CHEMISTRY
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To be cited as: *Chem. Asian J.* 10.1002/asia.202000783

Link to VoR: https://doi.org/10.1002/asia.202000783

A Journal of
ACES Asian Chemical Editorial Society

A sister journal of Angewandte Chemie and Chemistry – A European Journal

WILEY-VCH

10.1002/asia.202000783

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Phenoxazine-based near-infrared fluorescent probes for the specific detection of copper (II) ion in living cells

Yang Shen[+],[a] Wubin Zheng[+],[a] Yusi Yao,[a] Dongmei Wang,[a] Guanglei Lv,*[a] and Chunxia Li*[a], [b]

[a] Y. Shen, W. Zheng, Y. Yao, Dr. D. Wang, Dr. G. Lv, Prof. C. Li
Key Laboratory of the Ministry of Education for Advanced Catalysis Materials
Zhejiang Normal University
Jinhua 321004 (P. R. China)
E-mail: guanglei@zjnu.edu.cn

[b] Prof. C. Li
Institute of Frontier and Interdisciplinarity Science and Institute of Molecular Sciences and Engineering
Shandong University
Qingdao 266237 (P. R. China)
E-mail: cxli@sdu.edu.cn

[+] These authors contributed equally to this work.

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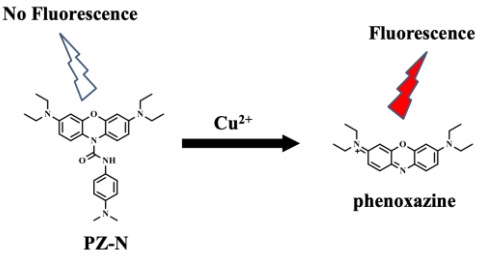
Abstract: It is well known that copper ions play a critical role in various physiological processes. However, a variety of human diseases are tightly correlated with copper overload. Although there are numerous fluorescent probes capable of detecting copper ions, most of them are "turn-off" probes owing to copper (II) ions fluorescence quenching effect, resulting in poor sensitivity. Herein, a novel "turn-on" near-infrared (NIR) fluorescent probe **PZ-N** based on phenoxazine was designed and synthesized for the selective detection of copper (II) ions (Cu^{2+}). Upon the addition of Cu^{2+}, the probe could quickly react with Cu^{2+} and emit strong fluorescence, along with colour change from colourless to obvious blue. Moreover, the probe **PZ-N** showed good water solubility, high selectivity, and excellent sensitivity with low limit of detection (1.93 nM) towards copper (II) ions. More importantly, **PZ-N** was capable of effectively detecting Cu^{2+} in living cells.

Among numerous indispensable trace elements, metal ions play a vital role in physiological processes, such as cellular energy production, catalytic cofactor, oxygen delivery and signal transduction.[1] Besides zinc and iron elements, copper also occupies a high proportion in human body.[2] Nevertheless, several studies reported that the excessive intake of copper contributed to a large number of diseases, including Menkes syndrome, Alzheimer's disease, Wilson's disease, Parkinson's disease and so on.[3] It is well known that rivers and soil are vulnerable for the pollution of copper ions due to the discharge of industry sewage, resulting in a significant increase in the risk of illness.[4] Therefore, it is significantly urgent to realize the specific and sensitive detection of copper ions.

It is well established that the fluorescence technique possesses several merits, such as high selectivity and sensitivity, real-time and non-invasive detecting as well as high resolution and so on.[5] So far, great effort has been made by numerous researchers to develop fluorescent probes for detecting copper ions.[6] However, most of them are "turn-off" fluorescent sensors owing to the fluorescence quenching effect of copper ions, which may cause false positive results.[7] Therefore, it is quite important to construct "turn-on" fluorescent probes for the selective detection of Cu^{2+}. Furthermore, (NIR, 650–900 nm) fluorescent probes, possessing high signal-to-noise ratio, deep tissue penetration, and low background fluorescence interference, have been widely used in living organisms.[8] Although there are some NIR fluorescent probes reported to detect Cu^{2+},[9] some challenges remain to overcome, including slow response and low detection sensitivity towards Cu^{2+}. Thus, it is of significant importance to develop novel "turn-on" near-infrared fluorescent probes for the selective, sensitive and rapid detection of Cu^{2+}.

