An Ultrasensitive Fluorescence Sensor with Simple Operation for Cu^{2+} Specific Detection in Drinking Water

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ABSTRACT: Whether short-term or long-term, overexposure to an abnormal amount of copper ion does significant harm to human health. Considering its nonbiodegradability, it is critical to sensitively detect copper ion. Herein, a novel fluorescent strategy with a “turn-on” signal was developed for highly sensitive and specific detection of copper ion (Cu^{2+}). In the present investigation, we found that Cu^{2+} exhibits excellent peroxidase-like catalytic activity toward oxidizing the nonfluorescent substrate of Amplex Red into the product of resorufin with outstanding fluorescence emission under the aid of H_2O_2. Thus, an enzyme-free and label-free sensing system was constructed for copper ion detection with quite simple operation. To ensure the detection sensitivity and reproducibility, the amount of H_2O_2 and incubation time were optimized. The limit of detection can reach as low as 1.0 nM. In addition, the developed assay demonstrated excellent specificity and could be utilized to detect copper ion in water samples including tap water and bottled purified water without standing recovery.

1. INTRODUCTION

As an essential element, copper ion (Cu^{2+}) plays critical roles for life. It can help to generate red blood cells and act as neurotransmitters for physiological functions and also as a cofactor of various enzymes including cytochrome oxidase, superoxide dismutase, and ferroxidases. However, an abnormal amount of copper ion has significant negative effects on human health. Excessive doses of Cu^{2+} can cause neurodegenerative diseases, such as Alzheimer’s disease, Parkinson’s disease, and Menkes and Wilson disease. It is also believed that excessive Cu^{2+} can lead to gastrointestinal disturbance and liver or kidney damage. Copper ion widely exists in the environment and may be finally accumulated in the human body via food chains. Because it is nonbiodegradable and remains in the ecological system indefinitely, it is important and necessary to develop an accurate and sensitive strategy to monitor copper ion for human health.

Traditional methods for detecting copper ion are based on graphite flame atomic absorption spectrometry, surface plasmon resonance sensor, inductively coupled mass atomic emission spectrometry, inductively coupled plasma optical emission spectroscopy, plasmon-resonance Rayleigh scattering spectroscopy, and so on. These methods exhibit considerable sensitivity while suffering limitations that the detection processes are rather complicated and require costly instruments and highly trained personnel. In recent years, fluorescent sensors have attracted ever-growing attention because of their advantages: nondestructive, cost-effective, and sensitive detection. It is worth noting that a few copper ion sensors are based on signal “turn-on” response and most of the developed fluorescent sensors for copper ion detection are signal “turn-off” response, which usually presents a high background signal. Meanwhile, some developed sensing systems could be used to simultaneously detect copper ion and multiple metal ions such as Fe^{3+}, Hg^{2+}, Zn^{2+}, Cd^{2+}, Cr^{6+}, or Mn^{2+}. On the point of specific detection, the detection of copper ion might suffer from interference from other metal ions. In the present work, we aimed to develop a signal “turn-on” sensor for highly sensitive, selective, and cost-effective detection of copper ion with simple operation.

Nonfluorescent Amplex Red (AR) can be oxidized into fluorescent substrate resorufin, which is widely used as a fluorogenic substrate to engineer fluorescence sensors for monitoring different targets. The AR oxidation in known assay systems relies on the coexistence of hydrogen peroxide and horseradish peroxidase (HRP). The use of enzyme greatly limits its further application due to high cost, high requirement for preservation, and easy denaturation of the enzymes. Researchers explored to replace HRP with diverse peroxidase-like materials such as nanomaterials and G-quadruplex combining hemin. In the present investigation, we found that Cu^{2+} could directly catalyze the oxidation of AR in the present of H_2O_2. According to the property, a “turn-on” fluorescence sensor for Cu^{2+} detection was developed with quite simple operation. The developed sensing system also presents excellent selectivity and has no obvious response on other metal ions, which could assay Cu^{2+} in drinking water with good recovery.

2. RESULTS AND DISCUSSION

2.1. Sensing Principle for Cu^{2+} Detection. In the present investigation, it was found for the first time that a nonfluorescent substrate of AR can be oxidized into a fluorescent...
response of the AR/H2O2 was activated in addition to that of Cu2+. By contrast, the phenomenon was not monitored in a Cu2+ ion-free system. Therefore, the sensing system presented excellent specificity for Cu2+ ion without being affected by other metal ions (Cr3+, Zn2+, Ca2+, Mg2+, Cd2+, Pb2+, Fe3+, and Hg2+).

