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Enzymatic Assay for Cu(II) with Horseradish Peroxidase and Its Application in Colorimetric Logic Gate

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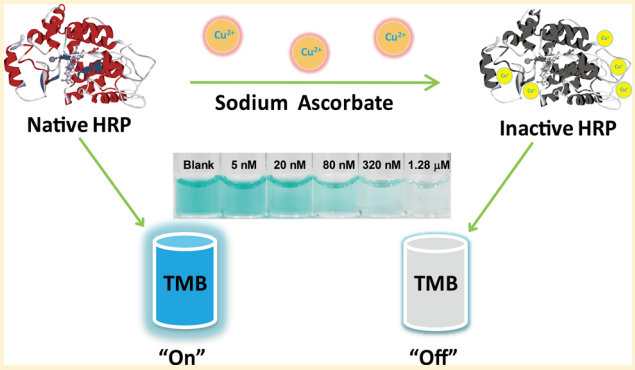
Supporting Information

ABSTRACT: We report an ultrasensitive and colorimetric assay for Cu(II) via enzymatic amplification strategy. The enzymatic activity of horseradish peroxidase (HRP) is strongly inhibited by Cu(I), which can be used indirectly to assay Cu(II). The limit of detection (LOD) is 0.37 nM, and the detection of 20 nM Cu(II) in solution can be achieved with naked eyes. This assay can be used to construct a colorimetric logic gate.



Copper plays a significant role in metabolic processes, ranging from the development of tissues to the formation of organs.[1] Both its deficiency and overdose will cause the imbalance of homeostasis, leading to severe diseases such as Wilson's disease and Alzheimer's disease.[2] As the majority of copper results from food and water intake, the U.S. Environmental Protection Agency (EPA) has set the guideline value of Cu(II) in drinking water as less than 20 μM. It is thus of considerable significance to develop a simple and efficient approach for Cu(II) assay in our daily life.

Conventional approaches, like atomic absorption spectrometry and inductively coupled plasma mass spectroscopy (ICPMS), are recognized as the gold standards for copper detection.[3] However, requirements of both advanced instruments and technicians limit their applications in resource-poor settings and point-of-care testing. The rapid development of nanotechnology offers potential solutions for this challenge. Owing to their unique properties, nanomaterials have been widely employed in the analytical field.[4] Methods without resorting to complex equipment have been reported for Cu(II) detection using various nanomaterials.[5] However, either synthesis or modification of nanomaterials is required, which impedes their practical applications. Herein, we describe a label-free method for visual detection of Cu(II) in aqueous media utilizing a commercially available enzyme called horseradish peroxidase (HRP). In addition, we have constructed a logic gate based on this colorimetric assay.

As intrinsic signal amplifiers, enzymes have well served biochemical assays because of their catalytic properties with high efficacy. Harnessing the remarkable catalytic ability, analytical signals can be amplified in an exponential manner, thus leading to the ultrasensitivity of analyte detection.[6] HRP is extensively employed because of its exceptional performances including good stability, rapid response, high catalytic efficiency, low cost, and biocompatibility.[7] When HRP is incubated with a colorless substrate such as tetramethylbenzidine (TMB), it can produce a colored and luminescent derivative, allowing for visual readout or quantification with a spectrometer.

In this paper, we report that the native HRP can selectively detect Cu(II) in aqueous solutions without any modification process or the addition of masking agents. This method relies on the fact that by reducing Cu(II) to Cu(I), Cu(I) can inhibit the catalytic ability of HRP, leading to a color change in solutions with the substrate (Scheme 1). The native HRP can catalyze TMB into a luminescent derivative with the absorption band around 405 nm, resulting in a light-blue color. Cu(I) can inhibit the catalytic activity of HRP, resulting in decreased color change to different degrees depending on the dose of Cu(I).

