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Ultrasensitive colorimetric detection of Cu^{2+} ion based on catalytic oxidation of L-cysteine

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ABSTRACT

As an essential element, copper ion (Cu^{2+}) plays important roles in human beings for its participation in diverse metabolic processes as a cofactor and/or a structural component of enzymes. However, excessive uptake of Cu^{2+} ion gives rise to the risk of certain diseases. So, it is important to develop simple ways to monitor and detect Cu^{2+} ion. In this study, a simple, facile colorimetric sensor for the ultrasensitive determination of Cu^{2+} ion was developed based on the following principle: L-cysteine and 1-chloro-2,4-dinitrobenzene (CDNB) could be conjugated to form the yellow product 2,4-dinitrophenylcysteine (DNPC), which was measurable at 355 nm; however, upon addition of Cu^{2+} ion, the absorbance of DNPC would be decreased owing to the Cu^{2+} ion catalytic oxidation of L-cysteine to L-cystine in the presence of O_2. Thus, the colorimetric detection of Cu^{2+} ion could be achieved. The optimal pH, buffer, temperature and incubation time for the colorimetric sensor were obtained of pH 6.8 in 0.1 M HEPES solution, 90 °C and 50 min, respectively. A good linearity within the range of 0.8–10 nM (r=0.996) was attained, with a high detectability up to 0.5 nM. Analyses of Cu^{2+} ion in drinking water, lake water, seawater and biological samples were carried out and the method performances were found to agree well with that obtained by ICP-MS. The developed simple colorimetric sensor proved applicable for Cu^{2+} ion determination in real samples with high sensitivity and selectivity.

1. Introduction

As one of the micronutrients, copper ion (Cu^{2+}) plays important roles in diverse metabolic processes of organisms, most importantly acting as an essential cofactor and/or a structural component of various enzymes such as cytochrome oxidase, nitrate reductase and superoxide dismutase in human bodies (Flemming and Trevors, 1989; Stasser et al., 2007). However, Cu^{2+} ion at elevated concentration gives rise to DNA damage (Trumbore et al., 2001), low-density lipoprotein oxidation (Witting et al., 1995) and membrane lipids damage (Chow, 1979), which will lead to serious neurodegenerative diseases such as Menkes disease, Wilson disease and Alzheimer's disease (Kim et al., 2008). Later, Cu^{2+} ion has been discovered to be required for oncogenic BRAF signaling which contributes to various kinds of cancers (Brady et al., 2014). In addition, higher levels of Cu^{2+} ion to the fishes, shellfishes and bacteria could significantly affect the self-purification ability of natural water systems (Aksuner et al., 2009). Since the hazardous effects of high level of Cu^{2+} ion, the maximum allowable level of Cu^{2+} ion in drinking water has been set at about 20 μM by U.S. Environmental Protection Agency (EPA) (Zhou et al., 2008). However, the concentrations of Cu^{2+} ion in certain local water area are much higher than this tolerance limit (Law, 1993). Therefore, it is of great importance to develop simple, highly sensitive and selective methods for the detection of Cu^{2+} ion.

Over the past decades, analytical methods were developed to detect Cu^{2+} ion including inductively coupled plasma mass spectrometry (ICP-MS), atomic absorption spectroscopy (AAS), electrochemical techniques, fluorescence methods and chromogenic sensors. Methods such as ICP-MS (Wu and Boyle, 1997; Dai et al., 2012) and AAS (Chan and Huang, 2000; Lima et al., 2012) can detect Cu^{2+} ion with high sensitivity and selectivity but with requirements of large instruments. Compared with electrochemical techniques (Liu et al., 1999; Salaun and van den Berg, 2006; Lin et al., 2012) and fluorescence methods (Chan et al., 2010; Yu et al., 2011; Yuan et al., 2013; Zhang et al., 2013), colorimetric methods

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are much simpler with detection by naked eyes and UV–vis spectroscopy (Lou et al., 2011; Liu et al., 2013; Shen et al., 2013; Wang et al., 2014). However, till now, the reported colorimetric methods usually present higher detection limit and cannot easily accomplish real samples analysis.

In this study, an ultrasensitive and selective sensor for detection of Cu^{2+} ion based on its catalytic activity on the oxidation of L-cysteine was developed. The concentration of Cu^{2+} ion was quantitatively determined by UV–vis absorbance of 2,4-dinitrophenylcysteine (DNPC), which was formed through the conjugation of L-cysteine and 1-chloro-2,4-dinitrobenzene (CDNB). The effects of pH, temperature and incubation time on the colorimetric sensor were investigated. Under the optimized conditions, good analytical performances were attained. Furthermore, the potential application of the sensor to real samples including drinking water, lake water, seawater and biological sample was also explored.

2. Materials and methods

2.1. Chemicals and Instruments

$CuSO_4 \cdot 5H_2O$, NaCl, KCl, LiCl, $CaCl_2$, $Cd(NO_3)_2$, $MgCl_2$, $Pb(NO_3)_2$, $ZnCl_2$, $HgCl_2$, $MnCl_2$, $FeCl_3$, $NaH_2PO_4 \cdot 2H_2O$, $Na_2HPO_4 \cdot 12H_2O$, phosphoric acid, boric acid, ethanol, acetic acid, $NaHSO_3$, $NaBH_4$, ethylenediaminetetraacetic acid (EDTA) and H_2O_2 were obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Amino acids including L-cysteine, L-cystine, glutathione (GSH), homocysteine (Hcy), tryptophan (Trp), glycine (Gly), threonine (Thr), proline (Pro), arginine (Arg), serine (Ser), methionine (Met) and valine (Val), 1-chloro-2,4-dinitrobenzene (CDNB), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), phosphate buffer saline (PBS), Britton-Robinson (B-R) and 3-(n-morpholino) propanesulfonic acid (MOPS) were purchased from Sangon Biotechnology Co. Ltd. (Shanghai, China). All other chemicals used in this study were of analytical reagent grade or better. L-cysteine was dissolved in 0.1 M HEPES solution to make the stock concentration of 2 mM (pH 6.8). CDNB was dissolved in ethanol at a concentration of 72 mM. UV–vis spectra were measured on a μ-Quant microplate reader Nanodrop 2000C (Thermo Scientific, USA) with a 1 cm quartz cell.