Initially, we focused on methylene blue (MB) scaffold to design fluorescent probes for the detection of Cu^{2+}. This mainly attributed to the significant difference in fluorescence between the reduced and oxidized forms of MB.[10] Therefore, a series of probes (**MB-N** and **MB-C**, Scheme S1) based on MB scaffold was synthesized to investigate the response to Cu^{2+}. Unfortunately, none of them showed quick response or high sensitivity towards Cu^{2+} (Figure S1). Subsequently, we turned our attention to the phenoxazine skeleton, which possesses similar photophysical properties to MB.[11] Its maximum absorption (λ_{abs} = 654 nm) and emission (λ_{em} = 669 nm) peaks both lie in the near-infrared region. In addition, the reduced form of phenoxazine (ROP) showed rare fluorescence, while the oxidized form of phenoxazine displayed intensive fluorescence emission in NIR region. These spurred us to develop novel "turn-on" fluorescent probes for the detection of Cu^{2+} on the basis of phenoxazine skeleton. Consequently, we for the first time developed several novel "turn-on" NIR fluorescent probes (**PZ-N**, **PZ-O** and **PZ-C**, Scheme S1) on the base of phenoxazine skeleton for detecting Cu^{2+}. Interestingly, among them, the probe **PZ-N** (Scheme 1) exhibited rapid response and higher sensitivity, along with dramatical fluorescence intensity increase. Another two fluorescent probes **PZ-O** and **PZ-C** did not exhibit remarkable fluorescence change in the presence of Cu^{2+} compared to **PZ-N** (Figure S1). Compared with some reported probes (Table S1), the probe **PZ-N** showed faster response, longer emission wavelength, and lower detection limit for Cu^{2+}. Apart from dramatic fluorescence intensity increase upon the



Scheme 1. The structure of **PZ-N** and the response towards Cu^{2+}.

addition of Cu^{2+}, the solution colour remarkably changed from colourless to blue, which could be directly obserbed by the naked eye. Moreover, the probe **PZ-N** exhibited repaid response and high sensitivity towards Cu^{2+} in living cells.

The probe **PZ-N** was obtained through a two-step reaction, as shown in Scheme S1. The compounds were characterized by IR spectroscopy, HR-MS, ^{1}H NMR, and ^{13}C NMR in supporting information. After that, we investigated the fluorescence response of **PZ-N** towards Cu^{2+}. The probe **PZ-N** itself showed relatively weak fluorescence (λ_{em} = 669 nm) and absorption, as shown in Figure S2. The addition of Cu^{2+} induced remarkable fluorescence intensity enhancement (Figure 1) in phosphate-buffered saline (PBS, pH = 7.4) solution containing **PZ-N** at 669 nm. The limit of detection (LOD) of the probe **PZ-N** towards Cu^{2+} was 1.93 nM, which could enable **PZ-N** to detect Cu^{2+} *in vivo*. Additionally, the probe **PZ-N** responded rapidly to Cu^{2+} and the fluorescence intensity significantly increased within approximately 40 s (Figure S3). More importantly, after the addition of Cu^{2+}, the colour of the solution changed from colorless to obvious blue, which made the detection of Cu^{2+} possible by naked eyes. These results indicated that the probe **PZ-N** exhibited fast response and high sensitivity towards Cu^{2+}.

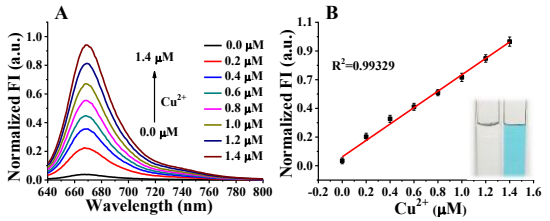


Figure 1. (A) Fluorescence spectra of **PZ-N** (10 μM in PBS, pH = 7.4) before/after the addition of Cu^{2+} (0, 0.2, 0.4, 0.6, 0.8, 1.0, 12 and 1.4 μM); (B) Fluorescence intensities (669 nm) and concentrations of Cu^{2+} (0-1.4 μM) showed good linear relationship. λ_{ex} = 620 nm, λ_{em} =669 nm.

