment, the fluorescence intensity ratio (I_{green}/I_{red}) showed no obvious difference in the absence and presence of 3-morpholinosydnonimine (SIN-1, a common $ONOO^-$ donor, Figures 4q and 4r), indicating **HMBT-Py** could not image $ONOO^-$ in living cells. As was reported, positively charged compounds could easily bind to negatively charged biomolecules like proteins and nucleic acid,[23] we suspected that those negatively charged biomolecule in cells would complex with **HMBT-Py** due to the positive charge of the pyridine salt, which might further hinder the sensing process.

In conclusion, we have developed a water-soluble ratiometric fluorescent probe **HMBT-Py** for $ONOO^-$ monitoring. **HMBT-Py** exhibited high reactivity and selectivity towards $ONOO^-$ over other reactive species in aqueous condition.

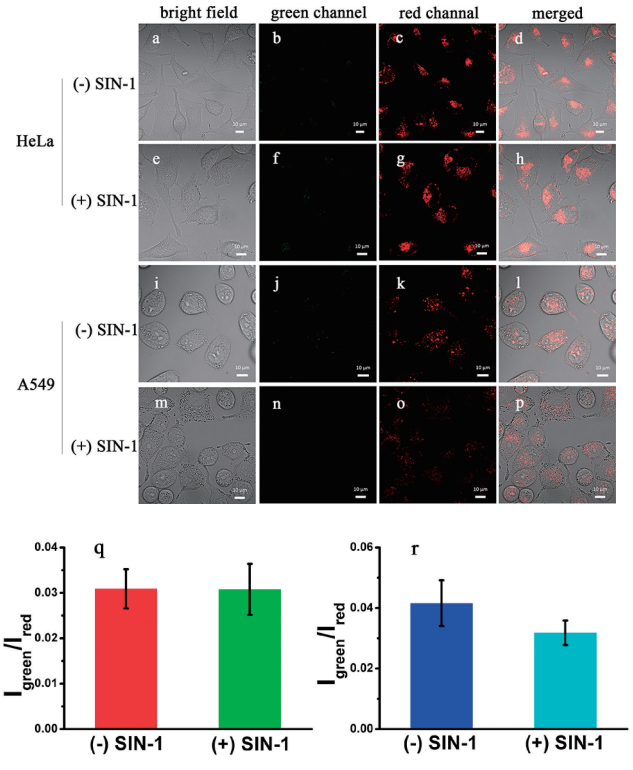


Figure 4. HeLa cells or A549 cells were incubated with **HMBT-Py** (5 μM) for 30 min (HeLa: a–d, A549: e–h), then incubated with 100 μM SIN-1 (HeLa: i–l, A549: m–p). Images of green channel (525–565 nm, ex@405 nm) and red channel (625–665 nm, ex@405 nm) were collected. Ratios of green channel and red channel (I_{green}/I_{red}) before and after incubating 100 μM SIN-1 in HeLa cells (q) or A549 cells (r) were calculated. The scale bar in the images (white bar) stands for 10 μm.

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References

1 a) Y. You, W. Nam, *Chem. Sci.* **2014**, *5*, 4123. b) Y.-Y. Huang, M.-J. Wang, Z. Yang, M.-Y. She, S. Wang, P. Liu, J.-L. Li, Z. Shi, *Chin. Chem. Lett.* **2014**, *25*, 1077.

2 B. C. Dickinson, C. J. Chang, *Nat. Chem. Biol.* **2011**, *7*, 504.

3 R. Radi, *J. Biol. Chem.* **2013**, *288*, 26464.

4 F. Yu, P. Song, P. Li, B. Wang, K. Han, *Analyst* **2012**, *137*, 3740.

5 N. Ashki, K. C. Hayes, F. Bao, *Neuroscience* **2008**, *156*, 107.

6 a) C. Szabó, H. Ischiropoulos, R. Radi, *Nat. Rev. Drug Discovery* **2007**, *6*, 662. b) J. P. Eiserich, M. Hristova, C. E. Cross, A. D. Jones, B. A. Freeman, B. Halliwell, A. van der Vliet, *Nature* **1998**, *391*, 393. c) P. Pacher, J. S. Beckman, L. Liaudet, *Physiol. Rev.* **2007**, *87*, 315.

7 T. Okamoto, T. Akaike, T. Sawa, Y. Miyamoto, A. van der Vliet, H. Maeda, *J. Biol. Chem.* **2001**, *276*, 29596.

8 P. Panizzi, M. Nahrendorf, M. Wildgruber, P. Waterman, J.-L. Figueiredo, E. Aikawa, J. McCarthy, R. Weissleder, S. A. Hilderbrand, *J. Am. Chem. Soc.* **2009**, *131*, 15739.

9 a) Y. Wang, Z. Chen, *Talanta* **2010**, *82*, 534. b) J. S. Cortés, S. G. Granados, A. A. Ordaz, J. A. L. Jiménez, S. Griveau, F. Bedioui, *Electroanalysis* **2007**, *19*, 61. c) W. C. A. Koh, J. I. Son, E. S. Choe, Y.-B. Shim, *Anal. Chem.* **2010**, *82*, 10075.

10 K. J. Bruemmer, S. Merrikhihaghi, C. T. Lollar, S. N. S. Morris, J. H. Bauer, A. R. Lippert, *Chem. Commun.* **2014**, *50*, 12311.