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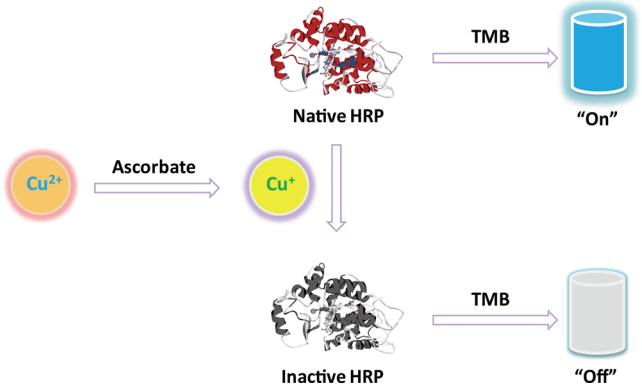
Scheme 1. Principle of HRP Inhibition-Based Assay for Cu(II)



The results can be read by either the naked eye or the spectrometer if quantification is needed.

In this assay, we developed a signal amplification strategy for a colorimetric readout of Cu(II) assay based on the catalytic capability of HRP. In a 96-well microplate, Cu(II) was reduced to Cu(I) by sodium ascorbate. After incubating Cu(I) and HRP in an aqueous solution, the catalytic activity was inhibited according to the color change of substrate solution. As Cu(I) was in situ produced by Cu(II) and sodium ascorbate, we examined their effects on the assay. The results showed that Cu(I) could inhibit the catalytic activity of HRP in a more efficient way than Cu(II) or sodium ascorbate (Figure S1, Supporting Information). In our approach, Cu(I) is in fact determined for Cu(II) assay because Cu(II) can be quantitatively transformed into Cu(I) in aqueous solutions at room temperature.

We investigated the selectivity of this method. Other metallic ions such as Co^{2+}, Cr^{3+}, Pb^{2+}, Ni^{2+}, Zn^{2+}, Ca^{2+}, Al^{3+}, Fe^{3+}, Hg^{2+}, Mg^{2+}, Ba^{2+}, Mn^{2+}, Cd^{2+}, and Ag^{+} were used at concentrations of 20 μM (Figure 1b). The concentration of Cu(II) was chosen to be 2 μM (10 times lower than the interfering ions). Sodium ascorbate was added to transform Cu(II) into Cu(I). Only Cu(II) significantly inhibited the catalytic activity of HRP, leading to a remarkable change in absorbance. Cu(II) (2 μM) inhibited more than 80% activity of HRP, while other metallic ions (20 μM) had no significant effects. It demonstrated good specificity of this enzyme inhibition-based method toward Cu(II) assay.

Table 1. Cu(II) Determination in Authentic Samples[a]

samples	ICP-OES, μM	this method, μM
1	10.09 ± 0.17	10.75 ± 0.53
2	3.63 ± 0.07	3.85 ± 0.09
3	2.55 ± 0.01	2.82 ± 0.04

[a]Sample 1: 10 μM Cu(II) into deionized water; sample 2: tap water; sample 3: Pu'er tea.

We also examined the sensitivity of this enzymatic amplification strategy-based assay. We optimized the conditions including incubation time, temperature, pH, and ionic strength of the solution (Figures S2–S5, Supporting Information). To obtain the calibration curve, we prepared Cu(II) samples in aqueous solution with concentrations ranging from 1.25 nM to 5.12 μM. The linear range of detection is from 5 nM to 1.28 μM with R^2 = 0.9943 (Figure 1d). The limit of detection (LOD) is about 0.37 nM (3 δ_{blank}). We attribute the high sensitivity to the catalytic property of HRP. As a commonly used enzyme in biochemical assays, HRP can catalyze a series of chemical reactions accompanied with color change, thus enhancing the detection signals dramatically.[8] For this colorimetric assay based on enzymatic catalysis, a sensitivity of 20 nM Cu(II) can be achieved with naked eyes, which is far below the guideline value in drinking water (20 μM) set by EPA. This naked-eye based detection, to the best of our knowledge, is the most sensitive Cu(II) assay with a label-free procedure and colorimetric readout (Table S1, Supporting Information).