Subsequently, we investigated the selectivity of the probe **PZ-N** towards Cu^{2+}. A variety of analytes, including reactive oxygen species (ROS)/reactive nitrogen species (RNS) (H_2O_2, TBHP, NO, ROO•, HO•, $ONOO^-$, O_2^-, and HClO), common cations (Al^{3+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Fe^{2+}, Fe^{3+}, K^+, Mg^{2+}, Na^+, Mn^{2+}, Ni^{2+}, NH_4^+, and Zn^{2+}), anions (CH_3COO^-, CO_3^{2-}, F^-, Cl^-, I^-, SO_4^{2-}, $S_2O_4^{2-}$ NO_2^-, and NO_3^-), and amino acids (Ala, Cys, Gln, Glu, Gly, Ile, Pro, Lys, Met, Phe, Ser, Thr, Tyr, Trp, Val, Hcy, GSH, and Cys), were chosen to perform the experiment. As shown in Figure 2 and S4, there were no remarkable fluorescence changes in the presence of these analytes except HClO. Although HClO induced slight fluorescence intensity increase, the intensity was very lower compared with the addition of Cu^{2+}. We found that only the addition of Cu^{2+} (PBS, pH= 7.4) could trigger significant fluorescence intensity increase, along with obvious colour change (Figure S5-8). Overall, the probe **PZ-N** exhibited high selectivity towards Cu^{2+}.

Based on previous literatures,[10, 11, 12] a possible detection mechanism was proposed. Initially, Cu^{2+} combined with the amide moiety from **PZ-N** to form a highly unstable four-membered ring (Scheme 2). And then, a water molecule attacked the carbon atom of carbonyl group, resulting in the cleavage of the four-membered ring to produce unstable carbamic acid. Finally, the carbamic acid released HCO_2^- to generate an unstable intermediate, which would quickly produce fluorophore phenoxazine. To confirm the detection

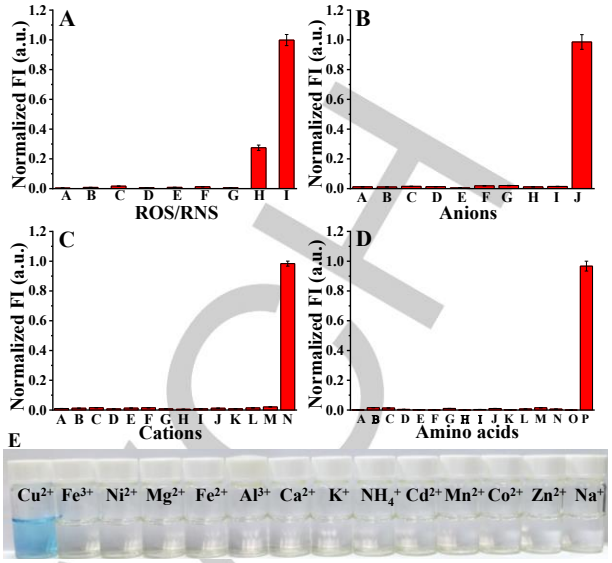
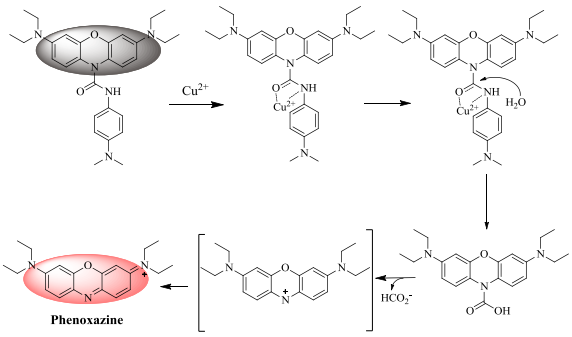


Figure 2. Fluorescence intensity changes of the probe **PZ-N** (10 μM in PBS, pH= 7.4) at 669 nm in the addition of 2 μM Cu^{2+} or other analytes. (A) Various ROS/RNS (2 μM): from A to H: H_2O_2, TBHP, NO, ROO•, HO•, $ONOO^-$, O_2^-, and HClO; (B) Some anions (20 μM): from A to I: CH_3COO^-, CO_3^{2-}, SO_4^{2-}, Cl^-, F^-, I^-, NO_2^-, $S_2O_4^{2-}$, and NO_3^-; (C) Several cations (20 μM): from A to M: Fe^{3+}, Ni^{2+}, Mg^{2+}, Fe^{2+}, Al^{3+}, Ca^{2+}, K^+, NH_4^+, Cd^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, and Na^+; (D) Varieties of amino acids (20 μM): from A to O: Pro, Ser, Ala, Gln, Val, Thr, Ile, Gly, Tyr, Met, Trp, Phe, Cys, Glu, and Lys; (E) Picture of **PZ-N** solutions in the presence of 2 μM Cu^{2+} or various anions.

mechanism, we performed the HPLC experiment. It can be clearly seen that the peak of fluorophore phenoxazine emerged after the reaction of **PZ-N** with Cu^{2+} (Figure S9). In addition, the molecular ion peak of phenoxazine at m/z = 324.2070 was found in HRMS (Figure S10). These results confirmed that **PZ-N** yielded the product phenoxazine after the addition of Cu^{2+}, further confirming the mechanism we proposed.



