A Sensitive and Selective Method for Visual Chronometric Detection of Copper(II) Ions Using Clock Reaction

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We report a visual chronometric method for the detection of copper(II) ions (Cu²⁺) using the clock reaction between methylene blue (MB) and hydrazine (N₂H₄). The addition of Cu²⁺ greatly accelerates the clock reaction due to the formation of the Cu cluster between Cu²⁺ and N₂H₄. By recording the change of the reaction time and solution color, different concentrations of Cu²⁺ in buffer and environmental water have been detected. As low as 300 nM Cu²⁺ can be visually identified by the naked eye without the assistance of any advanced instruments. This visual chronometric assay shows a dynamic range from 0.2 to 16 μM with a limit of detection of 20 nM. Additionally, it has good selectivity toward Cu²⁺ and against other common metal cations.

Keywords Copper(II), assay, detection, visualization, chronometry

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Introduction

The development of sensitive and selective methods for the detection of metal ions is of great importance for environmental protection and monitoring.⁴⁴ Among these metal ions, copper(II) ions (Cu²⁺) have high toxicity to humans when the uptake is excessive. For example, a high level of Cu²⁺ can induce cell apoptosis, amyotrophic lateral sclerosis, kidney and liver damage, Menkes syndrome, Wilson’s disease, and so on.⁵–⁷ Because copper-containing commercial products are widely used in the electronics industry and agriculture, copper contamination is an unneglectable problem.⁶⁷ Accordingly, the determination of Cu²⁺ in environmental water samples is an important issue. To this end, several approaches have been developed for detection of Cu²⁺, including inductively coupled plasma-atomic emission spectrometry, atomic absorption spectroscopy, voltammetry, and fluorometry.⁵₄–⁶₃ However, these methods often require sophisticated and expensive instrumentation.

On the contrary, visual detection methods are extremely appealing for the detection of Cu²⁺ because they can be directly read out with the naked eye. A variety of visual methods based on plasmonic nanoparticles and organic chromogenic reagents have been developed for visual detection of Cu²⁺.¹⁸–²⁶ These assays are based on the color change induced by Cu²⁺. Despite these advancements, there are still some limitations. For example, the methods using organic chromogenic reagents have low sensitivity, which requires multi-step organic preparations. Although the plasmonic nanoparticle-based assays are able to improve the sensitivity, the nanoparticle probes are easily affected by environmental conditions such as salts, temperature, and thiol-containing substances.¹⁸–²⁶ Moreover, the plasmonic nanoparticle is metastable, which gradually aggregates with time. These drawbacks seriously influence their analytical performance in terms of accuracy, reproducibility, and practicality. Therefore, there is an urgent need to develop a novel Cu²⁺ detection strategy.

Recently, the clock reaction of methylene blue (MB) and hydrazine (N₂H₄) has been receiving more and more attention. In this reaction, a reversible color change from blue to colorless and back has been observed upon the addition of Cu²⁺. In this work, we report a visual chronometric assay for Cu²⁺ detection based on the clock reaction between MB and N₂H₄.

Scheme 1 Schematic illustration of the visual chronometric detection of Cu²⁺ using the clock reaction of MB and N₂H₄.

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is observed based on redox reactions after an incubation time.27–29 The reaction time of the color change can be used for visual chronometric detection of analytes such as chromium(III) ions, glutathione and cysteine in the presence of nanocatalysts.30,31 However, the visual detection of Cu²⁺ based on the clock reaction has not been reported to date. Herein, we present a visual chronometric assay for ultrasensitive and selective detection of Cu²⁺ without the assistance of nanocatalysts. As illustrated in Scheme 1, the reduction of MB by N₂H₄ can not be effectively carried out in the absence of Cu²⁺. The color change requires a long time. However, after introduction of Cu²⁺, an obvious color change from blue to colorless is observed within a short incubation time, as the blue MB is reduced to the colorless leucomethylene blue (LMB). When shaking the reaction solution, the color of the solution becomes blue again due to the reoxidation of the LMB by oxygen (clock reaction). The variation of the reaction time (the time when the blue color of the test sample in the solution has completely disappeared) upon addition of Cu²⁺ provides a visual chronometric detection strategy. This method shows nanomolar level sensitivity, and the feasibility for determination of Cu²⁺ in real environmental water was also studied.