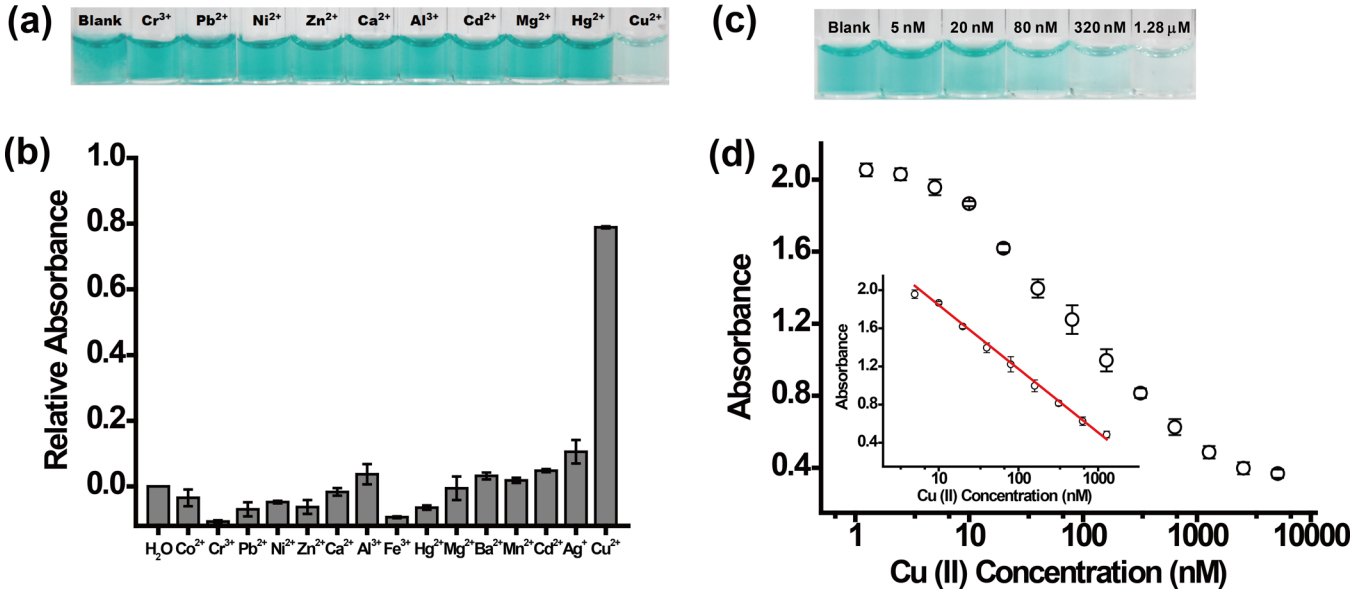


Figure 1. Selectivity and sensitivity of this assay. (a) Color change of the solution with typical metallic ions. (b) Response of absorbance with different metal ions. Cu(II) is 2 μM, and other metallic ions are 20 μM. Relative absorbance = $(A_0 - A)/A_0$, A_0 stands for absorbance of the blank control while A stands for that of the experimental group. (c) Color change with the increase of Cu(II) concentration from left to right. (d) Calibration curve and linear range of this assay. The error bars represent the standard deviation of three measurements.

