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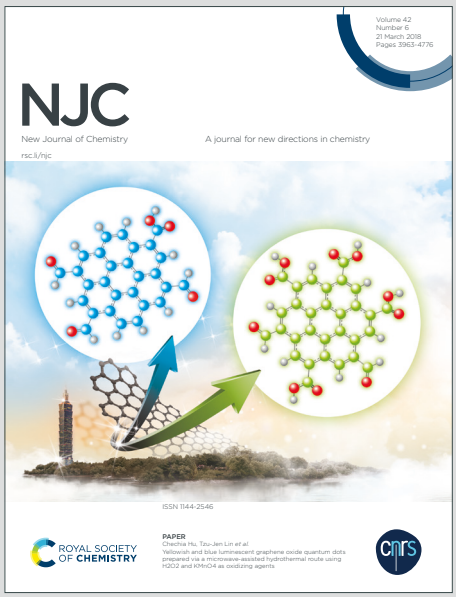

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COMMUNICATION

An HBT-based fluorescent probe for nitroreductase determination and its application in *Escherichia coli* cell imaging

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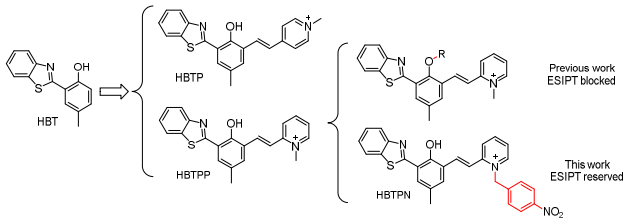
A novel HBT-based fluorescent probe HBTPN was reasonably designed for detecting nitroreductase by merging 4-nitrobenzyl as a sensing moiety to HBT-based dye HBTPP. Experiments showed that probe HBTPN displayed selective and sensitive "turn-on" response to nitroreductase and has been applied for sensing nitroreductase in living *Escherichia coli*.

The flavin-containing nitroreductase (NTR), which was found overexpressed in *Enterobacter cloacae* and hypoxic human tumours, could catalyse the reduction of nitroaromatic compounds to the corresponding hydroxylamines or amines with cofactor NADH.[1] This enzyme has been utilized in bioremediation of 2,4,6-trinitro toluene (TNT) and other nitroaromatic pollutants in the past years.[2] Nowadays, much attentions were paid to the development of NTR sensors, and most of them were focused on their applications in assessing hypoxic status of solid tumours.[3] In view of the high-expression of NTR in *Escherichia coli* (*E. coli*), we speculated that this enzyme was promising to be designed as a potential reporter to monitoring infections and food contamination caused by *E. coli*.[4, 5, 6]

Among all the NTR assays, fluorescence technology was desirable due to its high spatiotemporal resolution and nondamaging detection, and remarkable progress has been made in the development of NTR fluorescent probes recently.[7, 8] However, most of the fluorescent probes suffered from drawback of short wavelength emission or small Stokes shift (Table S1 in ESI†), which may lead to disadvantages such as poor penetration, severe photodamage and crosstalk between the excitation and emission spectra.[9] Hence, the development of novel probes for NTR with enlarged Stokes shift as well as long wavelength emission was becoming increasingly important. Recently, HBT (2-hydroxyphenyl-substituted benzothiazole) and its analogues have been frequently used in developing fluorophores with large Stokes shifts due to their intrinsic mechanism of ESIPT (excited-state intramolecular proton transfer), and the emission spectrum could be redshifted to long-wavelength region via extending the π-conjugation system with electron-accepting unit.[10, 11, 12] Exemplary HBT-based fluorophores (**HBTP** and **HBTPP**) have been developed by introducing cationic picoline salt as the electron acceptors, which were reported exhibiting large Stokes shift and long wavelength emission because of ESIPT coupled ICT (intramolecular charge transfer) mechanism (Scheme 1).[13] Through masking their phenolic hydroxyl with detecting group and thus blocking the ESIPT process, various probes were developed for monitoring analytes including hydrogen sulfide, hypochlorite, palladium, hydrogen peroxide, alkaline phosphatase and nitroreductase, etc.[13, 14]



Scheme 1. Design of fluorescent probes based on **HBTPP** scaffold.