2.2. Conjugation of L-cysteine and CDNB

Ten microliters of 2 mM L-cysteine and 10 μL of 72 mM CDNB were simultaneously added into the 980 μL HEPES solution. The absorbance of the product 2,4-dinitrophenylcysteine (DNPC) was measured at wavelength ranging from 200 to 700 nm. To determine the interference of other amino acids, GSH, Hcy, Trp, Gly, Thr, Pro, Arg, Ser, Met and Vin were also added with CDNB in HEPES solution. To investigate the effect of temperature on the conjugation, different temperatures within the range of 20–100 °C were used. To investigate the effect of pH, HEPES with different pH ranging from 5.0 to 9.0 were used.

2.3. Detection of Cu^{2+} ion based on the its inhibitory effect on the conjugation of L-cysteine and CDNB

Heavy metal ions were tested to see if they could affect the conjugation process of L-cysteine and CDNB, where Cu^{2+} ion was picked out for its obvious inhibitory effect. To further investigate the inhibitory effect of Cu^{2+} ion, different concentrations of Cu^{2+} ion ranging from 0 to 100 nM were separately added into HEPES (pH 6.8) containing 20 μM L-cysteine and the mixture was incubated at 90 °C for 40 min. Then CDNB was added at a final concentration of 0.72 mM and kept at 90 °C for another 10 min. Control was also carried out as described above but without the addition of Cu^{2+} ion. The absorbance of DNPC was measured at wavelength ranging from 200 to 700 nm with μ-Quant microplate reader.

2.4. The function of Cu^{2+} ion in inhibitory process

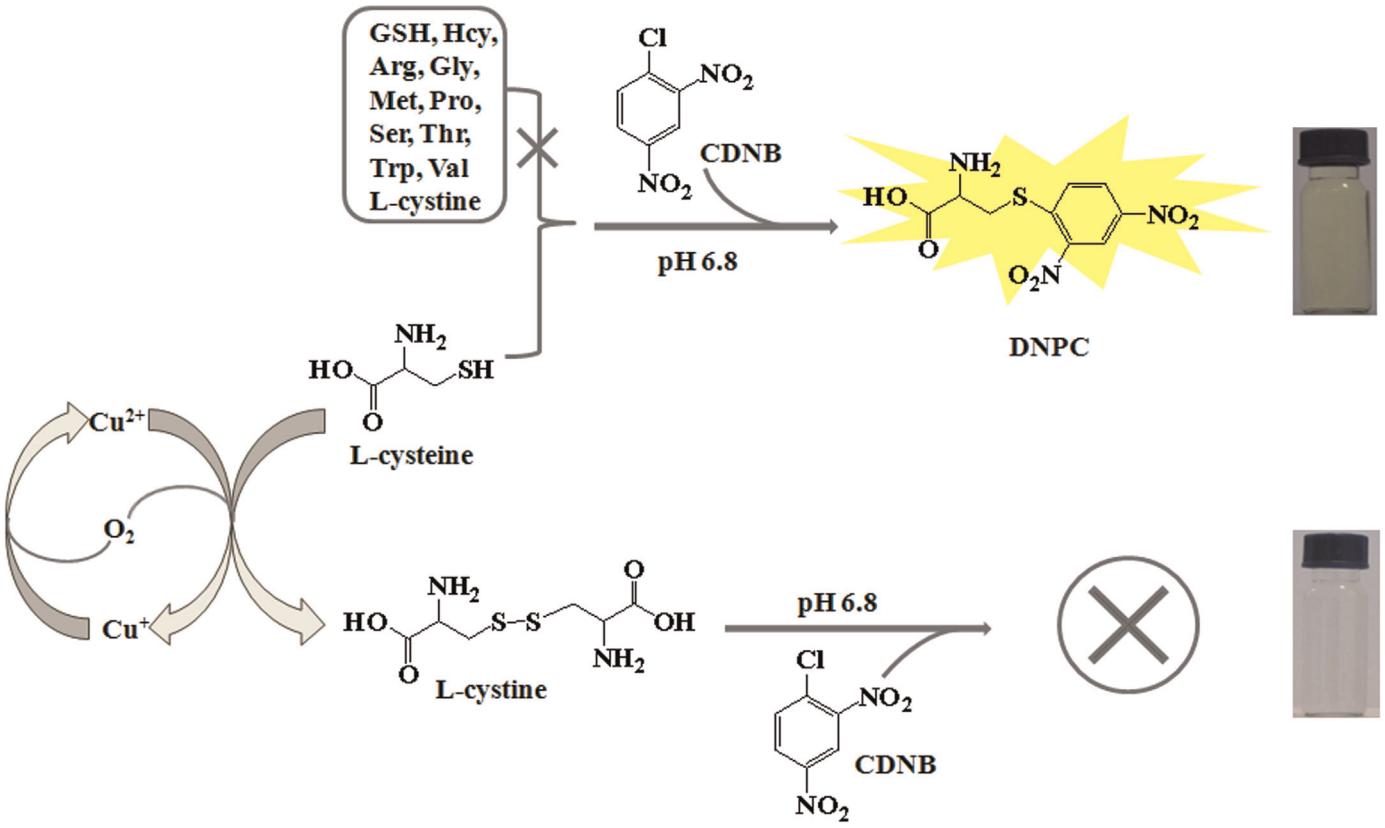
To investigate the mechanism of inhibitory effect of Cu^{2+} ion, experiments were carried out to detect whether Cu^{2+} ion was used as a catalyst or formed the composite with L-cysteine. 100 μM L-cysteine in HEPES solution was incubated at 90 °C for 40 min in the absence and presence of 50 nM Cu^{2+} ions, respectively, and then, HPLC-MS was employed to identify whether the L-cystine emerged. Furthermore, sodium sulfite was added into the system to confirm the function of O_2 as described by Meites and Meltes (1948). 100 μM L-cysteine with 50 nM Cu^{2+} ions in 0.2 M sodium sulfite pre-treated HEPES solution was incubated at 90 °C for 40 min and then 0.72 mM CDNB was added. Finally, the absorbance of the reaction product was determined. Control experiment was processed as the same but without the addition of sodium sulfite.