Experimental

Materials and instruments

MB was purchased from Shanghai Aladdin Bio-Chem Technology Co., Ltd. N₂H₄, copper(II) sulfate pentahydrate (CuSO₄·5H₂O) and other metal salts were obtained from Chengdu Kelong Reagent Co., Ltd. All the chemical reagents were directly used without further purification. Deionized water was employed to prepare various water solutions. Ultraviolet-visible (UV-vis) absorption spectra were recorded with a Shimadzu UV-1800 spectrophotometer.

Visual chronometric detection protocol for Cu²⁺

All the operations were carried out at room temperature. First, 2.6 mL of Britton-Robinson (BR) buffer (pH 10.4) was mixed with 0.1 mL of Cu²⁺ aqueous solution of different concentrations (0 - 0.5 mM). Then, 0.2 mL of 0.5 mM MB was added to the above solution. Finally, 0.1 mL of 1 M N₂H₄ was introduced to induce the color change, which was recorded with a video camera of a smartphone.

To demonstrate the practicality of this assay, river and lake water were collected, which were diluted 10-fold with deionized water. Subsequently, different concentrations of Cu²⁺ (0.5, 0.1, 0.01 mM) were added to these water samples. The spiked water samples were analyzed by using the same experimental procedures as mentioned above.

Results and Discussion

Cu²⁺ promoted MB-N₂H₄ clock reaction

The Cu²⁺ promoted MB-N₂H₄ clock reaction was investigated by UV-vis absorption. As shown in Fig. 1, the MB solution showed a maximum absorption wavelength at 664 nm. The reduction reaction between MB and N₂H₄ was relatively slow, and a slight change in absorbance was observed as shown in Fig. 1a. In contrast, the introduction of 16 μM Cu²⁺ remarkably accelerated the reaction speed of MB and N₂H₄, and the
characteristic absorption peak of MB at 664 nm completely disappeared within 90 s thanks to the generation of the colorless LMB, indicating that Cu$^{2+}$ can effectively promote the reaction between MB and N$_2$H$_4$. Moreover, the reaction speed of MB and N$_2$H$_4$ is dependent on the Cu$^{2+}$ concentration (Figs. S1 and S2, Supporting Information). With the increase of Cu$^{2+}$ concentration, the reaction speed increases as shown in Fig. 1c. Importantly, the MB color can be reproduced by directly shaking the reaction solution as depicted in Fig. 1d. The absorbance value at 664 nm gradually increases with the shaking time, suggesting that the colorless LMB is oxidized by oxygen from air. Thereafter, the MB is reduced by the excess N$_2$H$_4$ again. Therefore, a periodic color change between MB and LMB is found, confirming the clock reaction.

It has been demonstrated that the Cu cluster is capable of catalyzing the reduction of MB by N$_2$H$_4$ due to the formation of the Cu cluster-MB complex. In our reaction system, Cu$^{2+}$ and N$_2$H$_4$ coexist in the solution, in which a reduction reaction may occur to produce the Cu cluster in situ. To prove the production of the Cu cluster, the UV-vis absorption spectra of Cu$^{2+}$, N$_2$H$_4$, and their mixture were measured as displayed in Fig. S3 (Supporting Information). The UV-vis absorption results show that Cu$^{2+}$ and N$_2$H$_4$ have no obvious absorption in the wavelength range of 400 - 600 nm, while the mixture of Cu$^{2+}$ and N$_2$H$_4$ gives rise to a strong absorption band, which is attributed to the Cu cluster. In addition, a strong fluorescence emission is observed in the mixture of Cu$^{2+}$ and N$_2$H$_4$ (Fig. S4, Supporting Information), which further demonstrates the production of the Cu cluster. Thus, the Cu$^{2+}$ promoted MB-N$_2$H$_4$ clock reaction involves three steps as shown in Fig. S5 (Supporting Information). Cu$^{2+}$ is firstly reduced by N$_2$H$_4$ to generate Cu cluster which further interact with MB to produce the Cu cluster-MB complex. The resulted complex reacts with N$_2$H$_2$ to yield colorless LMB.