Inspired by those findings, a novel fluorescent probe (**HBTPN**) for NTR was designed by merging 4-nitrobenzyl group as responsive unit to dye **HBTPP** (Scheme 1). Different from the previous strategy of masking the hydroxyl group, the detecting group of probe **HBTPN** was merged with the pyridinium unit without blocking the ESIPT process.[15] We speculate that 4-nitrobenzyl moiety functioned not only as the NTR detecting unit, but also as alkylating agent which provide the probe characteristic of long wavelength emission. Furthermore, upon addition of NTR, nitroaromatic group might be reduced to hydroxylamines or amines, which would weaken the electron-withdrawing effect of the pyridinium moiety as well as the ICT process and the possible photoinduced electron transfer (PET) process from **HBTPP** moiety to the nitrobenzyl group, result in changes of fluorescence signal.[7, 16]

Probe **HBTPN** was synthesized following the procedures reported previously.[10, 15] To investigate the sensing mechanism,

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a novel HBT-based fluorophore **HBT-2Py** (Scheme 2) was synthesized for comparison.[17] Details of synthesis route and characterization were given in the Supporting Information (SI).

The optical properties of **HBTPN** were then measured in different conditions. Complex absorption spectra were observed in **HBTPN** with strong absorption bands around 300 and 410 nm in solvents (Fig. S1A in ESI†), and the absorption bands (in DMSO and CH_3OH) after 475 nm were reasonably ascribed to partial deprotonation of phenol unit (Fig. S2A in ESI†).[17, 18] In the fluorescence spectra, due to the mechanism of ESIPT coupled ICT process, longer emission wavelength (λ_{em}≥638 nm) and larger Stokes shift (Δλ≥232 nm) were obtained in probe **HBTPN** (Table S2 and Fig. S3 in ESI†). The fluorescent intensity decreased gradually from nonpolar solvent CH_2Cl_2 to polar solvent (CH_3CN, CH_3OH and DMSO) with the emission band becoming red-shifted (Fig. S1B in ESI†), which was in conformity with the report that the fluorescence property of HBT derivatives were sensitive to solvents,[12, 19] and notably, extremely weak fluorescence signal was detected in strong polar/protic water with a low quantum yield of Φ=0.0026.

Subsequently, the fluorescence responses of probe to NTR were evaluated in Tris-HCl (pH=7.4, 50 mM) with NADH (25 μM) as electron donor. The detecting solution of **HBTPN** (5 μM) displayed negligible emission signal in red/NIR region (Fig. 1). However, upon the addition of NTR, 22-fold increasement of fluorescence intensity at 633 nm was obtained, which validated **HBTPN** as a potential "turn-on" probe.

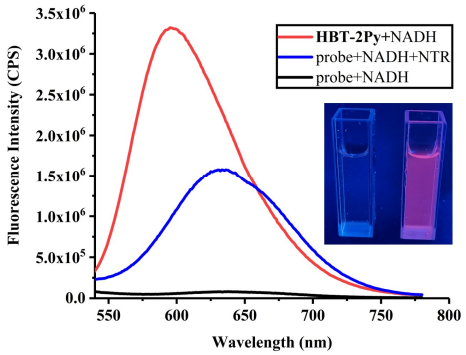
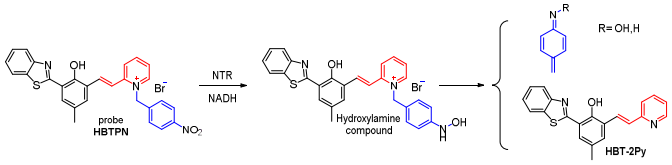


Fig. 1 Emission spectra of **HBTPN**, **HBT-2Py** (5 μM) and the reaction system (**HBTPN** incubated with 1.5 μg/ mL NTR), λ_{ex}=397 nm; Insert picture: fluorescence photograph of reaction solution.