11 a) R. M. Gatti, B. Alvarez, J. Vasquez-Vivar, R. Radi, O. Augusto, *Arch. Biochem. Biophys.* **1998**, *349*, 36. b) S. Bartesaghi, V. Valez, M. Trujillo, G. Peluffo, N. Romero, H. Zhang, B. Kalyanaraman, R. Radi, *Biochemistry* **2006**, *45*, 6813.

12 a) P. Chen, X. Zhou, H. Shen, N. M. Andoy, E. Choudhary, K.-S. Han, G. Liu, W. Meng, *Chem. Soc. Rev.* **2010**, *39*, 4560. b) Z.-Y. Wu, J.-N. Cui, X.-H. Qian, T.-Y. Liu, *Chin. Chem. Lett.* **2013**, *24*, 359. c) W.-Z. Zhang, Z.-B. Du, B. Song, Z.-Q. Ye, J.-L. Yuan, *Chin. Chem. Lett.* **2015**, *26*, 1465.

13 a) D. Yang, H.-L. Wang, Z.-N. Sun, N.-W. Chung, J.-G. Shen, *J. Am. Chem. Soc.* **2006**, *128*, 6004. b) Z.-N. Sun, H.-L. Wang, F.-Q. Liu, Y. Chen, P. K. H. Tam, D. Yang, *Org. Lett.* **2009**, *11*, 1887. c) J. Ma, J. Wu, W. Liu, P. Wang, Z. Fan, *Spectrochim. Acta, Part A* **2012**, *94*, 340.

14 a) A. Sikora, J. Zielonka, M. Lopez, J. Joseph, B. Kalyanaraman, *Free Radical Biol. Med.* **2009**, *47*, 1401. b) J. Kim, J. Park, H. Lee, Y. Choi, Y. Kim, *Chem. Commun.* **2014**, *50*, 9353. c) X. Sun, Q. Xu, G. Kim, S. E. Flower, J. P. Lowe, J. Yoon, J. S. Fossey, X. Qian, S. D. Bull, T. D. James, *Chem. Sci.* **2014**, *5*, 3368. d) J. Zhou, Y. Li, J. Shen, Q. Li, R. Wang, Y. Xu, X. Qian, *RSC Adv.* **2014**, *4*, 51589.

15 a) T. Peng, N.-K. Wong, X. Chen, Y.-K. Chan, D. H.-H. Ho, Z. Sun, J. J. Hu, J. Shen, H. El-Nezami, D. Yang, *J. Am. Chem. Soc.* **2014**, *136*, 11728. b) X. Li, R.-R. Tao, L.-J. Hong, J. Cheng, Q. Jiang, Y.-M. Lu, M.-H. Liao, W.-F. Ye, N.-N. Lu, F. Han, Y.-Z. Hu, Y.-H. Hu, *J. Am. Chem. Soc.* **2015**, *137*, 12296. c) H. Zhang, J. Liu, Y.-Q. Sun, Y. Huo, Y. Li, W. Liu, X. Wu, N. Zhu, Y. Shi, W. Guo, *Chem. Commun.* **2015**, *51*, 2721.

16 a) F. Yu, P. Li, G. Li, G. Zhao, T. Chu, K. Han, *J. Am. Chem. Soc.* **2011**, *133*, 11030. b) F. Yu, P. Li, B. Wang, K. Han, *J. Am. Chem. Soc.* **2013**, *135*, 7674. c) K. Xu, H. Chen, J. Tian, B. Ding, Y. Xie, M. Qiang, B. Tang, *Chem. Commun.* **2011**, *47*, 9468.

17 a) J.-T. Hou, J. Yang, K. Li, Y.-X. Liao, K.-K. Yu, Y.-M. Xie, X.-Q. Yu, *Chem. Commun.* **2014**, *50*, 9947. b) A. J. Shuhendler, K. Pu, L. Cui, J. P. Uetrecht, J. Rao, *Nat. Biotechnol.* **2014**, *32*, 373. c) X. Zhou, Y. Kwon, G. Kim, J.-H. Ryu, J. Yoon, *Biosens. Bioelectron.* **2015**, *64*, 285.

18 a) J.-C. Huang, D.-J. Li, J.-C. Diao, J. Hou, J.-L. Yuan, G.-L. Zou, *Talanta* **2007**, *72*, 1283. b) Q. Zhang, Z. Zhu, Y. Zheng, J. Cheng, N. Zhang, Y.-T. Long, J. Zheng, X. Qian, Y. Yang, *J. Am. Chem. Soc.* **2012**, *134*, 18479. c) K.-K. Lin, S.-C. Wu, K.-M. Hsu, C.-H. Hung, W.-F. Liaw, Y.-M. Wang, *Org. Lett.* **2013**, *15*, 4242.

19 T. Ueno, Y. Urano, H. Kojima, T. Nagano, *J. Am. Chem. Soc.* **2006**, *128*, 10640.

20 a) S. Pennathur, D. Maitra, J. Byun, I. Sliskovic, I. Abdulhamid, G. M. Saed, M. P. Diamond, H. M. Abu-Soud, *Free Radical Biol. Med.* **2010**, *49*, 205. b) J. Park, H. Kim, Y. Choi, Y. Kim, *Analyst* **2013**, *138*, 3368.

21 N. Karton-Lifshin, L. Albertazzi, M. Bendikov, P. S. Baran, D. Shabat, *J. Am. Chem. Soc.* **2012**, *134*, 20412.

22 M. H. Lee, J. S. Kim, J. L. Sessler, *Chem. Soc. Rev.* **2015**, *44*, 4185.

23 G. Han, D. Kim, Y. Park, J. Bouffard, Y. Kim, *Angew. Chem., Int. Ed.* **2015**, *54*, 3912.