**Feasibility and optimization of the visual chronometric method**

The feasibility of the visual chronometric method for detection of Cu$^{2+}$ is verified in BR buffer solution by chronometric measurements. As shown in Fig. 2, the color change of the MB-N$_2$H$_4$ reaction from blue to colorless requires about 1002 s. Nevertheless, the introduction of Cu$^{2+}$ induces a fast reduction process, and the MB solution becomes colorless after reaction for 43 s. The alteration of the reaction time in the absence and presence of Cu$^{2+}$ illustrates that the method is feasible to determine Cu$^{2+}$.

In order to obtain better analytical performance, some key experimental parameters including N$_2$H$_4$ concentration and the pH of the buffer solution were optimized. The reaction time of the Cu$^{2+}$ promoted MB-N$_2$H$_4$ clock reaction is related to the N$_2$H$_4$ concentration (Fig. S6a, Supporting Information). A high concentration of N$_2$H$_4$ results in a short reaction time. When the N$_2$H$_4$ concentration exceeds 0.03 M, the reaction time remains

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Figure 2: Typical photo images of the MB-N$_2$H$_4$ clock reaction in the absence and presence of 16 μM Cu$^{2+}$. The concentrations of MB and N$_2$H$_4$ are 33 μM and 33 mM, respectively.

Figure 3: (a - c) Visual detection and (d) calibration curve of the reaction time versus different concentrations of Cu$^{2+}$ (0 - 16 μM).
300 nM. The reaction time against Cu$^{2+}$ concentrations are lowest detection concentration by the naked eye is as low as simple, low-cost and does not require any advanced instruments.

Oppositely, our method is extremely complicated surface modification procedures for gold nanoparticles (AuNPs). Additionally, this sensitivity is comparable to the electrochemistry, colorimetric and fluorescence techniques orders of magnitude better than those of widely used methods. Taken together, these results suggest that this visual chronometric method offers the potential to be replaced to apply the traditional methods for detection of Cu$^{2+}$.

**Sensitivity, selectivity, and reproducibility of the visual chronometric method**

In order to evaluate the sensitivity of the visual chronometric method, the reaction time of the MB-N$_2$H$_4$ clock reaction was recorded after adding different concentrations of Cu$^{2+}$. Figures 3a - 3c show the typical optical image of the clock reaction at different reaction time points. Interestingly, the distinct color change allows visual detection of Cu$^{2+}$, and the lowest detection concentration by the naked eye is as low as 300 nM. The reaction time against Cu$^{2+}$ concentrations are displayed in Fig. 3d. The reaction time gradually decreases with the increasing of the Cu$^{2+}$ concentration from 0.2 to 16 μM, and a non-linear fitting method (ExpAssoc model) is utilized to fit the calibration curve with a correlation coefficient of 0.996. The limit of detection (LOD) is calculated to be 20 nM based on a signal-to-noise ratio of 3.2. This LOD is typically one to three orders of magnitude better than those of widely used electrochemistry, colorimetric and fluorescence techniques (Table 1). Additionally, this sensitivity is comparable to the lateral flow method that employs expensive DNA and complicated surface modification processes, and sample pretreatment, suggesting it as optimized experimental conditions for the determination of Cu$^{2+}$.

**Practical application of the visual chronometric method**

The practical application of the visual chronometric method was estimated by detecting Cu$^{2+}$ spiked environmental water samples. The river and lake water samples were collected and diluted 10-fold with deionized water without further treatment. As summarized in Table S1 (Supporting Information), good recoveries from 96.7 to 104% are obtained in the spiked samples by this method. In addition, the RSDs were less than 3%. These findings authenticate that this visual chronometric method is promising for the detection of Cu$^{2+}$ in real water samples.

**Conclusions**

In summary, a visual chronometric assay was developed for the detection of Cu$^{2+}$ based on the Cu$^{2+}$ promoted MB-N$_2$H$_4$ clock reaction. The promotion mechanism is attributed to the production of the Cu cluster between Cu$^{2+}$ and N$_2$H$_4$. The assay shows good sensitivity with a LOD of 20 nM, and selectivity against other metal cations, which is successfully applied to determine Cu$^{2+}$ in environmental water samples. Compared with the traditional methods, this assay does not involve any complicated instruments, nanomaterials preparation, surface modification processes, and sample pretreatment, suggesting it has a great potential as a facile, reliable, low-cost technique for on-site and ultrasensitive detection of Cu$^{2+}$, especially in remote regions.

**Supporting Information**

Figures S1 – S6 and Table S1 on the experiments are available free of charge on the Web at http://www.jsac.or.jp/analsci/

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