Notably, the emission band of reaction mixture was different with that of **HBT-2Py**, which exhibited strong fluorescence emission centred at 595 nm, indicating that probe **HBTPN** may not undergo the usual mechanism of NTR-catalysed reduction followed by rearrangement-elimination to liberate **HBT-2Py**.[20] To explore the sensing mechanism, ESI-HRMS experiments were conducted and results indicated that **HBTPN** converted into a molecular with ion peak at m/z 466.1566 (Fig. S6A in ESI†), which could be logically characterized as the hydroxylamine compound reduced from probe **HBTPN** (unfortunately, we failed to synthesize this compound). Subsequently, these species were further confirmed by HPLC (Fig. S7 in ESI†). After being incubated with nitroreductase, a decrease in the peak at 6.00 min on behalf of **HBTPN** was observed, together with the emergence of a new peak at 11.69 min ascribed to **HBT-2Py** and an uncharted peak at 3.56 min. The LC-MS spectrum of the uncharted peak (Fig. S6B in ESI†) provided a signal of hydroxylamine compound (m/z=466.0), suggesting that this peak could be assigned to hydroxylamine compound. Further experiments demonstrated that fluorescence signal of **HBT-2Py** would be partly inhibited by the coexistence of **HBTPN** and the addition of part of **HBT-2Py** could hardly change the emission curve of reaction mixture (Fig. S8 in ESI†). Based on these experiments, a plausible mechanism was proposed that probe **HBTPN** underwent NTR mediated nitro reduction to yield hydroxylamine compound (Scheme 2).[21] Inevitably, this compound might partly decompose into **HBT-2Py**.



Scheme 2 Probe **HBTPN** and its reaction mechanism with NTR.

Density functional theory (DFT) calculations were conducted to further investigate this response mechanism (Table S3 in ESI†).[16] As demonstrate in natural transition orbitals (NTOs), the lowest energy absorption band resides on the **HBTPP** moiety with the intramolecular charge transferred from HBT unit to pyridinium part for both probe and hydroxylamine compound. On the singlet emitting states the π electrons of **HBTPP** was delocalized over the electron-withdrawing nitro aromatic moiety in probe, while no electron transfer was observed for hydroxylamine compound. This confirms that the nitro group induced PET process, result in the weak fluorescence of probe in water, and that blocking of the PET effect lead to the strong fluorescence signal in hydroxylamine compound.

The fluorescence kinetic responses of probe **HBTPN** reacting with NTR were then investigated, which demonstrated that fluorescence spectra exhibited obvious changes along with reaction process (Fig. S9 in ESI†). Fluorescence intensity at 633 nm increased rapidly and reached to the maximum in about 10 min with 4-17 fold enhancement at various concentration, while hardly any change was observed without NTR during the same period. Moreover, negligible fluorescence intensity change was observed after being continuously illuminated for 75 minutes, and the absorption and emission spectra changed slightly at a temperature range of 0-60°C, suggesting the photostability of probe **HBTPN** (Fig. S4 and S10 in ESI†).

Subsequently, the selectivity of **HBTPN** to NTR was measured. A variety of interfering species including inorganic salts, reactive oxygen species, amino acids, biothiols, glucose and vitamin C, failed to trigger fluorescence response whereas only NTR could induce a remarkable increment of fluorescence signal (Fig. S11 in ESI†). Moreover, the fluorescence intensity

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incubated with dicoumarol (NTR inhibitor) decreased obviously and more dicoumarol would lead to a progressive decline. Those results demonstrate that **HBTPN** was catalysed by NTR specifically in the detective system. Besides, effects of pH or temperature on reaction system (Fig. S12 in ESI†) revealed that **HBTPN** functioned well under physiological conditions (37 °C, pH=7).