2.5. Sensitivity and specificity for the Cu^{2+} ion detection

We wonder that the Cu^{2+} ion could be quantitatively determined by the measurement of absorption of DNPC from which a colorimetric sensor for the determination of Cu^{2+} ion could be developed. In order to determine the linear range and detection limit of the Cu^{2+} ion sensor, the absorbance values of DNPC formed under different concentrations of Cu^{2+} ion (0, 0.4, 0.8, 2, 4, 6, 8, 10, 20, 40, 60 and 80 nM) were detected. The detection limit of the sensor was calculated by signal-to-noise (S/N) ratio equal to 3.0. To investigate whether the other ions could interfere with the detection of Cu^{2+} ion, the following ions were applied to the sensor: Na^+, K^+, Li^+, Ca^{2+}, Cd^{2+}, Mg^{2+}, Pb^{2+}, Zn^{2+}, Hg^{2+} and Mn^{2+}.

2.6. Detection of Cu^{2+} in real samples

Application of the colorimetric sensor to water and biological samples was conducted. Drinking water was collected from municipal water supply system (Yantai, China). Lake water was collected from Shandong Institute of Business and Technology (Yantai, China). The standard seawater was purchased from China Second Institute of Oceanography, State Oceanic Administration. All the water samples were only simply filtered through 0.22 μm membrane to remove particulate matters for use. After digestion using concentrated nitric acid as described by Fang et al. (2011), the shellfish samples without outshell were adequately washed with deionized water and then stored at −20 °C. Then, the frozen materials were dried in a freeze-dryer and grinded to powder by mortars. 10 mL concentrated nitric acid was added into 0.3 g powder sample, and the mixtures were incubated in a high pressure digestion tank at 150 °C for 6 h. Finally, the obtained colorless solution was diluted to 50 mL and filtered through 0.22 μm membrane. Before UV–vis detection, all the water and biological samples were diluted felicitously by deionized water to fit the linear range of the sensor.



Scheme 1. Schematic representation of the proposed colorimetric sensing principle for Cu^{2+} ion detection based on based on catalytic oxidation of L-cysteine.

3. Results and discussion

3.1. Sensing principle of the colorimetric strategy for Cu^{2+} ion detection

Scheme 1 illustrates the proposed colorimetric strategy for sensing of Cu^{2+} ion. As seen, L-cysteine could conjugate with CDNB to produce a yellow compound of DNPC at pH 6.8, which is measurable at 355 nm (Fig. S1); however, other amino acids such as GSH, Hcy, Arg, Gly, Met, Pro, Ser, Thr, Trp, Val and L-cystine could not react with CDNB at pH 6.8. As well known, in alkaline environment, CDNB can react with amino group of amino acids and thereby produce yellow conjugates, which has been used for the detection of amino acids (Sanger, 1945; Zhang et al., 2012). In our study, under the near neutral conditions (pH 6.8), it was firstly reported that CDNB could specifically conjugate with L-cysteine to produce the yellow conjugate DNPC, owing to the highly selective reaction between the thiol of L-cysteine and CDNB, as shown in Scheme 1. So, L-cysteine could be recognized specifically through its reaction to CDNB without the interference of other amino acids, even L-cystine. As shown in Fig. S2, in HEPES solution (pH 6.8) at 90 °C, the reaction between L-cysteine and CDNB was first-order, with a half-time of about 2.2 min and a rate constant k of 0.31, which was consistent with the obtained data by Burchfield (1958). Thus, a simple colorimetric strategy could be established for the sensing of L-cysteine.

More interestingly, on the other hand, in the presence of Cu^{2+} ion, the production of DNPC was reduced companied with the decrease of absorbance. As shown in Fig. 1, different concentrations of Cu^{2+} ions were added into 1 mL HEPES solution containing 100 μM L-cysteine. As observed, the color representing the concentration of DNPC became weak and even disappeared obviously, and could be distinguished with naked eyes when the concentration of Cu^{2+} ion was 50 nM. Hence, Cu^{2+} ion could be quantifiably determined by measuring the absorbance of DNPC, and therefore a colorimetric strategy could be established for the sensing of Cu^{2+} ion.