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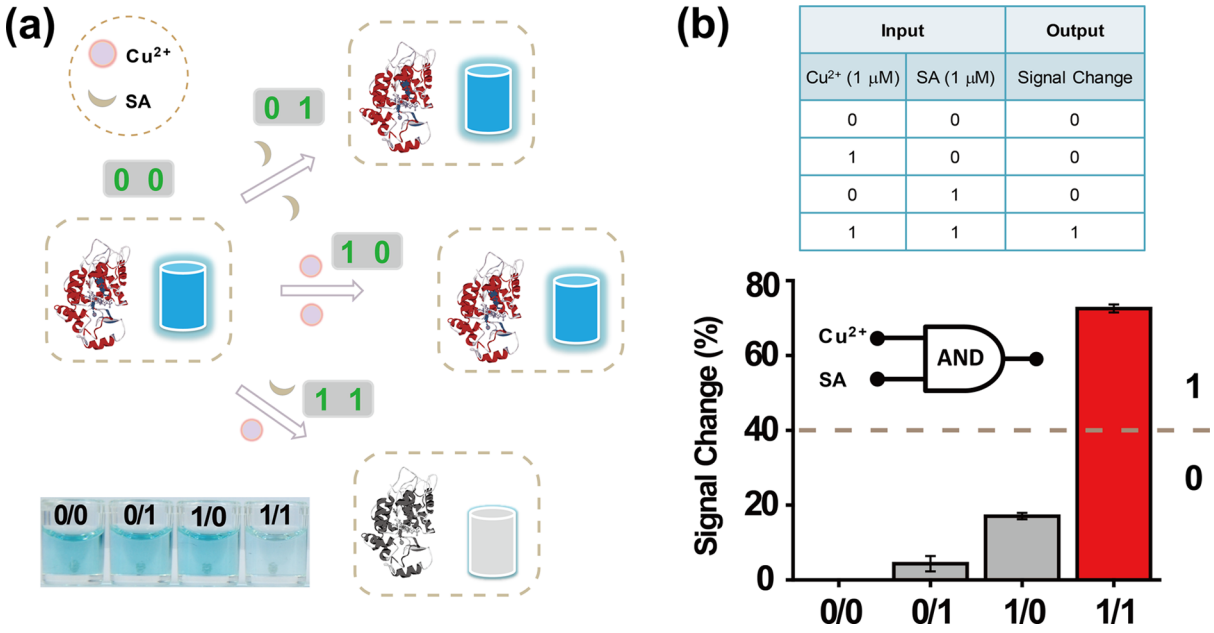

Figure 2. (a) Schematic illustration and photograph of the colorimetric "AND" logic gate. SA stands for sodium ascorbate. (b) Truth table of "AND" gate and the signal changes of different inputs. Signal change = $(A_0 - A)/A_0 \times 100\%$, A_0 is the absorbance of blank (0/0) and A is the absorbance of a certain situation (0/0, 0/1, 1/0, or 1/1). The error bars represent the standard deviation of three measurements.

To explore the mechanism that Cu(I) can dramatically inhibit the catalytic ability of HRP, inductively coupled plasma optical emission spectroscopy (ICP-OES) and X-ray photoelectron spectroscopy (XPS) were carried out. HRP was first incubated with Cu(I) or Cu(II) in solution, followed by centrifugation to remove unbound copper ions for ICP-OES and XPS analysis. ICP-OES results showed that the content of copper ions in Cu(I)-incubated HRP (29 ppb) was much more than that in Cu(II)-incubated HRP (6.4 ppb), suggesting that Cu(I) has a higher affinity with HRP than Cu(II) (Figure S6, Supporting Information). XPS characterizations verified that copper ions bound to HRP in the form of Cu(I), whether HRP was incubated with Cu(I) or Cu(II). The Cu 2p spectrum showed the characteristic satellite peaks of Cu(I), indicating the reduction of Cu(II) to Cu(I) (Figures S7 and S8, Supporting Information).[9] This reduction process might be associated with the potential reducibility of the protein.

HRP is a glycoprotein which consists of polypeptide and protoporphyrin. We inferred the interaction between Cu(I) and HRP occurred at amino acid residues rather than the porphyrin ligand by UV−vis spectrum and density functional theory (DFT) calculations. Aromatic amino acid (including phenylalanine, tyrosine, and tryptophan) residues have characteristic UV−vis absorption at around 275 nm, while the porphyrin ligand in HRP leads to the absorption at 403 nm. The UV−vis spectra showed that Cu(I)-incubated HRP presented remarkable changes in the absorption at 275 nm dependent on Cu(I) concentration (Figures S9 and S10, Supporting Information). The changes reflected the variations in the conjugated π-bond of benzene ring structure in the aromatic amino acids. However, the characteristic absorption of porphyrin ligand at 403 nm changed little. These data led us to believe that Cu(I) might not interact with the porphyrin ligand, even though Fe-porphyrin was generally considered as the active site of HRP.