According to the property, a signal “turn-on” strategy was constructed for highly sensitive and selective detection of copper ion. Ultraviolet absorption experiments were conducted to validate the oxidation reaction of AR, Figure 1A. No absorption spectrum was monitored in solutions containing only AR alone (a), both of AR and H2O2 (AR/H2O2) (b) or both of AR and Cu2+ (c). However, a significant absorption response was measured once Cu2+ was added into AR/H2O2 solution (d), which was the same as that of resorufin (e). The results are consistent with the previous report by Haugland’s group.

They confirmed that AR can be oxidized as resorufin under synergetic action of HRP and H2O2. Therefore, it was reasonable to deduce that Cu2+ can replace HRP to catalyze the oxidation of AR into resorufin. The feasibility of the proposed strategy was further investigated by directly monitoring the fluorescent response of the sensing system. As shown in Figure 1B, no obvious fluorescent response was detected in solution containing only AR (a). The addition of Cu2+ did not cause any obvious change (b). A slight fluorescence enhancement could be monitored in the coexistence of AR and H2O2 (c). Interestingly, the addition of Cu2+ into AR/H2O2 solution resulted in a significant fluorescence response (d). The results indicate that Cu2+ could convert nonfluorescent AR into fluorescent resorufin in the presence of H2O2 which was consistent with the ultraviolet absorption results. It was expected that the fluorescent response of the sensing system would be related to the concentration of Cu2+. Thus, a novel, enzyme-free, label-free, and cost-effective assay for Cu2+ detection with simple operation could be realized with the mixture of AR/H2O2 as a sensing system which can function as “detection kit”. To ensure detection sensitivity, the optimization of the experimental conditions was investigated, including H2O2 concentration and incubation time of the system.

2.2. Optimization of H2O2 Concentration. According to the results presented above (Figure 1B,c), it was found that H2O2 could slightly enhance the fluorescent response of AR. Noteworthy, if the concentration of H2O2 is high enough, it would cause a high background signal that would interfere in the detection. If the concentration of H2O2 is too low, however, it requires a larger amount of Cu2+ to generate an obvious fluorescence change of the sensing system, which would reduce the detection sensitivity. To ensure the detection is as sensitive as possible, the concentration of H2O2 was then optimized in the presence of Cu2+ and AR at room temperature. As illustrated in Figure 2A, the fluorescent response of the solution was significantly increased and reached a maximum with increasing concentration of H2O2, to 8 mM. This could be clearly seen by plotting fluorescent intensity at 583 nm against concentration of H2O2, Figure 2B. With further increasing amount of H2O2, the fluorescence intensity was gradually reduced. The reason might be that the oxidation product of AR, resorufin, could be further oxidized into a colorless and nonfluorescent substrate. Thus, 8 mM was chosen as the optimal concentration H2O2 in this experiment.

2.3. Optimization of the Incubation Time. In the investigation, it was found that the oxidation of AR catalyzed by the coexistence of H2O2 and Cu2+ was related with the incubation time. To enhance the sensitivity and reproducibility, the fluorescence responses of AR/H2O2 sensing system catalyzed by Cu2+ were investigated after incubating different time intervals. To learn the signal change caused by the added copper ion, the fluorescence responses of AR/H2O2 with incubation time were measured as control experiments. In the absence of Cu2+, a slight fluorescence change of AR/H2O2 solution was monitored with increasing incubation time, Figure 3A. After adding Cu2+, the fluorescence response of AR/H2O2 was increased with different time intervals, Figure 3B. The
relative fluorescence change, \((F - F_0)/F_0\) as a function of incubation time was plotted to help find the optimum reaction time, Figure 3C. Herein, \(F_0\) and \(F\) represent the fluorescence intensity of the AR/H\(_2\)O\(_2\) solution at 583 nm in the absence and presence of Cu\(^{2+}\) after incubating the same period, respectively. The result exhibits that the ratio of \((F - F_0)/F_0\) increases to a plateau after incubating 7 h and then gradually decreases after incubating 10 h. Thus, 7 h was selected as the optimal incubation time and used for the following experiments.