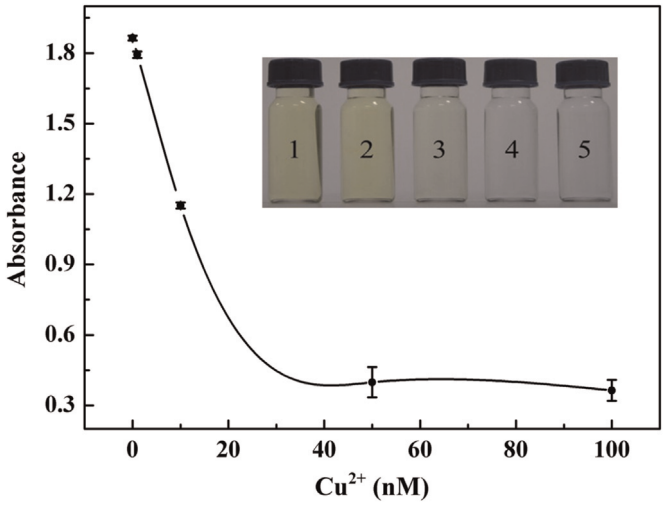


Fig. 1. The observation of inhibitory effect of Cu^{2+} ion by naked eyes. Bottles 1, 2, 3, 4 and 5 depict color of DNPC developed in the presence of 0, 1, 10, 50 and 100 nM of Cu^{2+} in the HEPES solution respectively. Data depict the means for three independent experiments and are presented as the mean ± standard error. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

As for the inhibiting effect of Cu^{2+} on L-cysteine, generally, there are mainly two kinds of principles including the chelation of Cu^{2+} ion with L-cysteine to generate L-cysteine–copper complexes (Bai et al., 1998), and the oxidization of L-cysteine to L-cystine by O_2 in the presence of Cu^{2+} ion (Pecci et al., 1997). According to the study of Rigo et al. (2004), when the molar ratio of L-cysteine to