Titration experiments were further carried out to test the performance of probe **HBTPN** for quantitatively detecting NTR. As depicted in Fig. 2, the fluorescence intensity at 633 nm enhanced accompany by the increasing concentrations of NTR, and good linearity was exhibited in the range of 0.05–1.5 μg/mL with detection limit determined as 2.9 ng/mL based on 3σ/k, suggesting **HBTPN** was able to detect NTR qualitatively and sensitively. The Michaelis constant (K_m) and the maximum of the initial reaction rate (V_{max}) for the NTR-activated reaction were determined to be 25.00 μM and 0.014 μM/s, respectively, which was comparable to the values reported previously (Fig. S13 in ESI†). [4, 7]

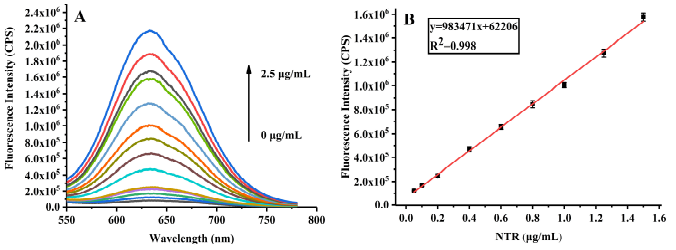

Fig. 2 (A) Fluorescence response of probe (5 μM) with NTR at varied concentrations; (B) Linear correlation between fluorescent intensities and NTR concentrations (0.05, 0.1, 0.2, 0.4, 0.6, 0.8 ,1.0, 1.25, 1.5 μg/mL); Error bars stand for the mean standard deviation of three results. λ_{ex} 397 nm.

Docking studies were carried out to explore molecular recognition procedure. In binding process, probe **HBTPN** tended to approach the catalytic core of NTR by π-π stacking force and the benzothiazole unit could bind to a hydrophobic pocket by hydrophobic interactions (Fig. S14A in ESI†). The 4-nitrobenzyl moiety access to a region delimited by residues Ser12, Arg172, Arg11, Arg10, and seven hydrogen bonds were constructed with these residues, which was in a similar binding mode with the probe reported previously (Fig. S14B in ESI†).[16] In the departure process, after being reduced to hydroxylamine compound, only three hydrogen bonds were formed (Fig. S14C in ESI†). The docking affinity was calculated as 8.99 kcal/mol for hydroxylamine compound, which was lower than that of probe **HBTPN** (10.59 kcal/mol). A low docking value indicated that enzyme could dissociate from the reduced compound easily and then prepare for the following catalytic process. As a consequence, the binding and departure process indicated hydrogen bond and docking affinity were significant in the NTR-mediated reduce reaction, and that probe **HBTPN** could be well catalysed by NTR.

It is known that NTR was abundant in *E. coli* and the assessment of this enzyme will be helpful to examine microbial contamination.[5] Compared with conventional methods like culture-based tests and PCR-based gene sequence identification, which need complicate equipment or time-costing and damaging pre-treatment, probe **HBTPN** should be promising to provide a facile, rapid and non-invasive method to assess NTR activity in live *E. coli*.[5, 6, 22] In this experiment, *E. coli* were cultured in Luria–Bertani media, washed with Tris-HCl buffer and then resuspended in Tris-HCl buffer without lysis to test the absorbance and emission spectra. Incubated probe **HBTPN** with bacteria solution for 10 min could trigger obvious increase in fluorescence intensity compared with only the probe or *E. coli* sample (Fig. 3A), indicating that **HBTPN** was able to diffuse across the rigid cell wall and the plasma membrane, and reacted with NTR within bacterial cytoplasm. However, the addition of dicoumarol (0.1 mM) to *E. coli* together with **HBTPN** led to significantly decrease in fluorescence signal, confirmed the specificity of **HBTPN** to endogenous NTR in *E. coli* cell (Fig. S15 in ESI†). Encouraged by these results, the capacity of probe **HBTPN** for dynamic detection the growth process of *E. coli* was investigated. As shown in Fig. 3, the concentration of *E. coli* (which was determined by the OD_{600} value) grew quickly at early stage and then increased slowly, and notably the change curve of fluorescence intensity fitted well with the growth status of *E. coli*, validating the **HBTPN** as a potential *E. coli* growth indicator.