Scheme 2. Possible detection mechanism of the probe **PZ-N** towards Cu^{2+}.

In order to ensure the effective applications of the probe **PZ-N** at the cellular level, the interference of several ubiquitous substances in living cells, including GSH (glutathione, 0-20 μM), NAC (*N*-acetylcysteine, 0-20 μM), aldehyde (0-200 μM) and glucose (0-200 μM) was studied. Interestingly, the presence of these species did not cause remarkable interference between **PZ-N** and Cu^{2+} (Figure 3A-D). In addition, we investigated whether different atmospheres, including air, nitrogen and oxygen, would interfere the detection of

Cu^{2+}. The fluorescence intensity of probe **PZ-N** considerably increased with prolonged time in the presence of Cu^{2+} under air, nitrogen and oxygen (Figure S11). It is worth noting that little fluorescence intensity changed when the **PZ-N** was only exposed to air, nitrogen or oxygen without the presence of Cu^{2+}, indicating Cu^{2+} did induce the fluorescence intensity increase. Moreover, the stability of the fluorophore phenoxazine was explored in PBS solution in the presence of high concentrations of Cu^{2+} (20 and 50 μM, Figure S12). The result displayed that there was rare fluorescence change in the presence of 50 μM Cu^{2+} even though the reaction time was up to one hour. Importantly, the fluorescence and absorption intensity of **PZ-N** showed little change in a wide pH range from 2 to 10 without the addition of Cu^{2+} (Figure 3, E and F), indicating that pH did not trigger the structure change of the probe **PZ-N**. Furthermore, the probe **PZ-N** exhibited the strongest fluorescence and absorption intensities (Figure 3, E and F) under the neutral environment (pH = 7.0), which is beneficial for the further application in living systems. Taken all together, these results showed that **PZ-N** exhibited high stability and sensitivity towards Cu^{2+}.

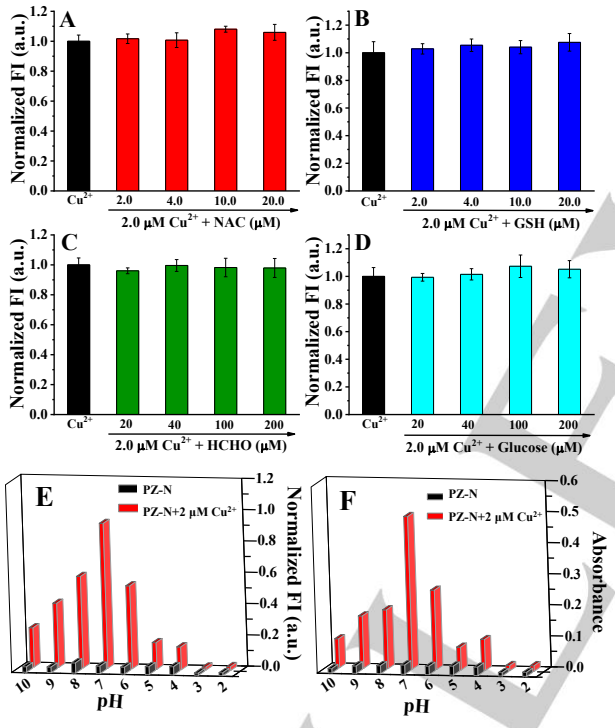


Figure 3. The anti-interference and stability of **PZ-N.** A series of substances, including NAC (A), GSH (B), HCHO (C) and Glucose (D), were added into **PZ-N** solution (10 μM). The fluorescence (E, λ_{em} = 669 nm) and absorption (F, λ_{abs} = 654 nm) intensity changes of **PZ-N** (10 μM) under different pH.