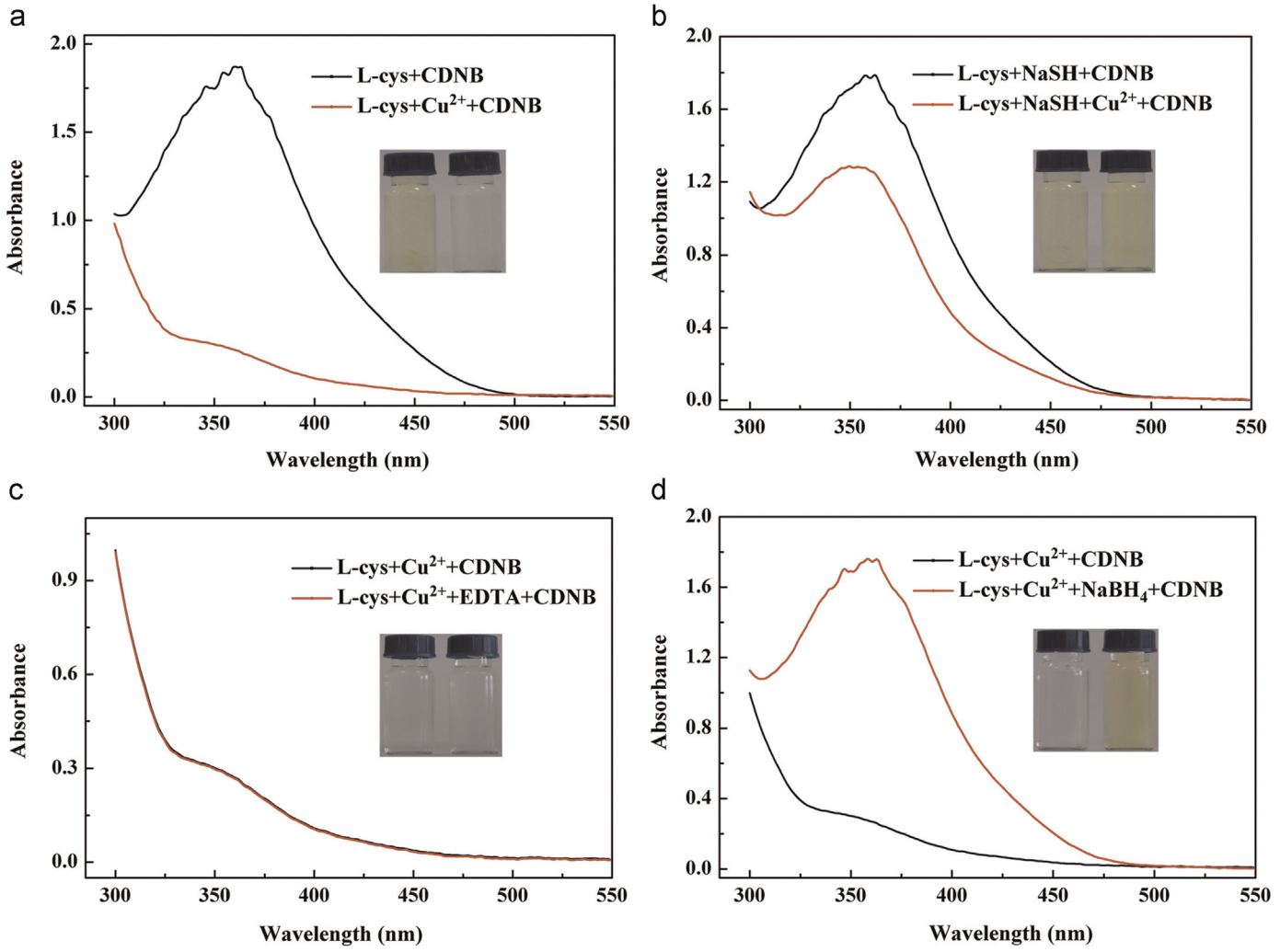


Fig. 2. Absorption spectra of DNPC which was generated by L-cysteine and CDNB (a) in the absence and presence of Cu^{2+} ion, (b) in the absence and presence of Cu^{2+} ion after O_2 was consumed by 0.2 M $NaHSO_3$, (c) in the absence and presence of 2 mM EDTA after L-cysteine reacted with Cu^{2+} ion, and (d) in the absence and presence of $NaBH_4$ after L-cysteine reacted with Cu^{2+} ion. Inset images show the responding color change. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Cu^{2+} ion was around 1:0.45, the Cu^{2+} ion would be reduced to Cu^{+} ion along with a stoichiometric production of L-cystine. In our reaction system, the molar ratio of L-cysteine to Cu^{2+} ion was much higher than 1:0.45, so it could be postulated that the L-cysteine was oxidized to L-cystine by O_2 via the Cu^{2+} ion catalytic effect, as shown in Scheme 1. To validate this assumption, several control experiments (Figs. 2 and S3–S6) were carried out from different aspects. The functions and effects of $NaHSO_3$, EDTA, $NaBH_4$ and H_2O_2 in the present reaction system were investigated. As shown in Fig. S3, L-cystine appeared and increased with L-cysteine decreasing in the presence of Cu^{2+} ion, which indicated that L-cysteine was oxidized to L-cystine. Furthermore, the yellow product DNPC appeared after L-cysteine reacted with CDNB, but disappeared in the presence of Cu^{2+} ion, as seen from Fig. 2a. After O_2 was consumed by 0.2 M $NaHSO_3$ (Meites and Meltes, 1948), in the presence of Cu^{2+} ion, the color of DNPC remained, as shown in Fig. 2b, indicating that the reaction was an oxidation reaction and the oxidization of L-cysteine only occurred in the presence of both dissolved O_2 as an oxidant and Cu^{2+} ion as a catalyst, as demonstrated in Scheme 1. It has been reported that L-cysteine would be released from L-cysteine–copper complexes in the presence of 2 mM EDTA (Fischer et al., 2011), but interestingly, the present reaction was not disturbed by EDTA, as observed in Fig. 2c. In addition, it is known that L-cystine can be reduced to L-cysteine by $NaBH_4$ (Graaf-Hess et al., 1999), so when 1 mM $NaBH_4$ was added into the reaction system after simultaneous incubation of L-cysteine and Cu^{2+} ion, followed by the addition of CDNB, the color of DNPC would be recovered, as seen in Fig. 2d. Therefore, it could be deduced that the present sensor was based on the principle: L-cysteine was oxidized to L-cystine by O_2 in the presence of Cu^{2+} ion, rather than the formation of L-cysteine–copper complexes.

The Cu^{2+}-catalyzed proceeding reaction could be presumed as the following steps (Lee and Notari, 1987; Ehrenberg et al., 1989; Pecci et al., 1997, Luo et al., 2005):

$$CSH \rightleftarrows CS^- + H^+ \quad (1)$$

$$CS^- + Cu^{2+} \rightarrow CS^{\bullet} + Cu^+ \quad (2)$$

$$2Cu^+ + O_2 \rightarrow 2Cu^{2+} + O_2^{2-} \quad (3)$$

$$2CS^{\bullet} \rightarrow CSSC \quad (4)$$

$$O_2^{2-} + 2H_2O \rightarrow H_2O_2 + 2OH^- \quad (5)$$

$$CS^- + H_2O_2 \rightarrow CSOH + OH^- \quad (6)$$

$$CSOH + CS^- \rightarrow CSSC + OH^- \quad (7)$$

$$4\,CSH + O_2 \xrightarrow{Cu^{2+}} 2\,CSSC + 2\,H_2O \quad (8)$$

where CSH was represented for cysteine, CSSC for cystine and CSOH for cysteine sulfenic acid (Luo et al., 2005). As seen, the presence of H_2O_2 could also influence this Cu^{2+}-catalyzed reaction. However, as shown in Fig. S4, the production of DNPC could be affected by H_2O_2 only when the concentration of H_2O_2 exceeded 10^{-6} M. Thus, herein, the influence of H_2O_2 to the reaction was not considered since H_2O_2 in the natural water system is normally much lower than 10^{-6} M (Uchida et al., 2008). The overall reaction for the oxidation of L-cysteine in the presence of Cu^{2+} ion could be summarized as Eq. (8). As a result of adding Cu^{2+} ion into the L-cysteine and CDNB solution, the L-cysteine was oxidized to L-cystine and thereafter DNPC was decreased leading to the absorbance decreasing, which allowed quantitation of the Cu^{2+} ion.

Furthermore, the possibility of Cu^{2+} ion decomposing DNPC and interfering the reaction kinetics was also excluded. As shown in Fig. S5, the absorbance was not decreased after adding Cu^{2+} ion, meaning that DNPC was not decomposed by Cu^{2+} ion. In addition, the reaction kinetics results (Fig. S6) indicated that the reaction between L-cysteine and CDNB was not influenced by Cu^{2+} ion. Because the Cu^{+} ion reduced from Cu^{2+} ion could be oxidized circulatory to Cu^{2+} ion by dissolved O_2, which catalyzed and recycled the whole reaction, the absorbance of DNPC decreased obviously even in the presence of quite low level Cu^{2+} ion. Consequently, the absorbance of DNPC was sensitive to even trace Cu^{2+} ion, which made the ultrasensitive detection of Cu^{2+} ion possible using the developed colorimetric sensor. So, the principle was conformed that the established colorimetric sensing strategy for Cu^{2+} ion was based on the catalytic oxidation effect of Cu^{2+} ion on the L-cysteine and CDNB conjugation system, as proposed and illustrated in Scheme 1.