We next used DFT calculations to show that Cu(I) does not interact with the porphyrin ligand. The calculations were performed with the Gaussian 09 program suite, and the method of B3LYP/Gencep was employed using the LANL2DZ basis set for the metal atoms and the 6-31G basis set for C, O, N, and H atoms in the molecule. All interaction energies were calculated by using the optimized geometries of the complex and were corrected for the basis set superposition error.[10] DFT results of Fe (III)-porphyrin and Cu(I)-porphyrin demonstrated that Cu(I) has a higher binding energy with porphyrin than Fe (III) (Figure S11 and S12, Supporting Information). The calculated binding energy of Fe (III)-porphyrin was −282.38 kcal mol^{-1}, while it was −163.15 kcal mol^{-1} for Cu(I)-porphyrin. The lower binding energy revealed that Fe (III)-porphyrin is more stable than Cu(I)-porphyrin. Therefore, the naturally existing Fe (III)-porphyrin complex would not be replaced by Cu(I)-porphyrin. Interestingly, it agreed well with ICP-OES results which showed that Fe remained at the same level in native HRP, Cu(I)-incubated HRP, and Cu(II)-incubated HRP. We thus infer that Cu(I) can inhibit the catalytic activity of HRP by interacting with amino acid residues, but the specific interaction site remains to be further investigated.[11]

As a proof of application, we employed the proposed method to detect Cu(II) in real samples. A result of 10.75 ± 0.53 μM was determined when 10 μM Cu(II) was added into deionized water. To investigate the practicality in a complicated environment, we assayed Cu(II) in tap water and tea samples. Compared with the ICP-OES result, the recovery rate was 106.06% and 110.59% for tap water and Pu'er tea, respectively (Table 1). The results showed that this facile assay could be used for qualitative analysis in practice (Table S2, Supporting Information).

This label-free assay, with straightforward readout and implementation, inspired us to apply it as an example of logic gate for the detection of metal ions.[12] We exploited this Cu(II) assay to construct a colorimetric "AND" gate (Figure 2). The two inputs were Cu(II) (1 μM) and sodium ascorbate (1 μM). We defined their absence as "0" and their presence as "1". The output was the color change of TMB solution. For output, the signal change below 40% was defined as "0", while that above

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40% was "1". Only the presence of both inputs (1/1) could lead to the inhibition of HRP, along with a dramatic color change (output = 1). When in the absence of both inputs (0/0) or in the presence of either input (1/0, 0/1), HRP continued to have catalytic activity and the color changed little (output = 0). This "AND" logic gate could be visualized by the naked eye, which might find potential applications as a biocomputing alternative.

In summary, we provide a colorimetric approach to detect Cu(II) with high sensitivity and selectivity. This label-free assay utilizes commercially available HRP to amplify the detection signals. The specificity relies on the interaction between Cu(I) (reduced from Cu(II)) and amino acid residues of HRP, resulting in the inhibition of the enzymatic activity. The linear range of the assay is from 5 nM to 1.28 μM, and 20 nM Cu(II) can be distinguished with naked eyes. The enzymatic Cu(II) assay is challenged with real samples, and we have successfully constructed a colorimetric logic gate based on this approach. This assay may become useful for indirect detections of proteins and small molecules, just as other Cu-based assays have enabled wide applications in those fields.[13]

■ ASSOCIATED CONTENT

Supporting Information

Description of the method and experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) (a) Uriu-Adams, J. Y.; Keen, C. L. *Mol. Aspects Med.* **2005**, *26*, 268. (b) Madsen, E.; Gitlin, J. D. *Annu. Rev. Neurosci.* **2007**, *30*, 317.