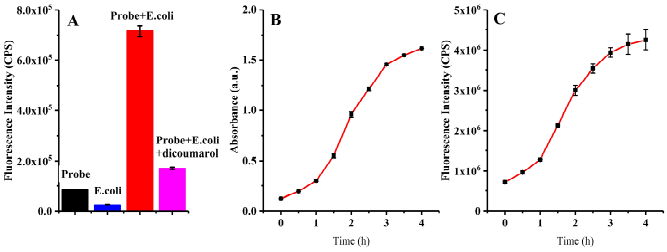

Fig. 3 (A) Fluorescence intensity of different reaction solution; bacterial concentration was OD_{600}=0.12. (B) Time-based absorbance change of *E. coli* in culture media. (C) Fluorescence intensity of **HBTPN** incubated with *E. coli* after growing for different periods of time. Error bars stand for the mean standard deviation of three results.

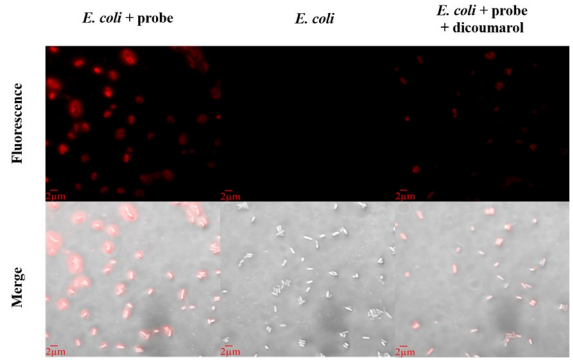

Fig. 4 Fluorescence images of *E. coli* cells incubated with **HBTPN** in the absence and presence of dicoumarol.

The evaluation of NTR activity in living *E. coli* was further conducted by confocal microscopy (Fig. 4). Strong fluorescence signal was detected after incubated with **HBTPN** for 10 min

while negligible fluorescence was observed in *E. coli* cells alone. Meanwhile, pre-treatment with dicoumarol before incubated with **HBTPN** led to obvious decline in fluorescence intensity, certified again that **HBTPN** could permeate the bacterial cell wall and be specially activated by the intracellular nitroreductase. These results demonstrated that probe **HBTPN** was potential to act as a useful indictor to rapidly assess *E. coli* caused infections.

In summary, we have developed a novel HBT-based fluorescent probe **HBTPN** for NTR by merging 4-nitrobenezyl into HBT derivative dye **HBTPP**, and its responses to NTR were evaluated experimentally and theoretically. Probe **HBTPN**, which exhibited excellent performance such as rapid response time, highly selectivity and sensitivity, large Stokes shift (Δλ=236 nm), as well as long-wavelength emission (λ_{em}=633 nm), was successfully applied to detect the intracellular nitroreductase produced by *E. coli* cell, and real-time monitor the growth process of *E. coli*.

Conflicts of interest

There are no conflicts to declare.

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Notes and references

1 C. A. Haynes, R. L. Koder, A. F. Miller and D. W. Rodgers, *J. Biol. Chem.*, 2002, **277**, 11513-11520; D. W. Bryant, D. R. McCalla, M. Leeksma and P. Laneuville, *Can. J. Microbiol.*, 1981, **27**, 81-86.

2 W. D. Won, R. J. Heckly, D. J. Glover and Hoffsomm.Jc, *Appl. Microbiol.*, 1974, **27**, 513-516; I. P. Solyanikova, B. P. Baskunov, M. A. Baboshin, A. I. Saralov and L. A. Golovleva, *Appl. Biochem. Micro+.*, 2012, **48**, 21-27; J. C. Spain, *Annu. Rev. Microbiol.*, 1995, **49**, 523-555.