2.4. Sensitive Detection of Cu\(^{2+}\) in Buffer Solution.

According to the above optimized experiment conditions, the developed sensing system for high sensitive detection of Cu\(^{2+}\) was explored. Figure 4A depicts the relationship between fluorescence responses of the "detection kit", AR/H\(_2\)O\(_2\), and copper ion within certain concentration range. The fluorescence response gradually increases with increasing copper ion. To evaluate the reproducibility of the "detection kit", error bars were calculated by plotting the degree of fluorescence intensity change \((F - F_0)/(F_{\text{max}} - F_0)\) against the concentration of copper ion, Figure 4B, where \(F\) and \(F_0\) represent fluorescence intensities at 583 nm in the presence and absence of Cu\(^{2+}\), respectively. \(F_{\text{max}}\) is the fluorescence intensity at 583 nm caused by the copper ion with its maximum detection concentration. A good linear relationship was found over the concentration range from 1.0 nM to 100 nM \((R^2 = 0.996)\). The error bars distributed within a very narrow range, indicating the excellent reproducibility of the provided assay. Limit of detection (LOD) reached as low as 1.0 nM by practical measurement rather than the conventional calculation of three times the ratio of signals-to-noise, confirming the high sensitivity. The LOD is comparable to that in recent reports about copper ion detection. Huang’s group constructed a "click chemistry" fluorescent sensor based on an intramolecular G-quadruplex formed by DNA-templated click reaction in the presence of Cu\(^{2+}\), which reported a LOD of 65 nM for Cu\(^{2+}\) detection.\(^{42}\) Zhang’s group developed a ratiometric fluorescent method for semiquantitative assay to detect Cu\(^{2+}\) down to 8.82 nM.\(^{43}\) On the basis of the specific interaction between Cu\(^{2+}\) and Cu\(^{2+}\)-dependent DNAzyme/substrate complex, a fluorophore-labeled fluorescent biosensor was provided, which could detect aqueous Cu\(^{2+}\) down to 0.6 nM.\(^{44}\) As compared with that in previous reports,\(^{45−47}\) the assay developed by us is cost-effective, label-free, and enzyme-free. Meanwhile, the detection procedure is quite simple.

2.5. Specific Detection of Cu\(^{2+}\). To evaluate the specificity of the as-prepared "detection kit", the effect of other metal ions on the sensing system was tested with Cr\(^{3+}\), Zn\(^{2+}\), Ca\(^{2+}\), Mg\(^{2+}\), Cd\(^{2+}\), Pb\(^{2+}\), Fe\(^{3+}\), and Hg\(^{2+}\). The experiments were conducted according to the same experimental procedure as Cu\(^{2+}\) detection. As shown in Figure 5, the fluorescence change of the sensing system caused by Cu\(^{2+}\) was significantly greater than that of the other metal ions, indicating that the sensing system responds specifically toward Cu\(^{2+}\). In recent reports, several groups developed fluorescent sensors with signal "turn-on" for Cu\(^{2+}\) detection. However, the Cu\(^{2+}\) detection was influenced by ions such as Hg\(^{2+}\), Fe\(^{3+}\), and Zn\(^{2+}\).\(^{30−34}\) In our investigation, the detection principle was based on the intrinsic catalytic property of Cu\(^{2+}\), which helps to eliminate the interference of other metal ions. It was noted that though Fe\(^{3+}\) has catalytic activity, it does not cause any fluorescence change of the "detection kit" since Fe\(^{3+}\) usually presents a catalytic property in acidic condition,\(^{35}\) while the Cu\(^{2+}\) determination in our investigation was conducted in neutral buffer solution that

**Figure 3.** Fluorescence spectra of the AR (0.1 μM)/H\(_2\)O\(_2\) (8 mM) solution in the absence (A) and presence (B) of Cu\(^{2+}\) (0.5 μM) after incubating different reaction time, 2 h (a), 4 h (b), 7 h (c), 10 h (d), 24 h (e). (C) Fluorescence changes of the AR/H\(_2\)O\(_2\) \((F - F_0)/F_0\) at 583 nm against different incubation time, \(F\) and \(F_0\) are the fluorescence intensities at 583 nm in the presence and absence of Cu\(^{2+}\) after incubating the same period, respectively. Error bars were obtained from three independent experimental results.