3.2. Conjugation of L-cysteine with CDNB

It is well known that L-cysteine is usually detected by derivatization methods through high performance liquid chromatography (Lindroth and Mopper, 1979; Zhang et al., 2012). However, derivatization processes are often quite complicated and easily interfered. In the present study, L-cysteine could specifically react with CDNB under pH 6.8 and produce the yellow conjugate of DNPC. And the color of the DNPC could be directly observed with naked eyes when the initial concentration of L-cysteine was 30 μM (Fig. S7). This result indicated that the visual detection of L-cysteine could be realized, which owned advantages over the derivatization methods for its simplicity and time effectiveness. The absorbance of DNPC was linearly dependent within the concentration of L-cysteine between 2 and 40 μM (Fig. S8). The lowest detection concentration for L-cysteine was 0.2 μM based on the signal-to-noise ratio of 3 (S/N=3), which was comparable to that of the previous methods (Wei et al., 2010; Hsiao et al., 2011; Lu et al., 2011). So, in the present sensing strategy, the special conjugation of L-cysteine with CDNB was confirmed, and thereby enabled the following Cu^{2+} ion detection.

3.3. Parameter optimization of the colorimetric sensor for Cu^{2+} ion detection

Performances of the sensor for Cu^{2+} ion based on the inhibitory process of Cu^{2+} ion to the conjugation of L-cysteine and CDNB strongly depended on the relevant experimental parameters including concentration of L-cysteine, buffer, pH, temperature and incubation time. Their effects for the DNPC absorbance were systematically investigated as follows. Firstly, different concentrations of Cu^{2+} ion were added into 1 mL of 20 μM L-cysteine HEPES solution. 20 μM L-cysteine was chosen because not only the color could be distinguished by naked eyes after conjugated with CDNB at this concentration but also the inhibitory process could be accomplished even in the presence of quite low concentration. And then, some more experiments were carried out for parameter optimization of the colorimetric sensor.

To examine the effect of buffer on the oxidization of L-cysteine in the presence of Cu^{2+} ion, the absorbance values of DNPC produced in different buffer systems including HEPES, PBS, B-R and MOPS were measured, respectively. The results showed that the best catalytic activity of Cu^{2+} ion on the oxidization of L-cysteine was obtained in HEPES solution, as shown in Fig. S9. Therefore, HEPES solution was chosen as buffer for the following experiments.

To evaluate the effect of pH on the catalytic efficiency of Cu^{2+} ion, the HEPES solutions with different pH values ranging from 5.0 to 9.0 were used. As observed in Fig. S10, the catalytic efficiency of Cu^{2+} ion increased from pH 5.0 to 6.8 and then decreased when the pH exceeded 6.8, showing that the catalytic efficiency of Cu^{2+} ion was significantly influenced by pH. It has been reported that better nucleophilic thiolateanion is received with pH value increasing from 5.0 to 6.8 (Tang and Chang, 1995). However, since amino group of amino acids could also react with CDNB when pH exceeded 6.8, higher concentration L-cystine generated from the oxidation of L-cysteine could interfere with the detection of Cu^{2+} ion. Moreover, the Fe^{3+} ion could also catalyze autoxidation and act as a direct oxidant in thiol oxidation under acidic conditions (Luo et al., 2005; Pirie, 1931), but the catalysis would be invalid at (near) neutral or alkaline conditions such as pH 7.3 (Elliott, 1930). As a result, pH 6.8 was chosen for the experiments.

To examine the effect of temperature, oxidization of L-cysteine by O_2 was performed at different temperatures ranging from 0 to 100 °C. As displayed in Fig. S11, the catalytic efficiency of Cu^{2+} ion increased with increasing temperature and reached the maximum at 90 °C. Thus, detection of Cu^{2+} ion using this sensor was carried out at 90 °C for the following experiments.

To test the effect of incubation time, the reaction system was divided into two phases. During the first phase, L-cysteine was oxidized by O_2 in the presence of Cu^{2+} ion for different time points, followed by the addition of CDNB, which was kept at 90 °C for another 10 min. As shown in Fig. S12a, 20 μM L-cysteine could be oxidized by O_2 within 40 min at the greatest extent. During the second phase, following the oxidization of L-cysteine by O_2 for 40 min, CDNB was added and reacted with L-cysteine at 90 °C for different time points. As illustrated in Fig. S12b, the complete conjugation of L-cysteine and CDNB could be obtained within 10 min. So, the incubation time was chosen, 40 min for L-cysteine oxidation and then 10 min for DNPC generation.

3.4. Specificity of the colorimetric sensor for Cu^{2+} ion

Specificity is a primary concern of a sensor, so we demonstrated the specificity of the developed sensor by carrying out two groups of experiments. One was to test the absorbance of DNPC in the individual presence of other metal ions, respectively, at 100 times concentration of Cu^{2+} ion. Another is to test the absorbance of DNPC in the presence of Cu^{2+} ion and 10 times the above other metal ions.