Subsequently, we further studied the practical application of **PZ-N** for monitoring Cu^{2+} in living cells. The cytotoxicity of **PZ-N** was firstly examined in living HeLa cells (Figure S13). The result showed that the probe **PZ-N** exhibited low cytotoxicity (cell viability > 90 %). And then the stability of **PZ-N** was tested under physiological condition (PBS, 37 ℃). As showed in Figure S14, the probe **PZ-N** did not show obvious absorption changes in physiological condition without the presence of Cu^{2+} within 120 minutes, indicating that **PZ-N** showed considerable stability. Then we chose HeLa cells to investigate whether the probe **PZ-N** could detect Cu^{2+} in living cells. As expected, no fluorescence was detected in absence of Cu^{2+} (Figure 4, A1-A3), while remarkable intracellular fluorescence signal could be observed upon the addition of the probe **PZ-N** and exogenous Cu^{2+} (Figure 4, B1-B3) in the fluorescence channel (700 ± 50 nm). Additionally, the probe showed concentration dependent towards Cu^{2+} at cellular level and the fluorescence signal became brighter with higher concentration of Cu^{2+} (Figure 4, B1-B3 and C1-C3). Taken together, the probe **PZ-N** was capable of permeating cell membrane and detecting Cu^{2+} in living cells and showing great potential for detecting Cu^{2+} in biological system.

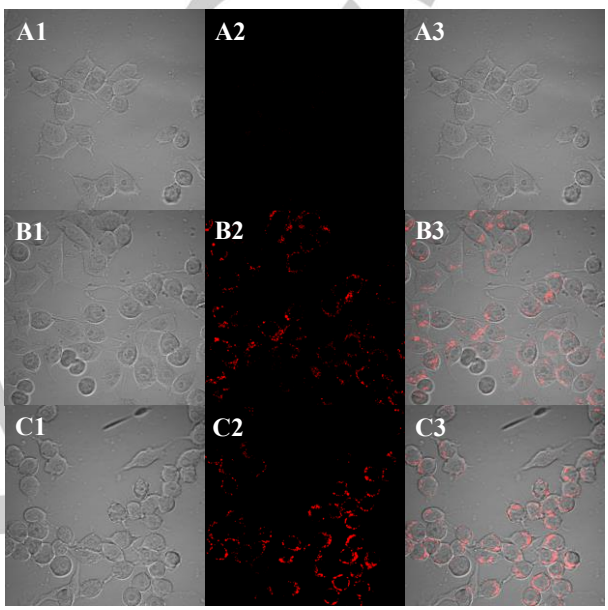


Figure 4. Fluorescence images of **PZ-N** for Cu^{2+} in HeLa cells. (A1-A3) The cells were only incubated with probe **PZ-N** (20 μM) for 20 min. The cells were preincubated with **PZ-N** (20 μM), and further cultured with 20 μM Cu^{2+} (B1-B3) and 50 μM Cu^{2+} (C1-C3) for 20 min, respectively. Images from left to right: bright field, fluorescence field and merged images, fluorescence channel: 700 ± 50 nm, λ_{ex} = 633 nm.

In summary, we designed and synthesized a novel near-infrared fluorescence "turn-on" probe **PZ-N** based on phenoxazine for detecting Cu^{2+}. The probe **PZ-N** exhibited high selectivity and fast response to Cu^{2+} in PBS solution. Furthermore, the probe **PZ-N** can be used to detect Cu^{2+} rapidly by the naked eye. More importantly, the probe showed low cytotoxicity and high specificity towards the detection of Cu^{2+} in living cells.

Acknowledgements

This research was funded by the National Natural Science Foundation of China (51872263), Taishan Scholars Project (ts20190911), and Zhejiang Provincial Natural Science Foundation of China (LZ19E020001 and LQ19B050003).

Keywords: fluorescent probes • near-infrared (NIR) • copper • phenoxazine

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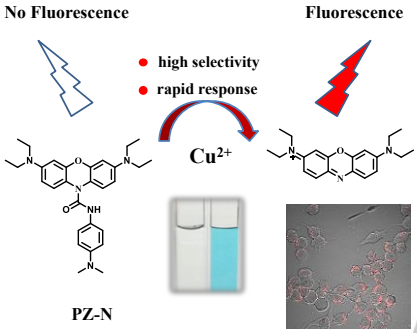

We developed a novel “turn-on” near-infrared (NIR) fluorescent probe **PZ-N** for the selective detection of copper (II) ions (Cu^{2+}). The probe **PZ-N** showed quick response and high sensitivity towards Cu^{2+}. Moreover, the probe **PZ-N** was also capable of detecting Cu^{2+} in living cells.