(2) (a) Que, E. L.; Domaille, D. W.; Chang, C. J. *Chem. Rev.* **2008**, *108*, 1517. (b) Pramanik, D.; Ghosh, C.; Dey, S. G. *J. Am. Chem. Soc.* **2011**, *133*, 15545.

(3) (a) Anthemidis, A. N.; Zachariadis, G. A.; Stratis, J. A. *Talanta* **2002**, *58*, 831. (b) Kosanovic, M.; Adem, A.; Jokanovic, M.; Abdulrazzaq, Y. M. *Anal. Lett.* **2008**, *41*, 406.

(4) (a) Xianyu, Y.; Sun, J.; Li, Y.; Tian, Y.; Wang, Z.; Jiang, X. *Nanoscale* **2013**, *5*, 6303. (b) Deng, J.; Yu, P.; Yang, L.; Mao, L. *Anal. Chem.* **2013**, *85*, 2516. (c) Zhang, Y.; Guo, Y.; Xianyu, Y.; Chen, W.; Zhao, Y.; Jiang, X. *Adv. Mater.* **2013**, DOI: 10.1002/adma.201301334.

(5) (a) Zong, C.; Ai, K.; Zhang, G.; Li, H.; Lu, L. *Anal. Chem.* **2011**, *83*, 3126. (b) Yang, X.; Wang, E. *Anal. Chem.* **2011**, *83*, 5005. (c) Guo, Y. M.; Wang, Z.; Shao, H. W.; Jiang, X. Y. *Analyst* **2012**, *137*, 301.

(6) (a) Lai, G.; Yan, F.; Wu, J.; Leng, C.; Ju, H. *Anal. Chem.* **2011**, *83*, 2726. (b) Zhao, Q.; Li, X. F.; Le, X. C. *Anal. Chem.* **2011**, *83*, 9234.

(7) Veitch, N. C. *Phytochemistry* **2004**, *65*, 249.

(8) Tang, D. P.; Yuan, R.; Chal, Y. Q. *Anal. Chem.* **2008**, *80*, 1582.

(9) Kirsch, P. D.; Ekerdt, J. G. *J. Appl. Phys.* **2001**, *90*, 4256.

(10) Rulisek, L.; Havlas, Z. *J. Phys. Chem. A* **2002**, *106*, 3855.

(11) Li, A. F.; Wang, J. H.; Wang, F.; Jiang, Y. B. *Chem. Soc. Rev.* **2010**, *39*, 3729.

(12) (a) Liu, D. B.; Chen, W. W.; Sun, K.; Deng, K.; Zhang, W.; Wang, Z.; Jiang, X. Y. *Angew. Chem., Int. Ed.* **2011**, *50*, 4103. (b) Du, J. J.; Jiang, L.; Shao, Q.; Liu, X. G.; Marks, R. S.; Ma, J.; Chen, X. D. *Small* **2013**, *9*, 1467. (c) Du, J. J.; Yin, S. Y.; Jiang, L.; Ma, B.; Chen, X. D. *Chem. Commun.* **2013**, *49*, 4196. (d) Du, J.; Zhu, B.; Chen, X. *Small* **2013**, DOI: 10.1002/smll.201300593.

(13) (a) Qu, W. S.; Liu, Y. Y.; Liu, D. B.; Wang, Z.; Jiang, X. Y. *Angew. Chem., Int. Ed.* **2011**, *50*, 3442. (b) Zhu, K.; Zhang, Y.; He, S.; Chen, W. W.; Shen, J. Z.; Wang, Z.; Jiang, X. Y. *Anal. Chem.* **2012**, *84*, 4267. (c) Lu, C. H.; Wang, Y. W.; Ye, S. L.; Chen, G. N.; Yang, H. H. *NPG Asia. Mater.* **2012**, *4*, No. e10.