In order to examine the specific catalytic ability of Cu^{2+} ion, other ions including Na^+, K^+, Li^+, Ca^{2+}, Cd^{2+}, Mg^{2+}, Pb^{2+}, Zn^{2+}, Hg^{2+} and Mn^{2+} were separately added into the same reaction solution containing 20 μM L-cysteine and 0.72 mM CDNB as Cu^{2+} ion. The concentration of Cu^{2+} ion was 10 nM, while 100-fold (1 μM) other metal ions were used. As observed in Fig. 3a, the absorbance of DNPC remained only 20% in the presence of Cu^{2+} ion, while the absorbance did not show significant decrease and difference in the presence of

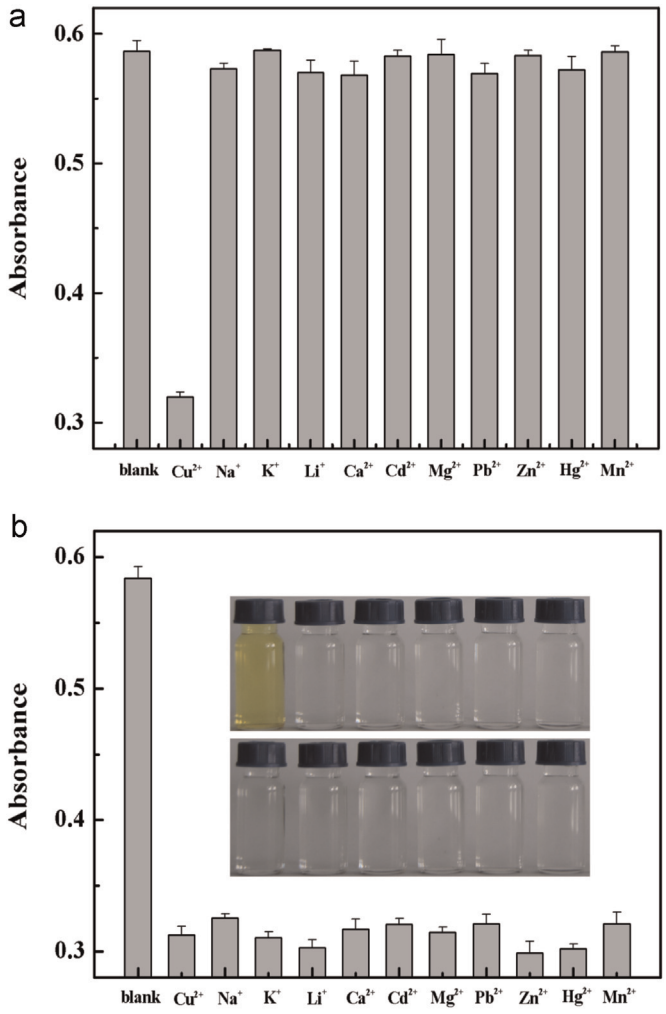


Fig. 3. Specificity of the colorimetric sensor. (a) Each metal ion was separately added into the reaction solution, and the absorption at 355 nm was quantitatively measured. The concentration of Cu^{2+} ion was 10 nM, and the concentrations of other metal ions were 1 μM. (b) Cu^{2+} ion was added into the reaction system in the presence of other metal ions. The concentration of Cu^{2+} ion was 10 nM, and the concentrations of other metal ions were 100 nM. Data depict the means for three independent experiments and are presented as the mean ± standard error. Inset images: from up to down and from left to right: blank, Cu^{2+}, Na^{+}, K^{+}, Li^{+}, Ca^{2+}, Cd^{2+}, Mg^{2+}, Pb^{2+}, Zn^{2+}, Hg^{2+} and Mn^{2+}, respectively.

other metal ions. Therefore, the developed sensor had a superior selectivity specialism for Cu^{2+} ion.

In order to further examine the specificity and reliability of the sensor for Cu^{2+}, the absorbance of DNPC in the presence of Cu^{2+} along with other possible interference ions including Na^{+}, K^{+}, Li^{+}, Ca^{2+}, Cd^{2+}, Mg^{2+}, Pb^{2+}, Zn^{2+}, Hg^{2+} and Mn^{2+}, respectively, was determined. Cu^{2+} ion and other metal ions were simultaneously added into the reaction system. The concentrations of other metal ions were 10-fold higher than that of Cu^{2+} ion. As shown in Fig. 3b, the sensor responded specifically toward Cu^{2+} ion without interference of other metal ions. Even though there is the fact that L-cysteine can form a chelate ring (usually 1:1) with divalent metal ions of Cd^{2+}, Pb^{2+}, Zn^{2+} and Hg^{2+} (Shindo and Brown, 1965), nevertheless, the concentrations of metal ions used in this work were much lower than the concentration of L-cysteine, and thus the presence of such heavy metals could not disturb the Cu^{2+} ion detection process. Moreover, L-cysteine can also be oxidized in the presence of Fe^{3+} ion in some study cases (Ehrenberg et al., 1989), but the solubility product constant (Ksp) of $Fe(OH)_3$ is 2.79×10^{-39}, and therefore, $[Fe^{3+}]$ is only 7×10^{-19} M under pH 6.8, which could not

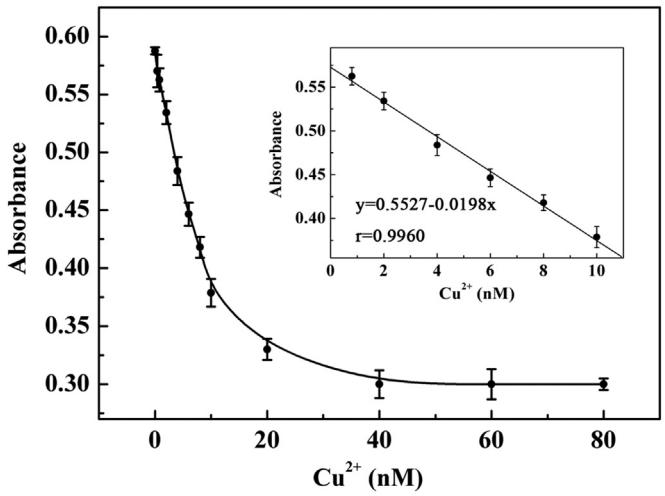


Fig. 4. Absorption values of DNPC in the presence Cu^{2+} ion with concentrations of 0, 0.4, 0.8, 2, 4, 6, 8, 10, 20, 40, 60 and 80 nM, respectively. Inset: a plot between 0.8 and 10 nM with linear regression equation $y = 0.5727 - 0.0198x$, $r = 0.9960$, where x and y represent the concentration of Cu^{2+} ion and the absorbance of DNPC, respectively. Data depict the means for three independent experiments and are presented as the mean ± standard error.

have obvious interferences with the reaction (Liu and Millero, 2002). In addition, the catalytic activity of Fe^{3+} ion has been proved to usually occur under acidic conditions (Pirie, 1931), and the catalysis of cysteine oxidation cannot be completed at pH 7.3 (Elliott, 1930). All the results indicated that the colorimetric sensor provided attractive specificity toward Cu^{2+} ion.