**Figure 5.** Selective investigation of the as-prepared sensing system for Cu\(^{2+}\) detection. The normalized fluorescence intensity of the sensing system at 583 nm in the presence of Cu\(^{2+}\) (0.1 μM) and other metal ions including Cr\(^{3+}\), Zn\(^{2+}\), Ca\(^{2+}\), Mg\(^{2+}\), Cd\(^{2+}\), Pb\(^{2+}\), Fe\(^{3+}\), and Hg\(^{2+}\) (0.5 μM). The error bars were obtained from three independent experimental results.
inhibits the catalytic property of Fe3+ and its interference correspondingly. This also provides a novel assay for specifically detecting Cu2+ in biological environments.

2.6. Determination Cu2+ in a Real Sample. To further investigate the potential application, the provided assay was utilized to detect Cu2+ in real samples including tap water and bottled purified water without any pretreatment. A standard addition method was adopted by adding Cu2+ into the water samples to reach final concentrations of 20 nM, 50 nM, and 80 nM. According to the experimental procedure described above, the recoveries of the proposed method were found within a range from 97% to 120% as illustrated in Table 1. The results validate the reliability of the developed assay for Cu2+ determination in real water samples.

| samples         | added (nM) | recovery (%) |
|-----------------|------------|--------------|
| tap water       | 20         | 120          |
|                 | 50         | 118          |
|                 | 80         | 105          |
| purified water  | 20         | 120          |
|                 | 50         | 115          |
|                 | 80         | 97           |

3. CONCLUSIONS
A novel enzyme-free and label-free strategy for Cu2+ detection with signal “turn-on” has been successfully developed. In our investigation, it was found for the first time that copper ion can catalyze nonfluorescent Amplex Red into fluorescent resorufin in the presence of H2O2, which was utilized to construct a sensing system for highly sensitive and specific detection of copper ion. The limit of detection reached 1.0 nM. The developed assay presents advantages as following. The detection procedure is quite simple and does not need organic synthesis, enzymatic reaction, chemical label, or sophisticated instrumentation. This greatly enhances the reproducibility and is highly critical for in situ analysis. Moreover, the provided assay is based on the intrinsic peroxidase-like activity of Cu2+ to AR/H2O2, which greatly eliminates the interference of other metal ions and presents outstanding selectivity for copper ion. In a real water sample, copper ion can be detected with excellent recovery. The provided approach is expected to have great potential application in environmental and food safety monitoring as well as in biological detection for human health.

4. EXPERIMENTAL SECTION

4.1. Materials and Chemicals. Amplex Red (AR) and resorufin were purchased from Sigma-Aldrich (St. Louis, MO). Hydrogen peroxide (H2O2) and cupric nitrate (Cu(NO3)2) were obtained from Sinopharm Chemical Reagent Co., Ltd. Other chemicals (Cr(NO3)3·9H2O, ZnSO4·(CH3COO)2Ca, (CH3COO)2Mg, CdCl2·2H2O, Pb(NO3)2, FeCl3·Hg(NO3)2) were purchased from Aladdin (Shanghai, China). All the chemicals were analytical reagent grade and used as received without further purification. Ultrapure water used throughout was prepared by the Milli-Q ultrapure water system (18.2 Ω·cm−1, Millipore System Inc.).

4.2. Detection of Cu2+. Cu(NO3)2 and H2O2 aqueous solution were freshly prepared before use. For each experiment, 20 μL of AR solution, 20 μL of H2O2, and 20 μL of different copper concentrations were mixed. Tris-HCl buffer solution (140 μL, 25 mM, pH = 7.4) was added to reach total volume of 200 μL as the sensing system. The final concentrations of AR and H2O2 were 0.1 μM AR and 8 mM, respectively. The fluorescence of the sensing system was detected after an incubation of 7 h. Fluorescence spectra were recorded from 570 to 650 nm under excitation at 540 nm at room temperature. Both emission and excitation spectra were scanned with 10 nm slit size, 650 V voltage, and 50 ms integration time.

4.3. Determination of Cu2+ in real sample. The real samples are daily drinking water including tap water and bottled purified water bought from the local supermarket. The samples were not further treated and used as received. The concentration of Cu2+ in a real sample was detected using the standard addition method. The final concentrations of added copper ion in the water sample were 20 nM, 50 nM, and 80 nM, respectively. The detection in water samples follows the same experimental procedure described in section 4.2.

4.4. Instruments. Fluorescence spectra were recorded with a LUMINA fluorescence spectrometer (Thermo, USA) at room temperature. Absorbance measurements were performed on a Cary 500 Scan UV/vis/NIR spectrophotometer (Varian, USA).

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Notes
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