3 A. Carnero and M. Lleonart, *Bioessays.*, 2016, **38**, S65-S74; K. Kiyose, K. Hanaoka, D. Oushiki, T. Nakamura, M. Kajimura, M. Suematsu, H. Nishimatsu, T. Yamane, T. Terai, Y. Hirata and T. Nagano, *J. Am. Chem. Soc.*, 2010, **132**, 15846-15848; J. Sun, Z. Hu, R. Wang, S. Zhang and X. Zhang, *Anal. Chem.*, 2019, **91**, 1384-1390; Z. Li, X. H. Li, X. H. Gao, Y. Y. Zhang, W. Shi and H. M. Ma, *Anal. Chem.*, 2013, **85**, 3926-3932.

4 Z. Li, X. H. Gao, W. Shi, X. H. Li and H. M. Ma, *Chem. Commun.*, 2013, **49**, 5859-5861.

5 R. H. F. Wong, T. Kwong, K. H. Yau and H. Y. Au-Yeung, *Chem. Commun.*, 2015, **51**, 4440-4442.

6 S. N. Xu, Q. H. Wang, Q. Y. Zhang, L. L. Zhang, L. M. Zuo, J. D. Jiang and H. Y. Hu, *Chem. Commun.*, 2017, **53**, 11177-11180.

7 J. Zhang, H. W. Liu, X. X. Hu, J. Li, L. H. Liang, X. B. Zhang and W. H. Tan, *Anal. Chem.*, 2015, **87**, 11832-11839.

8 H. C. Huang, K. L. Wang, S. T. Huang, H. Y. Lin and C. M. Lin, *Biosens. Bioelectron.*, 2011, **26**, 3511-3516; T. Guo, L. Cui, J. N. Shen, W. P. Zhu, Y. F. Xu and X. H. Qian, *Chem. Commun.*, 2013, **49**, 10820-10822; K. Xu, F. Wang, X. Pan, R. Liu, J. Ma, F. Kong and B. Tang, *Chem. Commun.*, 2013, **49**, 2554-2556.

9 X. D. Xue, S. B. Jin, Z. P. Li, C. Q. Zhang, W. S. Guo, L. M. Hu, P. C. Wang, J. C. Zhang and X. J. Liang, *Adv. Sci.*, 2017, **4**, 1700229; T. B. Ren, W. Xu, W. Zhang, X. X. Zhang, Z. Y. Wang, Z. Xiang, L. Yuan and X. B. Zhang, *J. Am. Chem. Soc.*, 2018, **140**, 7716-7722.

10 C. C. Hsieh, C. M. Jiang and P. T. Chou, *Acc. Chem. Res.*, 2010, **43**, 1364-1374.

11 J. E. Kwon and S. Y. Park, *Adv. Mater.*, 2011, **23**, 3615-3642; J. Wu, W. Liu, J. Ge, H. Zhang and P. Wang, *Chem. Soc. Rev.*, 2011, **40**, 3483-3495; J. Zhao, S. Ji, Y. Chen, H. Guo and P. Yang, *Phys. Chem. Chem. Phys.*, 2012, **14**, 8803-8817; Y. T. Zhang, C. X. Yan, C. Wang, Z. Q. Guo, X. G. Liu and W. H. Zhu, *Angew. Chem. Int Edit.*, 2020, **59**, 9059-9066; X. K. Li, W. J. Qiu, J. W. Li, X. Chen, Y. L. Hu, Y. Gao, D. L. Shi, X. M. Li, H. L. Lin, Z. L. Hu, G. Q. Dong, C. Q. Sheng, B. Jiang, C. L. Xia, C. Y. Kim, Y. Guo and J. Li, *Chem. Sci.*, 2020, **11**, 7292-7301; W. Fu, C. X. Yan, Y. T. Zhang, Y. Y. Ma, Z. Q. Guo and W. H. Zhu, *Front. Chem.*, 2019, **7** 291.