3.5. Sensitivity of the colorimetric sensor for Cu^{2+} ion

The ability of the developed sensor for quantitative analysis of Cu^{2+} ion was further evaluated. Under the optimal conditions, the concentration of Cu^{2+} ion ranging from 0.2 to 80 nM was added into the sensing system. As shown in Fig. 4, the absorbance of DNPC decreased with increasing concentrations of Cu^{2+} ion. A good linearity in the range of 0.8–10.0 nM was exhibited with relative standard deviations between 0.6% and 3.7% (inset of Fig. 4). The limit of detection for Cu^{2+} ion was attained of 0.5 nM based on the signal to noise ratio of 3 (S/N = 3) which was much lower than the other existing colorimetric methods (Zhou et al., 2008; Shen et al., 2013). So, the colorimetric strategy was demonstrated to be ultrasensitive and highly reliable for the detection of trace Cu^{2+} ion.

3.6. Applications of the sensor to real water and biological samples

To demonstrate the applicability of the developed colorimetric strategy with high selectivity, sensitivity and reliability for practical sample analysis, Cu^{2+} ion was measured in real water and biological samples. The concentrations of Cu^{2+} ion in the tested drinking water, lake water, seawater and shellfish samples are presented in Table 1. It was found that the results obtained by the present colorimetric method were in excellent agreement with those obtained by ICP-MS. In the drinking and lake water samples, the detected concentrations of Cu^{2+} ion by this sensor were 14.96 and 27.34 nM, respectively, which were approximate to the values of 15.38 and 26.75 nM detected by ICP-MS, respectively. In the seawater sample, 87.39 nM Cu^{2+} ion was detected using this sensor, which was slightly higher than but still comparable to that obtained using ICP-MS. The results revealed that it is possible to detect Cu^{2+} ion in aquatic environments by the developed sensor. Additionally, the Cu^{2+} ion in the shellfish sample detected was 33.38 nM, which was also comparable to the result obtained by

Table 1
Comparison of the results obtained by the developed colorimetric sensing method and ICP-MS for determination of Cu^{2+} in real water and biological samples.

Sample	This method (nM)	ICP-MS (nM)
Drinking water	15.96 ± 0.57[a]	15.38
Lake water	29.34 ± 1.85	26.75
Seawater	87.39 ± 2.39	78.13
Shellfish	33.38 ± 1.63	38.28

[a] Mean value of three determinations ± standard error.

ICP-MS. All these data indicated the potential application of the developed Cu^{2+} ion colorimetric sensor in real samples. Hence, the present sensing method was proved practically feasible for real sample analysis with high accuracy and good reliability.

3.7. Method performance comparison

The performance of the developed colorimetric method for the determination of Cu^{2+} ion was compared with some reported approaches such as ICP-MS, electrochemistry, fluorometry and other colorimetric methods. As seen from Table S1, methods such as ICP-MS (Wu and Boyle, 1997; Dai et al., 2012) and AAS (Chan and Huang, 2000; Lima et al., 2012) can detect Cu^{2+} ion with high sensitivity and selectivity, but they require large-scale instruments and highly trained operators restricting the practical on-site detection. The fluorometry methods also own good selectivity, but they are often troubled with complicated synthesis (Chan et al., 2010; Yu et al., 2011; Yuan et al., 2013; Zhang et al., 2013). The electrochemistry methods show low detection limit and broad linear range, but possibly with the disadvantages of instability and interference of organics in anodic/catholic stripping voltammetry (Liu et al., 1999; Salaun and van den, Berg, 2006; Lin et al., 2012). The reported colorimetric methods can be realized by naked eyes but without ideal sensitivity (Lou et al., 2011; Liu et al., 2013; Shen et al., 2013; Wang et al., 2014). Our established method presented ideal sensitivity and simple operation processes. More importantly, the method can be successfully applied for the detection of Cu^{2+} ion in complex matrices, especially in seawater samples and biological samples. As a result, comparison with the previous methods for Cu^{2+} ion determination, the present method has remarkable advantages such as simplicity and universality, high selectivity and sensitivity, good reliability and practicability.

4. Conclusions

In summary, a simple facile colorimetric sensor was successfully developed for ultrasensitive detection of Cu^{2+} ion based on the catalytic oxidization of L-cysteine. By taking advantages of the specific conjugation reaction of L-cysteine with CDNB at pH 6.8, and the specific catalytic ability of Cu^{2+} ion to L-cysteine, excellent analytical performances were attained such as high sensitivity (0.5 nM), high selectivity, and desirable practical applicability in seawater and biological samples. Since various amino acids/proteins or other biological molecules (e.g., L-cysteine) as well as specific interactions (e.g., conjugation and catalysis) have been exploited, such a sensing strategy can open up new opportunities and provide promising potentials for analysis of heavy metals.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.08.058.

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