12 A. C. Sedgwick, L. Wu, H. H. Han, S. D. Bull, X. P. He, T. D. James, J. L. Sessler, B. Z. Tang, H. Tian and J. Yoon, *Chem. Soc. Rev.*, 2018, **47**, 8842-8880.

13 T. Gao, P. F. Xu, M. H. Liu, A. Y. Bi, P. Z. Hu, B. Ye, W. Wang and W. B. Zeng, *Chem-Asian. J.*, 2015, **10**, 1142-1145; P. F. Xu, M. H. Liu, T. Gao, H. L. Zhang, Z. W. Li, X. Y. Huang and W. B. Zeng, *Tetrahedron. Lett.*, 2015, **56**, 4007-4010; Y. Liu, L. Bai, Y. H. Li, Y. Ni, C. Q. Xin, C. W. Zhang, J. H. Liu, Z. P. Liu, L. Li and W. Huang, *Sensor. Actuat. B-Chem.*, 2019, **279**, 38-43.

14 Y. H. Chen, T. W. Wei, Z. J. Zhang, W. Zhang, J. Lv, T. T. Chen, B. Chi, F. Wang and X. Q. Chen, *Chinese. Chem. Lett.*, 2017, **28**, 1957-1960; P. Zhang, C. Fu, Q. Zhang, S. Li and C. Ding, *Anal. Chem.*, 2019, **91**, 12377-12383; Q. Yang, S. Wang, D. Li, J. Yuan, J. Xu and S. Shao, *Anal. Chim. Acta.*, 2020, **1103**, 202-211; S. Paul, S. Goswami and C. Das Mukhopadhyay, *New. J. Chem.*, 2015, **39**, 8940-8947.

15 L. He, X. J. Liu, Y. Zhang, L. Yang, Q. Fang, Y. N. Geng, W. Q. Chen and X. Z. Song, *Sensor. Actuat. B-Chem.*, 2018, **276**, 247-253.

16 Y. H. Li, Y. Sun, J. C. Li, Q. Q. Su, W. Yuan, Y. Dai, C. M. Han, Q. H. Wang, W. Feng and F. Y. Li, *J. Am. Chem. Soc.*, 2015, **137**, 6407-6416.

17 Q. Z. Yang, Y. J. Wen, J. Xu and S. J. Shao, *Talanta.*, 2020, **216**, 120982.

18 D. Dahal, K. R. Ojha, N. Alexander, M. Konopka and Y. Pang, *Sensor. Actuat. B-Chem.*, 2018, **259**, 44-49.

19 A. P. Demchenko, K. C. Tang and P. T. Chou, *Chem. Soc. Rev.*, 2013, **42**, 1379-1408.

20 J. Xu, S. Sun, Q. Li, Y. Yue, Y. Li and S. Shao, *Analyst.*, 2015, **140**, 574-581.

21 M. K. Lee, J. Williams, R. J. Twieg, J. H. Rao and W. E. Moerner, *Chem. Sci.*, 2013, **4**, 220-225.

22 G. X. Feng, Y. Y. Yuan, H. Fang, R. Y. Zhang, B. G. Xing, G. X. Zhang, D. Q. Zhang and B. Liu, *Chem. Commun.*, 2015, **51**, 12490-12493.

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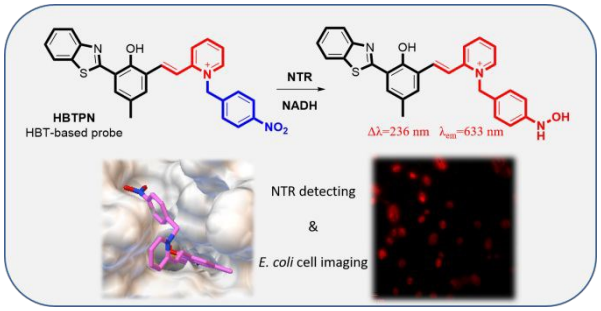

HBTPN exhibited excellent performance such as rapid response time, large Stokes shift highly selectivity and sensitivity, and long-wavelength emission.